Differential dose-response effect of cyclosporine A in regulating apoptosis and autophagy markers in MCF-7 cells

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Abstract

Cyclosporine A (CsA) is an immunosuppressant primarily used at a higher dosage in transplant medicine and autoimmune diseases with a higher success rate. At lower doses, CsA exhibits immunomodulatory properties. CsA has also been reported to inhibit breast cancer cell growth by downregulating the expression of pyruvate kinase. However, differential dose-response effects of CsA in cell growth, colonization, apoptosis, and autophagy remain largely unidentified in breast cancer cells. Herein, we showed the cell growth-inhibiting effects of CsA by preventing cell colonization and enhancing DNA damage and apoptotic index at a relatively lower concentration of 2 μM in MCF-7 breast cancer cells. However, at a higher concentration of 20 μM, CsA leads to differential expression of autophagy-related genes ATG1, ATG8, and ATG9 and apoptosis-associated markers such as Bcl-2, Bcl-XL, Bad, and Bax, indicating a dose-response effect on differential cell death mechanisms in MCF-7 cells. This was confirmed in the protein-protein interaction network of COX-2 (PTGS2), a prime target of CsA, which had close interactions with Bcl-2, p53, EGFR, and STAT3. Furthermore, we investigated the combined effect of CsA with SHP2/PI3K-AKT inhibitors showing significant MCF-7 cell growth reduction, suggesting its potential to use as an adjuvant during breast cancer therapy.

Keywords: Cyclosporine A, Apoptosis, Autophagy, Breast cancer, DNA damage, COX-2

Introduction

Cyclosporin A (CsA), first discovered as an anti-fungal agent in Switzerland in 1972, isolated from the fungus *Tolypocladium inflatum* and *Cylindrocarpon lucidium*, was soon identified as a potent immunosuppressant, with wide clinical use (Borel et al. 1976, 1977). CsA comprises 11 amino acids possessing a cyclic lipophilic structure and exerts selective immunoregulation of T cells, reducing T cell activation (Mukherjee and Mukherjee 2009; Patocka et al. 2021). CsA is used orally, topically, or intravenously following organ transplantation and in autoimmune diseases and is often associated with adverse side effects, including the development of malignancies (Durnian et al. 2007; Flores et al. 2019). Further, a recent analysis has shown that higher doses of CsA cause severe signs and symptoms of poisoning in patients (Ershad et al. 2023).

The role of CsA in cancer progression is controversial and depends on the dose and duration of the treatment (Landewé et al. 1999; Flores et al. 2019). Masuo et al. first demonstrated that CsA acts as an anticancer agent against colon cancer cells by downregulating c-myc and the proliferating cell nuclear antigen (PCNA) (Masuo et al. 2009). Notably, c-Myc is an oncogene, and PCNA is a marker of proliferation for colonic epithelium (Kubben et al. 1994). Furthermore, p21 controls the PCNA-dependent DNA replication and repair, whereas c-myc overexpression inhibits the expression of p21. Interestingly, CsA is known to selectively inhibit cyclooxygenase-2 (COX-2, i.e., PTGS2), which on upregulation along with Prostaglandin E2 (PGE-2) expression is known to induce cytochrome P450 family 19 (CYP-19) transcription and biosynthesis of estrogen thereby stimulating unbridled mitogenesis (Harris 2014; Groenendyk et al. 2018).

In contrast, CsA promotes carcinogenesis by inducing transforming growth factor (TGF)-β production or by acting as a specific inhibitor of the nuclear factor of activated T cells (NFAT) (Werneck et al. 2012; Kawahara et al. 2015). Moreover, PI3K/AKT-regulated kinases such as glycogen synthase kinase 3 beta (GSK3β) and mammalian target of rapamycin (mTOR) inhibit NFAT, suggesting crosstalk between calcium signaling pathways and growth factors. Further, it was shown that CsA led to cell cycle arrest and necroptosis in colon cancer cells independent of TGFβ, NFAT, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and PI3K/mTOR pathways (Werneck et al. 2012). Interestingly, calcineurin overexpression and NFAT activation have been previously reported in human colorectal cancer, suggesting the possible role of calcineurin-mediated activation of NFAT in the pathogenesis of colorectal cancer (Lakshmikuttyamma et al. 2005). CsA also promotes cell cycle arrest, apoptosis, and necroptosis, inhibiting cell growth in colon and bladder cancer cells (Werneck et al. 2012; Kawahara et al. 2015).

In breast cancer, CsA modulates the expression and activity of pyruvate kinase M2 (PKM2), an embryonic and cancer-specific enzyme involved in the final step of glycolysis. PKM2 is highly

upregulated in breast cancer cells, and treatment with CsA downregulates PKM2, reduces ATP synthesis, and induces necrosis (Jiang et al. 2012). However, the dose-response effect of CsA in breast cancer cells and its mechanism in regulating apoptosis and autophagy remains elusive. Moreover, understanding the mechanism of CsA dose-response is crucial, as its general administration to patients with breast cancers may impair the host anti-tumor immune responses (Jiang et al. 2012). Therefore, this study aimed to investigate the dose-response effect of CsA on different modes of cell death using a human breast cancer cell line, MCF-7. Our study showed that treatment with low and high doses of CsA resulted in differential regulation of apoptosis and autophagy-related markers. Furthermore, the combinational effect of CsA with inhibitors of Src homology region 2 (SH2) containing protein tyrosine phosphatase-2 (SHP2)/PI3K-AKT cell proliferation pathways showed a remarkable reduction in cell colonization, suggesting the beneficial effects of CsA in breast cancer management.

Materials and methods

Cell culture and drug preparation

Human breast cancer MCF-7 cells were maintained in the Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin, and 1% streptomycin. The cells were grown at 37°C with 5% CO_2 in a humidified incubator. CsA was dissolved in dimethyl sulfoxide (DMSO), with the final DMSO volume never exceeding 1% (v/v).

MTT cytotoxicity assay

An MTT kit (Sigma Aldrich, Missouri, USA) based assay was used to assess the cytotoxicity of CsA. The cells were counted using LUNA-FL™ automated fluorescence cell counter, and 10,000 cells were seeded per well in the 96-well plates at 200 µL and incubated overnight. Following incubation, cells were treated with different concentrations (0, 2, 5, 10, and 20 µM) of CsA and incubated for 48 h. After incubation, 10 µL of the MTT substrate was added to each well. The plate was covered with aluminum foil and placed in the dark at 37°C incubator for 3 h. A hundred µL of solubilization buffer was then added to each well and incubated further for 10 min in the dark. The absorbance was measured using Biotek ELx800 ELISA plate reader at 490 nm and the percentage cell viability was analyzed.

Colony-forming assay

The colony-inhibiting effect of CsA was analyzed by a method described elsewhere (George et al. 2013) with minor modifications. One thousand cells were seeded in a 6-well plate, grown overnight, then treated with different concentrations of CsA, and incubated for 48 h. After incubation, the drug-containing media was removed, and cells were washed twice with phosphate-buffered saline (PBS) and incubated for 5–7 days with fresh media until enough colonies were observed in the control group. For staining, the cells were fixed with 2 mL of

methanol + PBS (1:1 ratio) followed by 2 mL of absolute methanol for 10 min. After removing the excess fixative, the cells were washed twice with PBS and stained with 0.1% crystal violet solution. The cells were rewashed twice with PBS, and the plate was allowed to dry at room temperature for 2 h. The pictures of the colonies were taken at 4× and 30–50 cells in the group were assumed as one colony.

Scratch assay

The cell migrating inhibitory effect of CsA was studied using a protocol described elsewhere with minor modifications (Vazhappilly et al. 2019). MCF-7 cells were counted using LUNA-FL™ automated fluorescence cell counter, and 80,000 cells were seeded in 2 mL media in 6-well plates. The cells were incubated overnight in a 37°C incubator allowing cell adhesion. A 10 µL pipette tip was used to create a vertical scratch and scrape off cells from the monolayer in each well. The wells were washed with PBS twice to remove dislodged cells and were treated with different concentrations of CsA. The area of migrated cells was then recorded at different time points (0, 24, and 48 h) during growth at 4× magnification using a microscope.

DNA fragmentation assay

DNA fragmentation assay was carried out to check whether CsA can induce DNA damage in MCF-7 cells using a method described elsewhere (Vazhappilly et al. 2020). Briefly, after CsA treatment, cells were trypsinized, lysed with lysis buffer, and incubated with 0.5 µg/mL Proteinase K at 37°C overnight. Proteinase K was then deactivated, and RNAse-A (0.5 µg/mL) was added and incubated at 37°C for 2 hrs. DNA was isolated using the phenol-chloroform-isoamyl alcohol and separated on 2% agarose gel. The gel was then stained with ethidium bromide, and images were recorded by Chemidoc MP (BioRad, Germany) for DNA laddering.

Real-time PCR

For the proapoptotic, anti-apoptotic, and autophagy gene expression studies at the mRNA level, we performed real-time quantitative chain reaction (RT-qPCR) under the following conditions. Briefly, the MCF-7 cells were treated with DMSO (control), 2 µM, and 20 µM of CsA in T25 flasks and incubated for 48 hrs, followed by RNA extraction using RNA-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Korea). RNA was converted into cDNA using Firescript RT cDNA synthesis kit (Solis Biodyne, Estonia) as per the supplier's protocol. The quantity and quality of cDNA were analyzed using Denovix DS-11 Series Spectrophotometer/Fluorometer. The RT-qPCR was performed for p21, Bak, Bad, ATG1, ATG8, and ATG9 genes using GAPDH as the internal control. The expression level of the markers was quantified with respect to the control and was plotted using Graph-Pad Prism 5 software (San Diego, CA, USA).

Western blot

Western blot was performed to analyze the expression at the protein level for various apoptotic and DNA damage markers as described elsewhere. Briefly, following treatment, the total lysate was prepared using 1X cell lysis buffer (Cell Signaling Technology, Danvers, USA), and the protein was quantified by the Bicinchoninic Acid (BCA) kit (Sigma Aldrich, Missouri, USA). Equal amounts of each protein sample (30 µg) were loaded and separated using SDS-PAGE gel electrophoresis. After transferring into the nitrocellulose membrane, the membranes were blocked using 5% non-fat milk solution and probed with different primary antibodies (1:1000) overnight. The membrane was then washed with 1X TBST, incubated with respective secondary antibodies (1:2000) for 1 h, and developed to detect proteins using the enhanced chemiluminescence (ECL) method. The gel images were documented with Chemidoc MP (BioRad, Germany). Beta-actin was used as the internal control.

Protein-protein network analysis

The protein-protein interactions (PPI) between PTGS2 and the related partners were curated from the STRING v11 database at high confidence scores (STRING, https://string-db.org/ (Szklarczyk et al. 2015)). Cytoscape software was used to visualize the established PPI network, while the MCODE plugin was used for cluster analysis, Cytohubba and CentiScaPe in Cytoscape were used to select significant modules and calculate the closeness of genes from the PPI network (Scardoni et al. 2015).

Statistical analysis

All the experiments were carried out in triplicates, and data were presented as mean ± standard deviation (SD). Two-way ANOVA or unpaired student's t-test was used for analysis, and a p-value less than 0.05 was considered significant.

Results

Antiproliferative effects of CsA

The dose-response effect of CsA should be studied prior to investigating its effect at a molecular level. MCF-7 cells were treated with different concentrations of CsA, and morphological changes and cytotoxicity were analyzed using microscopic analysis and MTT assay, respectively (Figure 1a & b). Morphologically, cell shrinkage, cell detachment, and cell number reduction were observed in CsA-treated cells, with 57.18% and 43.56% reduced cell viability at 10 and 20 µM concentrations, respectively. However, lower CsA concentrations of 2 and 5 µM did not affect the cell viability. Further, 10 and 20 µM CsA treatment significantly inhibited MCF-7 cell colony formation indicating antiproliferative effects of CsA, especially at higher doses (Figure 1c). We also performed a scratch assay to determine whether CsA can inhibit cell migration. As shown in

Supplementary Figure 1, treatment with CsA inhibited cell migration and reduced cell growth up to 48 h compared to control cells.

DNA damaging effect of CsA on MCF-7 cells

DNA fragmentation and western blotting were performed to investigate whether CsA can induce DNA fragmentation, leading to DNA damage. An increase in DNA fragmentation with high molecular weight smear (with fragmented DNA ladders) was observed with increasing doses of CsA compared to control cells which were further confirmed by analyzing the expression of γ-H2AX (Figure 2a & b), an early marker for identifying DNA double-strand breaks (DSBs) (Kinner et al. 2008). Western blot data showed an increased expression of γ-H2AX at the serine 139 in MCF-7 cells following CsA treatment. Interestingly, lower and higher doses of CsA induced the expression of γ-H2AX compared to the control group. We also performed an RT-PCR gene expression study to analyze p53 and p21 levels since many studies have reported a direct correlation between γ-H2AX and p53 expression (George et al. 2017; Siddiqui et al. 2020). Treatment with lower and higher CsA concentrations significantly inhibited the p53 expression, suggesting a p53-independent DNA damage mechanism in MCF-7 cells (Figure 2c). However, inhibition of p21 was only observed at 2 µM concentration of CsA (Figure 2d).

CsA induces apoptotic index in MCF-7 cells

DNA damage often correlates with cell death mechanisms (Lomphithak and Fadeel 2023), and analyzing the dose-response effect of CsA at protein and mRNA levels is crucial. Accordingly, we investigated the dose-response effect of CsA on MCF-7 cell apoptotic markers (Figure 3a). CsA treatment activated caspase-9 in MCF-7 cells. Treatment with all doses of CsA could cleave caspase 9, with slightly higher activation observed at 5 µM concentration. Treatment with CsA showed no caspase-3 expression in MCF-7 cells, as they are known to be caspase-3-deficient cells (Wang et al. 2016). A dose-dependent inhibition of DNA methyltransferase 1 (DNMT1) expression was observed further, indicating CsA's role in DNA methylation, thereby dysregulating DNA synthesis. However, a low dose of CsA could not affect the anti-apoptotic protein Bcl-XL expression, while a high dose of 20 µM inhibited Bcl-XL expression, suggesting its role in apoptotic activation. Furthermore, expression profiles of Bcl-2, Bak, and Bad at low and high doses of CsA were studied at the mRNA level using RT-qPCR (Figure 3b-d). At a low dose of 2 µM, the expression of Bcl-2 and Bak was upregulated compared to control cells suggesting apoptotic cell death. However, 20 µM CsA dosage reversed the Bcl-2 expression indicating a possible alternate cell death mechanism in MCF-7 cells. No significant difference was observed for Bak and Bad at a high dose of 20 µM compared to control cells.

CsA regulates autophagy markers in MCF-7 cells

To analyze other cell death mechanisms apart from apoptosis, we analyzed the expression of autophagy markers in MCF-7 cells upon treatment with low and high doses of CsA. Our results showed that at a high concentration of 20 µM, CsA enhanced ATG8 expression at the mRNA level compared to control cells (Figure 4a). A slight increase in the expression of ATG1 was observed at low and high doses of CsA treatment (Figure 4b). A differential expression of ATG9 was observed upon CsA treatment, where the low dose enhanced and the high dose inhibited its expression (Figure 4c). To summarize, we observed a differential effect of autophagy markers at low and high doses of CsA treatment suggesting its role in mediating autophagy.

The synergetic effect of CsA with SHP2/PI3K-AKT inhibitors reduces cell growth

Further, we investigated the combined effect of CsA with growth inhibitors that regulates SHP2 and AKT pathways in cancer cells. The cellular phosphatase SHP2 is a highly explored protein as a master regulator or mediator of cancer progression (Chen et al. 2016; Rehman et al. 2019). Therefore, we used the SHP2 inhibitor, SHP099 (Vazhappilly et al. 2019), alone or combined with CsA to observe its effects on MCF-7 cell colonization. As expected, the colonization was reduced when the cells were treated with CsA or SHP099 alone (Figure 5a). Interestingly, we observed no colonies when the cells were treated with CsA and SHP099. We also analyzed CsA's effect with AKT inhibitor miransertib (Forde et al. 2021), alone or in combination. We observed a few colonies in the wells treated with miransertib; combined treatment showed no colonies (Figure 5b). Together, these results indicate that CsA could effectively halt the progression of breast cancer cells when combined with growth inhibitors.

Protein-protein interaction (PPI) between the marker and related genes

Further, to predict the targets of CsA, we performed PPI studies using PTGS2, aka COX-2, a known target protein of CsA (Groenendyk et al. 2018). A PPI network was analyzed with PTGS2 as the central node. The proteins were analyzed for their interactions with related proteins and their involvement in any known metabolic pathways (Figure 6a). Accordingly, PTGS2, aka COX-2, closely interacts with STAT3, P53, Bcl2, EGFR, and its co-regulators PTGES and ALOX proteins. Furthermore, we performed more PPI studies with the target proteins of CsA identified in this study, such as PPIA (cyclophilin A), DNMT1, ATG9A, and BCL2L1 (Bcl-XL). These proteins interacts with known oncogenes and tumor suppressor proteins (Figure 6b-e). The statistics of their interactions with initial and extended networks were presented in Supplementary Table 1-5.

Clustering analysis using the Cytoscape-MCODE tool revealed three different clusters (Figure 7a). The protein PTGS2 (ENSP00000356438) is included in cluster 1, along with 14 other nodes having 54 edges. BCL2, CDK2, TP53, and 8 other nodes were included in cluster 2 with 36 edges. PTGIS, PTGES, and TBXAS1 were included in cluster 3 with three nodes. Twelve proteins were not

included in any of the clusters. The top 10 direct interactors of PTGS2 in all clusters are highlighted in Figure 7b, where C1 had eight interactors, including EGFR and STAT3, while C2 had two direct interactors, including Bcl2. We further used the extended interaction network and identified the hub genes, as highlighted in (Figure 7c). This revealed the important proteins p53, STAT3, CDK2, and HSP90AA1 as hub genes in this network of PTGS2.

Discussion

CsA suppresses immune responses by inhibiting calcineurin and is widely used for facilitating organ transplantation (Miach 1986). However, its role in cancer initiation/progression remains controversial. Several studies presumed that overdose or higher concentrations of CsA could cause unwanted toxic effects, leading to cancer initiation (Hojo et al. 1999; Landewé et al. 1999; Thoms et al. 2011; Ershad et al. 2023). Therefore, understanding the dose-response mechanism of CsA during various treatments is essential. In the present study, we investigated the dose-response effect of CsA in MCF-7 breast cancer cells using various *in vitro* assays.

To investigate the effect of CsA, we initially analyzed the cytotoxic effect of CsA on breast cancer cells, especially at higher doses. Even though the cytotoxic nature of CsA has been reported before in many cancer cells (Jiang et al. 2012; Kawahara et al. 2015), it is essential to understand its effects at various lower and higher doses. Our results indicated that CsA was non-cytotoxic at lower concentrations (2 and 5 µM) and affects cell viability significantly at higher concentrations. As reported previously, this observed cytotoxicity could be attributed to the downregulation of PKM2, c-Myc, and PCNA in breast cancer cells leading to less ATP generation (Jiang et al. 2012). In addition, the expression of c-Myc and PCNA has been shown to be downregulated by CsA through inhibition of the calcineurin/NFAT pathway. c-Myc and PCNA are essential regulators of cell proliferation in cancer cells (Masuo et al. 2009). The non-cytotoxic low dose of CsA was previously reported for its beneficial effects in treating rheumatoid arthritis and psoriasis (Lowe et al. 1996; Tanaka and Tsujimura 2002). Further, our observed cytotoxic effect of CsA was consistent with colony-inhibiting potential at a higher dose in MCF-7 cells, confirming its significant anti-proliferative effect, particularly at higher concentrations. The cytotoxic nature of many anticancer drugs is often associated with anti-migratory properties (Gandalovičová et al. 2017; Dhyani et al. 2022), and CsA was found to inhibit cell migration slightly at lower doses, and higher doses showed dysregulated cell morphology suggesting cell death. Previous studies have shown that CsA can inhibit human prostate cancer growth and migration by inhibiting COX-2 expression, a pro-metastatic protein overexpressed in cancer tissues (Guo et al. 2015; Cevik et al. 2019).

Anticancer drugs induce cell death using different mechanisms in cancer cells (Olivier et al. 2021; Ioele et al. 2022). Accordingly, we observed a differential expression of DNA damage and

apoptotic index with CsA treatment in MCF-7 cells. Increased DNA fragmentation and enhanced γ-H2AX were observed with increasing concentrations of CsA, indicating signs of early DSBs. A caspase 9-mediated cell death was observed at all concentrations, particularly at 5 µM, suggesting its role in inducing cell death. Caspases trigger mitochondrial-mediated cell death mechanisms, such as apoptosis (Tait and Green 2013). Interestingly, the pro-apoptotic protein Bcl-XL was inhibited only at a higher dose of 20 µM, indicating a higher dose requirement to activate mitochondria-mediated apoptosis in MCF-7 cells (Popgeorgiev et al. 2018). In contrast, CsA was reported earlier for inhibiting apoptosis by preventing the release of cytochrome C in human endothelial cells at 10 µM (Walter et al. 1998), indicating the possibility of using a higher dose to induce mitochondria-mediated apoptosis.

Furthermore, DNA methylation inhibition, specifically DNMT1, was associated with impaired DNA synthesis, which correlated with p53-mediated DNA damage and apoptosis (Jin and Robertson 2013; Siddiqui et al. 2020; Laranjeira et al. 2023). Interestingly, a lower dose of 2 µM indicated signs of apoptotic-mediated cell death and enhanced expression of anti-apoptotic proteins, such as Bcl2, Bak, and Bad. However, Bcl2 expression was inhibited at a higher dose, suggesting an apoptotic mode of cell death. A similar observation was also noted by Kim et al. in rat pituitary GH3 cells (Kim et al. 2014). We also observed p21 upregulation at low concentrations indicating the cell cycle arresting potential of CsA (Lally et al. 1999; Flores et al. 2019). However, CsA showed no differential p53 expression at tested lower or higher doses, implying a p53-independent mechanism of cell death in MCF-7 cells in response to DNA damage/stress (Etti et al. 2017).

It is interesting to note that phenotypically upregulated Bcl-2 was identified as a close interactor of PTGS2, a target of CsA, through PPI networking analysis. This confirms the role of PTGS2 in regulating apoptotic pathways in response to CsA activity in MCF-7 cells. Among the three clusters identified, EGFR and STAT3 were in the main cluster along with PTGS2. EGFR is known for its involvement in various downstream signaling pathways, such as MEK-ERK, PI3K-AKT, p38, STAT, and JNK (Wee and Wang 2017). In addition, STAT3 activation could also occur independent of EGFR signaling, enhancing the importance of STAT3 activation in tumor growth (Tan 2011). Therefore, inhibition of STAT3 and EGFR limits cancer cell proliferation.

Cluster 2 consisted of proteins, including Bcl2, CDK2, and p53. The phenotypic studies proved the dose-dependent effect of CsA on Bcl2 and the dose-independent inhibition of p53. These results were reflected in the PPI network, confirming the effect of CsA on Bcl-2 and p53 via PTGS2. Similarly, PTGIS, PTGES, and TBXAS1 were included in cluster 3 with three nodes. PTGES is a classical ER target gene in MCF-7 cells (Frasor et al. 2008). PTGIS and TBXAS1 are involved in prostaglandin synthesis and need to be studied further for their role in the CsA mechanism of action in MCF-7 cells. To the best of our knowledge, this is the first study to analyse the PPI

network of PTGS2 and its associated interacting proteins for the effect of CsA. Interestingly, when we performed the protein interaction network analysis with the identified targets of COX-2, we found the association of these targets with the known oncogenes. For example, PPIA (Cyclophilin A) interacted with a known oncogene BRCA1, and DNMT1 interacted with cell cycle regulators such as CDK4 and E2F3 and anti-apoptosis marker TP53. ATG9A interacted with BCL2 and BCL2L1 (apoptosis regulators). Lastly, BCL2L1 interacted with cell cycle regulator CDK2, anti-apoptosis marker TP53, and its negative regulator MDM2. These results showed that CsA regulates genes and proteins interacting with cell cycle regulators and apoptosis.

We also investigated the expression of autophagy markers since CsA is known to regulate apoptosis and autophagy (Ciechomska et al. 2013; Kim et al. 2014). CsA enhanced the expression of ATG8 and ATG9 at a low dose of 2 µM. ATG8 and ATG9 play a crucial role in autophagy by regulating the outgrowth of autophagosomal membranes (Slobodkin and Elazar 2013; Zhuang et al. 2017). Further, a slight increase in the expression of ATG1 was observed with CsA treatment suggesting its involvement in activating ATG9 (Papinski et al. 2014). The induction of ATG1 was known to inhibit cell growth and induce cell death (Scott et al. 2007). However, inhibition of the ATG9 gene was observed at a higher dose of 20 µM and could be associated with apoptotic induction rather than autophagy in MCF-7 cells, and was evident earlier, as 20 µM of CsA treatment showed DNA damage, caspase 9 activation, and Bcl-2 inhibition.

Various reports have described the possible synergetic effect of CsA with other drugs in tumor inhibition (Kim 2010; Wu et al. 2022). SHP2 activation via PI3K/AKT pathways is well-studied in breast cancer cells and remains a challenge in breast cancer treatment (Zhou et al. 2008; Yuan et al. 2020). Therefore, we investigated the combined effect of CsA on growth inhibitors. Treatment with CsA and SHP099 or miransertib inhibitors showed no colony growth in MCF-7 cells suggesting its potential use as an adjuvant during breast cancer therapy. Moreover, a similar study combining CsA with crizotinib inhibited Ca^{2+}/calcineurin/Erk pathway and proposed its use for the targeted treatment of patients with MET-amplified lung cancer (Liu et al. 2019). However, further studies are required to extend its use into animal and clinical settings to prove its synergetic effect.

Conclusions

Our results indicated that CsA, an immunosuppressant, possesses anticancer properties and can inhibit MCF-7 cell growth by inducing apoptosis and autophagy. However, the dose-response mechanism of CsA should be considered when used for different treatments. Our results suggested that CsA exhibits differential effects on apoptotic and autophagy markers at different concentrations. Moreover, using CsA as an adjuvant, along with growth inhibitors may provide a better therapeutic strategy for managing different breast cancer phenotypes.

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Conflict of interest
Authors declare no conflict of interest in this article.

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Data availability

We confirm that all the data supporting above findings are available in the article and its Supplementary materials.

References

Borel JF, Feurer C, Gubler HU, Stähelin H (1976) Biological effects of cyclosporin A: A new antilymphocytic agent. Agents and Actions 6:468–475. https://doi.org/10.1007/BF01973261

Borel JF, Feurer C, Magnée C, Stähelin H (1977) Effects of the new anti-lymphocytic peptide cyclosporin A in animals. Immunology 32:1017–1025

Cevik O, Turut FA, Acidereli H, Yildirim S (2019) Cyclosporine-A induces apoptosis in human prostate cancer cells PC3 and DU145 via downregulation of COX-2 and upregulation of TGFβ. Turkish Journal of Biochemistry 44:47–54. https://doi.org/10.1515/tjb-2017-0355

Chen Y-NP, LaMarche MJ, Chan HM, et al (2016) Allosteric inhibition of SHP2 phosphatase inhibits cancers driven by receptor tyrosine kinases. Nature 535:148–152. https://doi.org/10.1038/nature18621

Ciechomska IA, Gabrusiewicz K, Szczepankiewicz AA, Kaminska B (2013) Endoplasmic reticulum stress triggers autophagy in malignant glioma cells undergoing cyclosporine A-induced cell death. Oncogene 32:1518–1529. https://doi.org/10.1038/onc.2012.174

Dhyani P, Quispe C, Sharma E, et al (2022) Anticancer potential of alkaloids: a key emphasis to colchicine, vinblastine, vincristine, vindesine, vinorelbine and vincamine. Cancer Cell Int 22:206. https://doi.org/10.1186/s12935-022-02624-9

Durnian JM, Stewart RMK, Tatham R, et al (2007) Cyclosporin-A associated malignancy. Clin Ophthalmol 1:421–430

Ershad A, Taziki S, Ebrahimian M, Abadi SSD (2023) Acute cyclosporine overdose: A systematic review. Medicina Clínica Práctica 6:100358. https://doi.org/10.1016/j.mcpsp.2022.100358

Etti I, Rasedee A, Mohd Hashim N, et al (2017) Artonin E induces p53-independent G1 cell cycle arrest and apoptosis through ROS-mediated mitochondrial pathway and livin suppression in MCF-7 cells. DDDT Volume11:865–879. https://doi.org/10.2147/DDDT.S124324

Flores C, Fouquet G, Moura IC, et al (2019) Lessons to Learn From Low-Dose Cyclosporin-A: A New Approach for Unexpected Clinical Applications. Front Immunol 10:588. https://doi.org/10.3389/fimmu.2019.00588

Forde K, Resta N, Ranieri C, et al (2021) Clinical experience with the AKT1 inhibitor miransertib in two children with PIK3CA-related overgrowth syndrome. Orphanet J Rare Dis 16:109. https://doi.org/10.1186/s13023-021-01745-0

Frasor J, Weaver AE, Pradhan M, Mehta K (2008) Synergistic Up-Regulation of Prostaglandin E Synthase Expression in Breast Cancer Cells by 17β-Estradiol and Proinflammatory Cytokines. Endocrinology 149:6272–6279. https://doi.org/10.1210/en.2008-0352

Gandalovičová A, Rosel D, Fernandes M, et al (2017) Migrastatics—Anti-metastatic and Anti-invasion Drugs: Promises and Challenges. Trends in Cancer 3:391–406. https://doi.org/10.1016/j.trecan.2017.04.008

George VC, Dellaire G, Rupasinghe HPV (2017) Plant flavonoids in cancer chemoprevention: role in genome stability. The Journal of Nutritional Biochemistry 45:1–14. https://doi.org/10.1016/j.jnutbio.2016.11.007

George VC, Kumar DRN, Suresh PK, et al (2013) Comparative Studies to Evaluate Relative in vitro Potency of Luteolin in Inducing Cell Cycle Arrest and Apoptosis in HaCaT and A375 Cells. Asian Pacific Journal of Cancer Prevention 14:631–637. https://doi.org/10.7314/APJCP.2013.14.2.631

Groenendyk J, Paskevicius T, Urra H, et al (2018) Cyclosporine A binding to COX-2 reveals a novel signaling pathway that activates the IRE1α unfolded protein response sensor. Sci Rep 8:16678. https://doi.org/10.1038/s41598-018-34891-w

Guo Z, Jiang J-H, Zhang J, et al (2015) COX-2 Promotes Migration and Invasion by the Side Population of Cancer Stem Cell-Like Hepatocellular Carcinoma Cells. Medicine 94:e1806. https://doi.org/10.1097/MD.0000000000001806

Harris RE (2014) Cyclooxygenase-2 and the inflammogenesis of breast cancer. WJCO 5:677. https://doi.org/10.5306/wjco.v5.i4.677

Hojo M, Morimoto T, Maluccio M, et al (1999) Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature 397:530–534. https://doi.org/10.1038/17401

Ioele G, Chieffallo M, Occhiuzzi MA, et al (2022) Anticancer Drugs: Recent Strategies to Improve Stability Profile, Pharmacokinetic and Pharmacodynamic Properties. Molecules 27:5436. https://doi.org/10.3390/molecules27175436

Jiang K, He B, Lai L, et al (2012) Cyclosporine A inhibits breast cancer cell growth by downregulating the expression of pyruvate kinase subtype M2. International Journal of Molecular Medicine 30:302–308. https://doi.org/10.3892/ijmm.2012.989

Jin B, Robertson KD (2013) DNA Methyltransferases, DNA Damage Repair, and Cancer. In: Karpf AR (ed) Epigenetic Alterations in Oncogenesis. Springer New York, New York, NY, pp 3–29

Kawahara T, Kashiwagi E, Ide H, et al (2015) Cyclosporine A and tacrolimus inhibit bladder cancer growth through down-regulation of NFATc1. Oncotarget 6:1582–1593. https://doi.org/10.18632/oncotarget.2750

Kim (2010) Cyclosporin A and sanglifehrin A enhance chemotherapeutic effect of cisplatin in C6 glioma cells. Oncol Rep 23:. https://doi.org/10.3892/or_00000732

Kim HS, Choi S-I, Jeung E-B, Yoo Y-M (2014) Cyclosporine A Induces Apoptotic and Autophagic Cell Death in Rat Pituitary GH3 Cells. PLoS ONE 9:e108981. https://doi.org/10.1371/journal.pone.0108981

Kinner A, Wu W, Staudt C, Iliakis G (2008) -H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Research 36:5678–5694. https://doi.org/10.1093/nar/gkn550

Kubben FJ, Peeters-Haesevoets A, Engels LG, et al (1994) Proliferating cell nuclear antigen (PCNA): a new marker to study human colonic cell proliferation. Gut 35:530–535. https://doi.org/10.1136/gut.35.4.530

Lakshmikuttyamma A, Selvakumar P, Kanthan R, et al (2005) Increased expression of calcineurin in human colorectal adenocarcinomas. J Cell Biochem 95:731–739. https://doi.org/10.1002/jcb.20437

Lally C, Healy E, Ryan MP (1999) Cyclosporine A-induced cell cycle arrest and cell death in renal epithelial cells. Kidney International 56:1254–1257. https://doi.org/10.1046/j.1523-1755.1999.00696.x

Landewé RBM, van den Borne BEEM, Breedveld FC, Dijkmans BAC (1999) Does cyclosporin A cause cancer? Nat Med 5:714–714. https://doi.org/10.1038/10417

Laranjeira ABA, Hollingshead MG, Nguyen D, et al (2023) DNA damage, demethylation and anticancer activity of DNA methyltransferase (DNMT) inhibitors. Sci Rep 13:5964. https://doi.org/10.1038/s41598-023-32509-4

Liu Z, Jiang L, Li Y, et al (2019) Cyclosporine A sensitizes lung cancer cells to crizotinib through inhibition of the Ca2+/calcineurin/Erk pathway. EBioMedicine 42:326–339. https://doi.org/10.1016/j.ebiom.2019.03.019

Lomphithak T, Fadeel B (2023) Die hard: cell death mechanisms and their implications in nanotoxicology. Toxicological Sciences 192:141–154. https://doi.org/10.1093/toxsci/kfad008

Lowe NJ, Wieder JM, Rosenbach A, et al (1996) Long-term low-dose cyclosporine therapy for severe psoriasis: Effects on renal function and structure. Journal of the American Academy of Dermatology 35:710–719. https://doi.org/10.1016/S0190-9622(96)90726-4

Masuo T, Okamura S, Zhang Y, Mori M (2009) Cyclosporine A inhibits colorectal cancer proliferation probably by regulating expression levels of c-Myc, p21WAF1/CIP1 and proliferating cell nuclear antigen. Cancer Letters 285:66–72. https://doi.org/10.1016/j.canlet.2009.05.001

Miach PJ (1986) Cyclosporin A in organ transplantation. Medical Journal of Australia 145:146–150. https://doi.org/10.5694/j.1326-5377.1986.tb113775.x

Mukherjee S, Mukherjee U (2009) A Comprehensive Review of Immunosuppression Used for Liver Transplantation. Journal of Transplantation 2009:1–20. https://doi.org/10.1155/2009/701464

Olivier T, Haslam A, Prasad V (2021) Anticancer Drugs Approved by the US Food and Drug Administration From 2009 to 2020 According to Their Mechanism of Action. JAMA Netw Open 4:e2138793. https://doi.org/10.1001/jamanetworkopen.2021.38793

Papinski D, Schuschnig M, Reiter W, et al (2014) Early Steps in Autophagy Depend on Direct Phosphorylation of Atg9 by the Atg1 Kinase. Molecular Cell 53:471–483. https://doi.org/10.1016/j.molcel.2013.12.011

Patocka J, Nepovimova E, Kuca K, Wu W (2021) Cyclosporine A: Chemistry and Toxicity – A Review. CMC 28:3925–3934. https://doi.org/10.2174/0929867327666201006153202

Popgeorgiev N, Jabbour L, Gillet G (2018) Subcellular Localization and Dynamics of the Bcl-2 Family of Proteins. Front Cell Dev Biol 6:13. https://doi.org/10.3389/fcell.2018.00013

Rehman AU, Rahman MU, Khan MT, et al (2019) The Landscape of Protein Tyrosine Phosphatase (Shp2) and Cancer. CPD 24:3767–3777. https://doi.org/10.2174/1381612824666181106100837

Scardoni G, Tosadori G, Faizan M, et al (2015) Biological network analysis with CentiScaPe: centralities and experimental dataset integration. F1000Res 3:139. https://doi.org/10.12688/f1000research.4477.2

Scott RC, Juhász G, Neufeld TP (2007) Direct Induction of Autophagy by Atg1 Inhibits Cell Growth and Induces Apoptotic Cell Death. Current Biology 17:1–11. https://doi.org/10.1016/j.cub.2006.10.053

Siddiqui SS, Rahman S, Rupasinghe HPV, Vazhappilly CG (2020) Dietary Flavonoids in p53—Mediated Immune Dysfunctions Linking to Cancer Prevention. Biomedicines 8:286. https://doi.org/10.3390/biomedicines8080286

Slobodkin MR, Elazar Z (2013) The Atg8 family: multifunctional ubiquitin-like key regulators of autophagy. Essays in Biochemistry 55:51–64. https://doi.org/10.1042/bse0550051

Szklarczyk D, Franceschini A, Wyder S, et al (2015) STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Research 43:D447–D452. https://doi.org/10.1093/nar/gku1003

Tait SWG, Green DR (2013) Mitochondrial Regulation of Cell Death. Cold Spring Harbor Perspectives in Biology 5:a008706–a008706. https://doi.org/10.1101/cshperspect.a008706

Tan Y (2011) Effect of bisphenol A on the EGFR-STAT3 pathway in MCF-7 breast cancer cells. Mol Med Report. https://doi.org/10.3892/mmr.2011.583

Tanaka Y, Tsujimura S (2002) [Clinical implication of cyclosporin for rheumatoid arthritis]. Nihon Rinsho 60:2345–2350

Thoms K-M, Kuschal C, Oetjen E, et al (2011) Cyclosporin A, but not everolimus, inhibits DNA repair mediated by calcineurin: implications for tumorigenesis under immunosuppression: Cyclosporin A inhibits DNA repair mediated by calcineurin. Experimental Dermatology 20:232–236. https://doi.org/10.1111/j.1600-0625.2010.01213.x

Vazhappilly CG, Hodeify R, Siddiqui SS, et al (2020) Natural compound catechol induces DNA damage, apoptosis, and G1 cell cycle arrest in breast cancer cells. Phytotherapy Research ptr.6970. https://doi.org/10.1002/ptr.6970

Vazhappilly CG, Saleh E, Ramadan W, et al (2019) Inhibition of SHP2 by new compounds induces differential effects on RAS/RAF/ERK and PI3K/AKT pathways in different cancer cell types. Invest New Drugs 37:252–261. https://doi.org/10.1007/s10637-018-0626-5

Walter DH, Haendeler J, Galle J, et al (1998) Cyclosporin A Inhibits Apoptosis of Human Endothelial Cells by Preventing Release of Cytochrome C From Mitochondria. Circulation 98:1153–1157. https://doi.org/10.1161/01.CIR.98.12.1153

Wang S, He M, Li L, et al (2016) Cell-in-Cell Death Is Not Restricted by Caspase-3 Deficiency in MCF-7 Cells. J Breast Cancer 19:231–241. https://doi.org/10.4048/jbc.2016.19.3.231

Wee P, Wang Z (2017) Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. Cancers 9:52. https://doi.org/10.3390/cancers9050052

Werneck MBF, Hottz E, Bozza PT, Viola JPB (2012) Cyclosporin A inhibits colon cancer cell growth independently of the calcineurin pathway. Cell Cycle 11:3997–4008. https://doi.org/10.4161/cc.22222

Wu P-J, Hsin I-L, Hung W-L, et al (2022) Combination treatment with cyclosporin A and arsenic trioxide induce synergistic cell death via non-apoptotic pathway in uterine cervical cancer cells. Chemico-Biological Interactions 368:110177. https://doi.org/10.1016/j.cbi.2022.110177

Yuan Y, Fan Y, Gao Z, et al (2020) SHP2 promotes proliferation of breast cancer cells through regulating Cyclin D1 stability *via* the PI3K/AKT/GSK3β signaling pathway. Cancer Biol Med 17:707–725. https://doi.org/10.20892/j.issn.2095-3941.2020.0056

Zhou X, Coad J, Ducatman B, Agazie YM (2008) SHP2 is up-regulated in breast cancer cells and in infiltrating ductal carcinoma of the breast, implying its involvement in breast oncogenesis. Histopathology 53:389–402. https://doi.org/10.1111/j.1365-2559.2008.03103.x

Zhuang X, Chung KP, Cui Y, et al (2017) ATG9 regulates autophagosome progression from the endoplasmic reticulum in *Arabidopsis*. Proc Natl Acad Sci USA 114:. https://doi.org/10.1073/pnas.1616299114

Figure legends:

Figure 1: Antiproliferative effect of CsA. A) Morphological changes after treatment with different concentrations of CsA. B) Cell viability effects of CsA on MCF-7 cells with different concentrations as analyzed by MTT assay. C) Quantified data for the number of colonies in MCF-7 after CsA treatment. Data presented M ± SD. Asterisk indicated statistical significance ($p < .05$)

Figure 2: CsA induces DNA fractions and damage. A) Agarose gel electrophoresis image showing DNA fractions/laddering after treatment with CsA with different concentrations for 48 hrs and control untreated cells. B) Western blot for p-H2AX DNA damage marker protein and p53 after treating MCF-7 cells with different concentrations of CsA along with beta-actin as a loading control. C&D) Relative mRNA expression levels of p53 and p21 in MCF-7 cells after treatment with low and high doses of CsA over GAPDH as an internal loading control. Data presented M ± SD. Asterisk indicated statistical significance ($p < .05$)

Figure 3: CsA alters apoptotic index in MCF-7 cells. A) Western blot data for cleaved caspase-9, caspase-3, DNMT-1, and Bcl-XL expression and beta-actin as a loading control. B, C&D) Relative mRNA expression levels of Bcl2, Bad, and Bak, respectively, in MCF-7 cells after treatment with low and high doses of CsA over GAPDH as an internal loading control. Data presented M ± SD. Asterisk indicated statistical significance ($p < .05$)

Figure 4: CsA regulates different autophagy markers. A, B &C) Relative mRNA expression levels of ATG8, ATG1, and ATG9 in MCF-7 cells after treatment with low and high doses of CsA over GAPDH as an internal loading control. Data presented M ± SD. Asterisk indicated statistical significance ($p < .05$)

Figure 5: Synergetic growth inhibition potential of CsA. A&B) CsA treatment alone or in combination with SHP099 or miransertib growth inhibitors showed significant inhibition of colonies in MCF-7 cells. Data presented M ± SD. Asterisk indicated statistical significance ($p < .05$)

Figure 6: Protein-protein interaction network analysis of A) PTGS2 (ENSP00000356438), B) cyclophilin a, C) Bcl-XL, D) DNMT1, and E) ATG9A.

Figure 7: Gene interaction network clustering analysis of PTGS2 (ENSP00000356438) protein: A) Three clusters were identified using Cytoscape-MCODE tool. The gene PTGS2 is included in cluster 1 along with 14 other nodes having 54 edges. The cluster included other direct interactors such as EGFR and STAT3. B) The ten direct interactors of the PTGS2 gene were highlighted. Cluster C1 has eight interactors, followed by C2 with two nodes. C) The top 10 genes with a maximum number of direct interactors were identified using the Cytoscape-Cytohubba tool.