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Domperidone Induces Apoptosis through Suppression of STAT3 Signaling in Human Renal Cancer Caki-2 Cells

Geumi Park[1,2,*], Manoj Kumar Baniya[1,*], Eun-Jeong Cha[1,*], So Jin Sim[1], Joon-Seok Choi[3,**], Kyung-Soo Chun[1,**]

[1]College of Pharmacy, Keimyung University, [2]Preclinical Research Center, Daegu-Gyeongbuk Medical Innovation Foundation (K-MEDIhub), Daegu, [3]College of Pharmacy, Daegu Catholic University, Gyeongsan, Korea

Renal cancer continues to offer a great challenge for its successful therapy today, thus underscoring the need for effective chemotherapeutic agents. In the current study, we explored the anticancer effects of domperidone, a dopamine D2 receptor (DRD2) antagonist, in renal cancer Caki-2 cells. Domperidone induced dose and time-dependent cytotoxic effects in Caki-2 cells, triggering intrinsic apoptosis via the stimulation of the caspase cascade and PARP cleavage. The cytotoxic effect of domperidone was found to be partially DRD2-dependent. Domperidone treatment markedly augmented the production of intracellular reactive oxygen species which induced the cell death of Caki-2 cells. In addition, domperidone suppressed Janus kinase 2 and STAT3 phosphorylation, leading to inhibition of survival and proliferation of these cells. Hence, domperidone can be considered a promising candidate for renal cancer treatment.

Key Words Domperidone, Apoptosis, STAT3 transcription factor, Reactive oxygen species, Kidney neoplasms

INTRODUCTION

Renal cancer continues to be the highly incident form of kidney cancer and the most fatal among all types of genitourinary cancers representing around 3% of total cancer occurrence. The asymptomatic nature of the disease at its initial stages and its highly metastatic potential contribute to a lower survival rate of renal cancer patients [1,2]. In addition, resistance to available conventional chemotherapeutics and radiotherapy poses a significant challenge for successful therapy of renal cancer [3,4]. Therefore, it is crucial to develop new therapeutic drugs for the better clinical outcomes of renal cancer.

Apoptosis represents a programmed cell demise mechanism proceeding through two different biochemical pathways. One involves the mitochondria often called an intrinsic pathway while the other is an extrinsic pathway that incorporates activation of various membrane-associated death receptors [5]. The Bcl-2 family proteins including anti-apoptotic Bcl-2 and Bcl-xl, and proapoptotic Bax regulate the mitochondrial membrane integrity. Any imbalance in their equilibrium can destabilize the mitochondrial membrane potential thus causing the transfer of mitochondrial contents in the cytoplasm [6]. In the intrinsic pathway, the discharge of mitochondrial cytochrome *c* in the cytoplasm triggers the initiation of the caspase cascades which fuels the cleavage of PARP and DNA damage ultimately contributing to cell death [7].

Aberrant production of intracellular reactive oxygen species (ROS) in cancer cells is associated with the initiation of apoptosis. In addition, failure of the cancer cells to cope with excessive ROS production leads to suppression of unregulated oncogenic signals including STAT3 signaling and the mitogen-activated protein kinase (MAPK) kinase pathway [8,9]. This strategy has been utilized to combat different types of cancer.

STAT3 is reported to be a major oncogenic signaling in renal cancer that is constitutively activated by non-receptor tyrosine kinases such as Janus kinase (JAK), Src family kinase, and epidermal growth factor receptor (EGFR) [10,11]. Phosphorylation of STAT3 leads to dimerization and trans-

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Correspondence to Kyung-Soo Chun, E-mail: chunks@kmu.ac.kr, https://orcid.org/0000-0001-9933-2897
Joon-Seok Choi, E-mail: joonschoi@cu.ac.kr, https://orcid.org/0000-0002-9569-8055
*These authors contributed equally to this work as co-first authors.
**These authors contributed equally to this work as co-correspondence authors.

location into the nucleus which regulates the transcription of various cell survival genes ultimately contributing to the proliferation and metastasis of cancer cells [12]. In addition, MAPK cascades also represent key signaling that modulates the cellular processes including proliferation, differentiation, and apoptosis of cancer cells through the STAT3 axis [12,13]. Interestingly, simultaneous inhibition of multiple oncogenic signals such as PI3K/Akt and STAT3 is associated with enhanced cytotoxicity to cancer cells highlighting the requirement for broad-spectrum anticancer drugs [14].

Dopamine is a neurotransmitter that displays various types of brain functions. Recent studies suggest the intricate interplay of dopamine and dopamine receptors in cancer [15]. Therapeutic approaches for the treatment of schizophrenia involving dopamine receptor antagonists revealed a decreased incidence of cancer [16]. Additionally, a strong correlation has been observed with the prevalence of Parkinson's disease and reduced incidence of cancers [17]. In particular, the expression of dopamine D2 receptor (DRD2) is found to be aberrant both at mRNA and protein levels in various types of cancer [15,18]. These results shed light on DRD2 as a promising target for cancer therapy. Domperidone is a DRD2 antagonist used for the treatment of nausea and vomiting which is now considerably explored for its anticancer properties. Contrary to its notable anticancer effects in triple-negative breast cancer [19] and colorectal cancer [18], a study found that domperidone facilitated human small-cell lung cancer tumorigenesis by abrogating the effects of DRD2 agonist [20]. These studies indicate the controversial role of domperidone in cancer which depends on the cancer type.

In the current research, we uncovered the effect of domperidone on renal cancer Caki-2 cells and unveiled the underlying mechanism for its anticancer effects. The effects of domperidone on the apoptosis initiation and distortion of STAT3 pathway was explored.

MATERIALS AND METHODS

Chemicals and reagents

Sigma-Aldrich supplied domperidone and *N*-acetyl cysteine (NAC). Cell Signaling Technology Inc. provided antibodies directed against cleaved caspase-9, caspase-3, caspase-7, PARP, Bcl-2, Bax, cytochrome *c*, STAT3, p-STAT3 (Y705), p-STAT3 (S727), JAK2, and p-JAK2 and secondary antibodies as well. Novus Biologicals provided anti-survivin antibody. Santa Cruz Biotechnology delivered antibodies against cyclin D2 and cyclin D3. Promega supplied MTS reagent. Pierce Biotechnology provided the bicinchoninic acid protein assay kit. BD Biosciences supplied the FITC-Annexin V staining kit. Invitrogen supplied 2,7-dichloroflourescin diacetate (DCF-DA). Super Signal WesternBright™ ECL HRP Substrate was acquired from Advansta. Supersignal™ West Femto Maximum Sensitivity Substrate was procured from Thermo Scientific.

Cell culture

Korean Cell Bank (Seoul, South Korea) supplied human renal cancer Caki-2 cells. The cells were cultured in Dulbecco's modified Eagle medium mixed with 10% (v/v) fetal bovine serum, and 1% (v/v) penicillin-streptomycin. Caki-2 cells were incubated in a humidified condition well facilitated with 5% CO_2 supply and temperature control at 37°C.

Assessment of cell viability

The viable population of cells after drug treatment was assessed using the MTS assay method as previously described [7]. The cells were grown in 96 well plates and incubated for 24 hours followed by domperidone treatment with or without NAC for given time points. Thereafter, the cells were treated with MTS reagent dissolved in the culture media for 1 hour and absorbance was determined using a Versamax microplate reader (Molecular Devices) at a maximum wavelength of 490 nm. The viability of the cells was calculated and expressed relative to the control.

Quantification of apoptosis using Annexin V/ propidium iodide staining

The proportion of dead cells undergoing apoptosis was estimated using the Annexin V staining kit following the supplier's instructions. Briefly, the cells were incubated with various strengths of domperidone for the indicated time point. The cells were harvested and washed with PBS followed by incubation with Annexin V and propidium iodide (PI) in binding buffer for 15 minutes. Finally, the cells were analyzed by flow cytometry (BD Biosciences).

Western blot analysis

Cells treated with domperidone were collected and lysed in the presence of radio immunoprecipitation assay lysis buffer to obtain the total cellular protein. The obtained protein was quantified using a bicinchoninic acid protein assay kit, and appropriate quantities of protein were separated on SDS-PAGE gels. The resolved proteins were moved to polyvinylidene difluoride membrane which was followed by incubation with skim milk (5%) for 1 hour, and incubation with suitably diluted primary antibodies overnight at 4°C. Thereafter, the membranes were washed with TBS with tween and incubated with respective secondary antibodies for 1 hour under shaking at 25 rpm at room temperature. Finally, the chemiluminescent images were captured using ImageQuant™ LAS 4000 (Fujifilm Life Science) after treating the membranes with Super Signal WesternBright™ ECL HRP Substrate or Supersignal™ West Femto Maximum Sensitivity Substrate.

Determination of cellular ROS levels

The production of ROS in the cell was determined using the DCF-DA as a fluorescence probe [9]. Briefly, the cells were incubated with different strengths of domperidone in the presence or absence of NAC. The cells were washed with PBS

and incubated with DCF-DA (25 μM) diluted in culture media for 30 minutes in the dark at 37°C. The cells were washed with PBS and analyzed by measuring the fluorescence intensity of oxidized DCF at excitation and emission wavelengths of 480 nm and 525 nm respectively, using fluorescence microscopy or flow cytometry.

Statistical analysis

Values are shown as the mean ± SD of at least three independent experiments. One-way analysis of variance followed by Tukey's multiple comparison test was employed for the evaluation of data. A *P*-value < 0.05 was assumed statistically significant.

RESULTS

Domperidone provokes apoptosis in renal cancer Caki-2 cells

Firstly, we investigated the effects of domperidone on renal cancer Caki-2 cells. The results demonstrated that domperidone treatment exerted dose-dependent and time-dependent cytotoxic effects on Caki-2 cells as shown in Figure 1A. Domperidone at a dose of 50 μM suppressed the viability of cells by more than 90% when treated for 72 hours. In addition, to uncover the mechanism of cell death induced by domperidone in Caki-2 cells, Annexin V/PI staining was performed which revealed that domperidone treatment increased the number of cells undergoing apoptotic death. This highlights the crucial role of apoptosis in the initiation of cell death by domperidone in Caki-2 cells (Fig. 1B and 1C).

Domperidone modulates the expression of apoptotic markers in Caki-2 cells

Bcl-2 and Bax play a pivotal role in the induction of intrinsic apoptosis mediated via mitochondrial outer membrane permeabilization. Therefore, we determined the changes in the expression levels of Bcl-2 and Bax following domperidone treatment by Western blot analysis. Interestingly, domperidone treatment downregulated the expression of Bcl-2 while increasing the levels of Bax (Fig. 2A). Moreover, the expression level of cytochrome *c* was also elevated following domperidone treatment (Fig. 2A). Additionally, domperidone increased the fragmentation of caspase-9, -7, and -3 and escalated the breakdown of PARP as shown in Figure 2B.

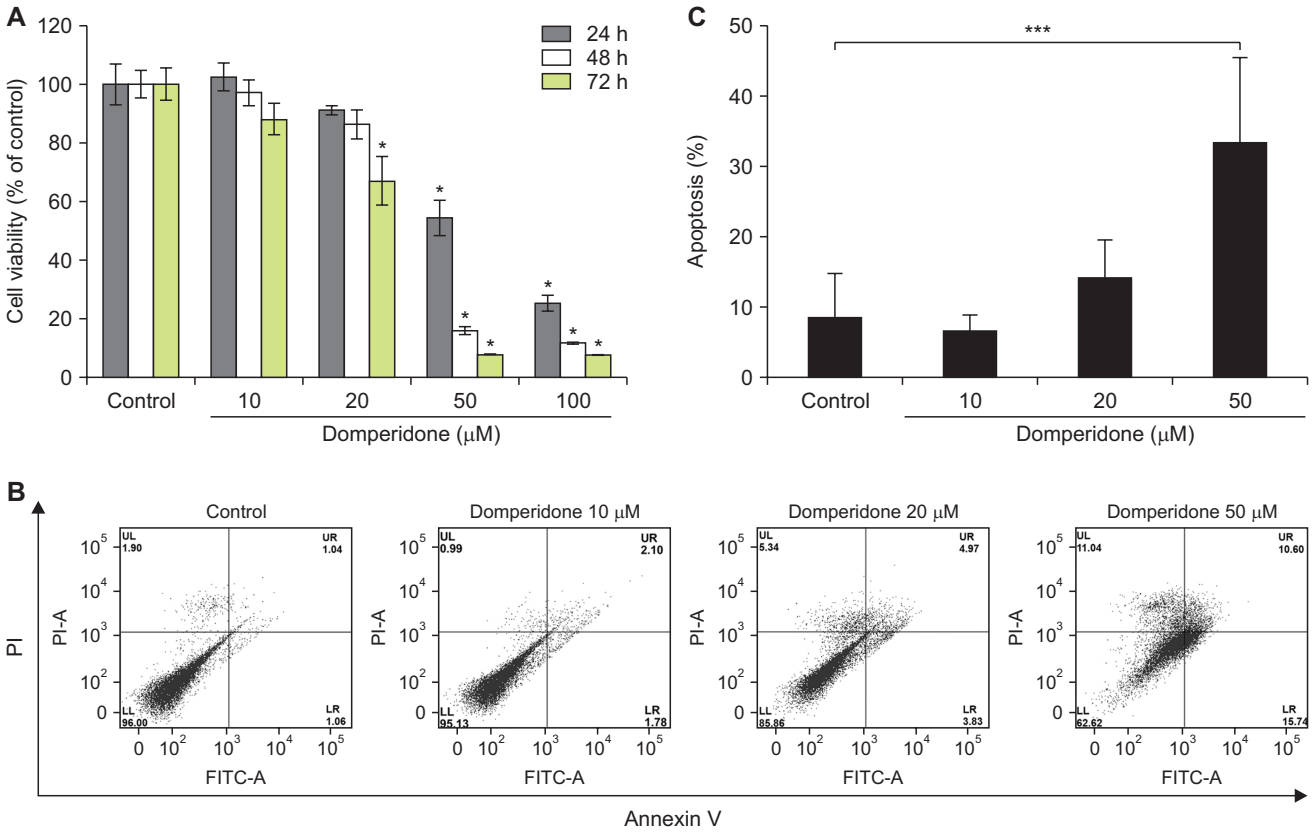

Figure 1. Cytotoxic effect of domperidone in Caki-2 cells. (A) Caki-2 cells were treated with various strengths of domperidone for indicated time points. Cell viability was studied using the MTS assay method. Data are presented relative to control as the mean ± SD (n = 3). **P* < 0.05 vs. control. (B, C) Cells were incubated with domperidone in varying concentrations for 48 hours. (B) Annexin V/PI staining was performed to measure apoptosis by flow cytometry. (C) Statistical data showing apoptosis. Data are expressed as the mean ± SD (n = 3). ****P* < 0.001 vs. control. PI, propidium iodide.

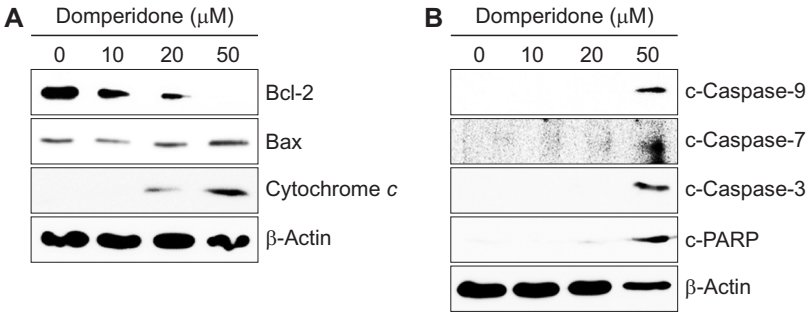

Figure 2. Effect of domperidone on the expression of apoptotic markers in Caki-2 cells. Caki-2 cells were treated with different concentrations of domperidone as indicated for 48 hours. (A) Immunoblot analysis of Bcl-2, Bax, and cytochrome *c* and (B) cleaved forms of caspase-3, -7, and -9 and PARP. β-Actin was used as a loading control.

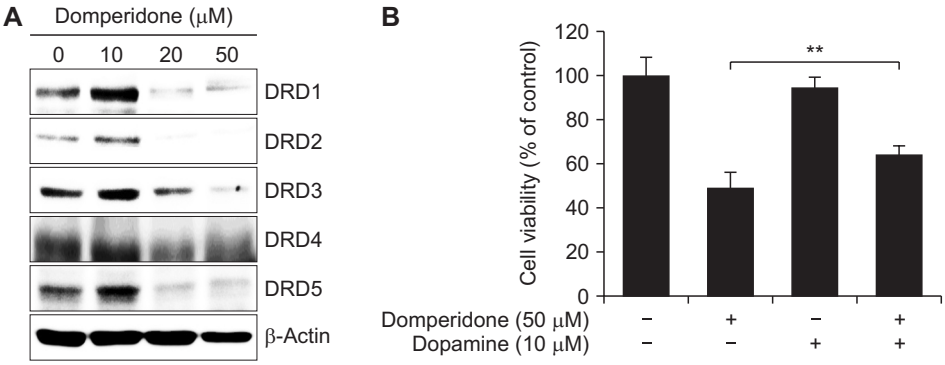

Figure 3. Involvement of dopamine receptors in domperidone-mediated cytotoxic effects. (A) Caki-2 cells were treated with domperidone (10, 20, 50 μM) for 48 hours and expression of dopamine receptors, DRD1, DRD2, DRD3, DRD4, and DRD5 was determined by immunoblot analysis. (B) Cells were pretreated with dopamine for 1 hour followed by treatment with domperidone. Viability of cells was assessed by the MTS method. Results are expressed as the mean ± SD (n = 3). $^{**}P < 0.01$ vs. domperidone alone. DR, dopamine receptor.

Domperidone-induced cytotoxicity is mediated through DRD2 in Caki-2 cells

The expression of DRD2 is abundantly higher in various types of cancer that is likely to affect diverse cellular processes including proliferation, apoptosis, and angiogenesis [18,21-23]. Our research demonstrates that domperidone treatment markedly downregulated the levels of DRD2 in Caki-2 cells (Fig. 3A). Furthermore, the expression of other types of dopamine receptors except DRD4 was also suppressed (Fig. 3A). As domperidone belongs to a class of DRD2 antagonists, we sought to uncover whether the cytotoxic effect of domperidone is umpired through DRD2. For this, we pretreated the cells with dopamine, the physiological agonist of dopamine receptors. The results demonstrate that co-treatment of domperidone with dopamine abrogated the cytotoxic effects of domperidone in Caki-2 cells as illustrated in Figure 3B.

Domperidone induces ROS generation in Caki-2 cells

Aberrant production of ROS by various anticancer agents has been linked to the induction of apoptosis in cancer cells [9,24]. Thus we determined the effect of domperidone on the generation of ROS in Caki-2 cells. Results from fluorescence microscopy show that domperidone treatment increased the production of ROS to a higher extent than control (Fig. 4A). In addition, we examined the ROS production by domperidone using fluorescence activated cell sorting (FACS) analysis. As shown in Figure 4B and 4C, FACS analysis also demonstrated a markedly increased generation of ROS in Caki-2 cells upon treatment of cells with domperidone at higher concentration.

Domperidone-induced ROS contributes to cell death in Caki-2 cells

To elucidate the crosstalk between domperidone-actuated ROS and cell death of Caki-2 cells, we pretreated the cells with NAC and measured the domperidone-mediated ROS production. We observed that the ROS produced by domperidone was abolished when the cells were co-treated with domperidone and NAC (Fig. 5A-5C). In addition, the cytotoxic effect of domperidone was reversed upon co-treatment with domperidone and NAC which suggests that domperidone-induced ROS production leads to the toxicity of cancer cells (Fig. 5D).

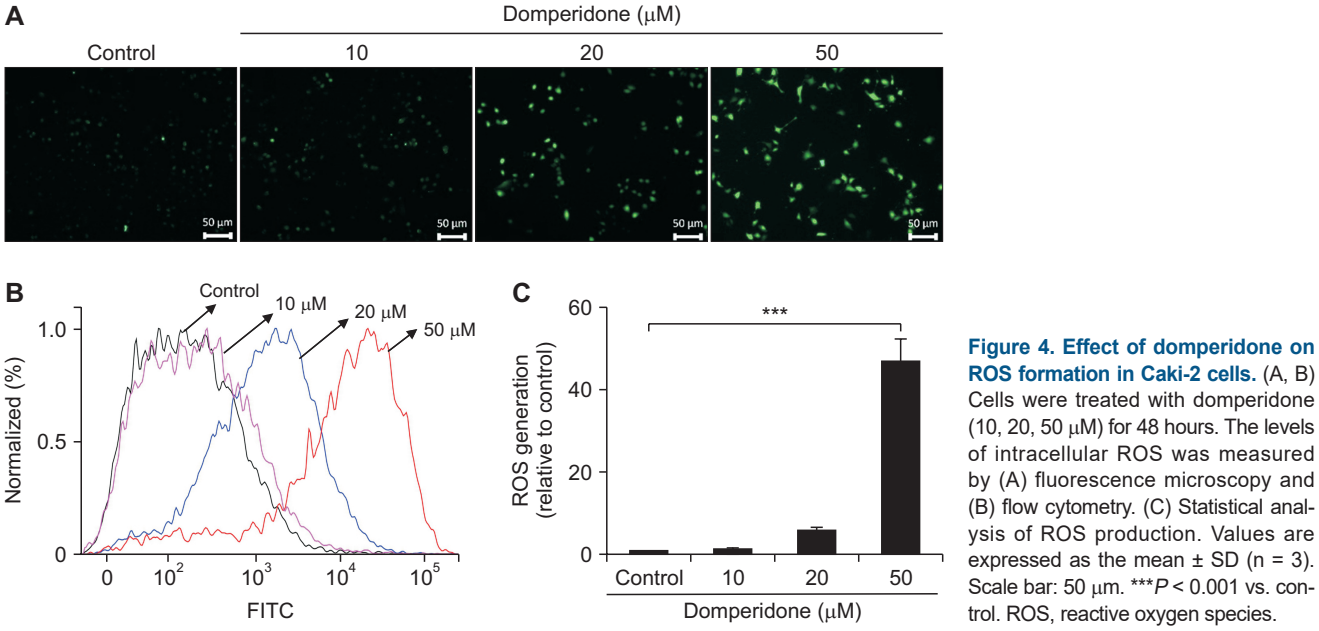

Figure 4. Effect of domperidone on ROS formation in Caki-2 cells. (A, B) Cells were treated with domperidone (10, 20, 50 μM) for 48 hours. The levels of intracellular ROS was measured by (A) fluorescence microscopy and (B) flow cytometry. (C) Statistical analysis of ROS production. Values are expressed as the mean ± SD (n = 3). Scale bar: 50 μm. ***P < 0.001 vs. control. ROS, reactive oxygen species.

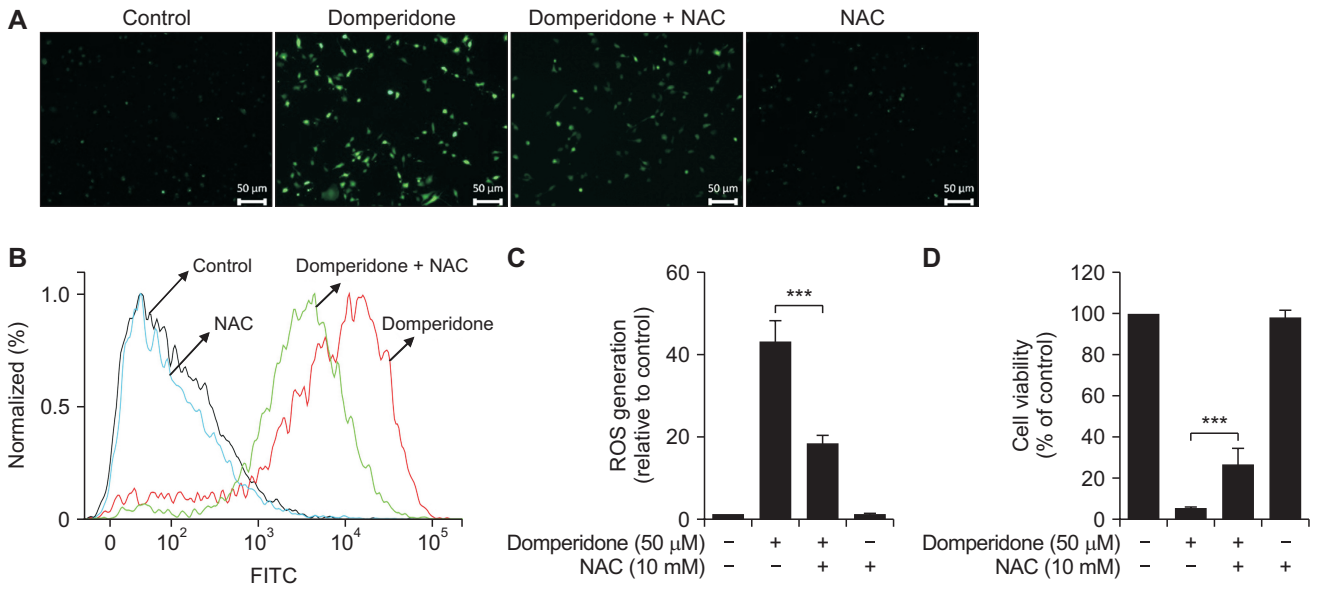

Figure 5. Role of domperidone-induced ROS in cell death of Caki-2 cells. Cells were treated with domperidone in the presence of NAC (10 mM) for 48 hours. The level of intracellular ROS was determined by (A) fluorescence microscopy and (B) flow cytometry. Scale bar: 50 μm. (C) Statistical analysis of ROS production. (D) Cell viability was determined using the MTS method. Data are expressed as the mean ± SD (n = 3). ***P < 0.001 vs. domperidone. ROS, reactive oxygen species; NAC, *N*-acetyl cysteine.

Domperidone downregulates STAT3 phosphorylation and expression of its gene products

Our results demonstrate that domperidone suppressed the phosphorylation of JAK2, an upstream signaling of STAT3 in Caki-2 cells. Furthermore, the activation of STAT3 at serine 727 and tyrosine 705 residues was inhibited by domperidone (Fig. 6A). Moreover, the expression of various STAT3-regulated genes i.e. cyclin D2, cyclin D3, and survivin was also suppressed when the cells were treated with domperidone (Fig. 6B).

DISCUSSION

Repurposing clinically approved drugs for the therapy of cancer represents an efficient approach to developing new chemotherapeutic agents [25,26]. Domperidone, a potent DRD2 antagonist, is used for the management of nausea and vom-

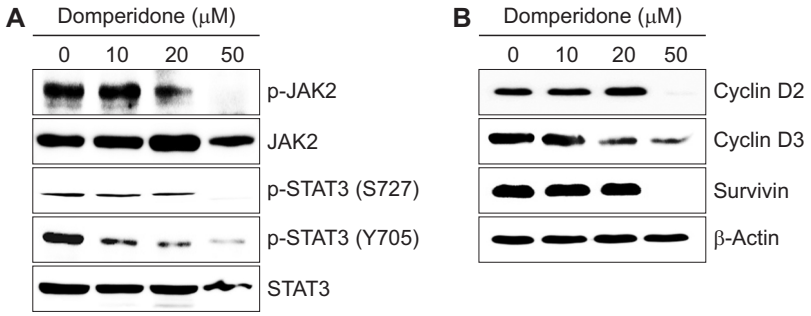

Figure 6. Effect of domperidone on modulation of STAT3 signaling pathway in Caki-2 cells. Cells were treated with domperidone (10, 20, 50 μM) for 48 hours. Western blot analysis for detecting (A) JAK2 and STAT3 phosphorylation and (B) cyclin D2, cyclin D3 and survivin expression. β-Actin was used as a loading control. JAK, Janus kinase.

iting. Results from interventional studies and some preclinical trials illuminate the crucial role of DRD2 in cancer progression [15,19], therefore, we designed and conducted this study to unravel the impact of domperidone in cancer cells. To the best of our knowledge, this is the first study reporting the antitumor effect of domperidone against renal cancer cells.

Our results indicate promising antitumor effects of domperidone in renal cancer Caki-2 cells as revealed by the dose-dependent and time-dependent cell cytotoxicity and marked increment in the percentage of apoptotic cells in response to domperidone. These outcomes resonate with the results of previous studies showing potential antitumor effects in breast cancer [19] and colorectal cancer cells [18] which illuminate the broad-spectrum activity of domperidone against cancer cells.

One of the key aspects mediating apoptosis is the rupture of the integrity of the mitochondrial membranous structure and the discharge of mitochondrial contents in the cytoplasm which is often regulated coordinately by the Bcl-2 family proteins such as Bcl-2 and Bax [27]. Thus, we determined the impact of domperidone on this phenomenon. The result demonstrates that domperidone suppressed the expression of Bcl-2 and upregulated the Bax expression which aids the translocation of cytochrome *c* from mitochondria to the cytoplasm thereby fueling caspase cascade and contributing to PARP damage. This provides compelling insights into the initiation of intrinsic apoptosis by domperidone in Caki-2 cells.

A close association between DRD2 expression and tumorigenesis has been elucidated by various research studies suggesting its oncogenic nature [15]. In our study, we found that domperidone suppressed the expression levels of DRD2. This decrease in DRD2 levels stirs a debate whether the anticancer effect of domperidone is mediated through its antagonistic effect or downregulation of DRD2 expression levels. Interestingly, pretreatment with dopamine to saturate DRD2 receptors on cancer cells, followed by domperidone treatment, led to a reduction in the cytotoxic effects of domperidone. This suggests that domperidone's cytotoxicity may occur as a result of its antagonistic effects against dopamine receptors. Cancer cells may show plastic behavior in response to drug-induced stress leading to adaptive response to chemotherapeutic agents [28]. In this study, we observed that a low dose of domperidone showed an increase in the expression of dopamine receptors in contrast to the suppressive effect at higher doses. This could be attributed to the plastic behavior of renal cancer cells in response to domperidone at lower dose to compensate the dopaminergic signaling which is overwhelmed at higher toxic doses.

Although, domperidone is well known as a DRD2 antagonist, surprisingly the expression of other subclass of dopamine receptors was decreased upon domperidone treatment. In contrast, a previous study from our lab demonstrated that domperidone treatment selectively decreased the expression of DRD2 in colorectal cancer HCT116 cells [18]. Based on this, it can be speculated that the mechanistic insights of domperidone at the molecular level may differ depending on the cancer type which underscores the necessity for further investigation to delve into the underlying mechanism for direct regulation of various dopamine receptor expression by domperidone in Caki-2 cells.

It is suggested that aberrant formation of ROS in cancer cells triggers apoptotic cell death by disrupting various cell survival and proliferation pathways in renal cancer Caki-1 cells [4]. Herein, domperidone treatment resulted in marked induction of ROS in Caki-2 cells which was declined in co-treatment with NAC, a ROS scavenger. In addition, combinatorial treatment of NAC and domperidone rescued the cells from domperidone-induced cytotoxicity suggesting the central role of ROS in cell death. Recently, it has been reported that the domperidone-mediated anticancer effect in triple-negative breast cancer cells relies on the excessive formation of ROS which aligns with the results of our study [19]. However, we did not investigate the sources of ROS production triggered by domperidone which requires further research work.

STAT3 participates in the growth, development, metastasis, and angiogenesis of various types of tumors including renal cancer [29]. Apart from this, it has been reported to modulate antitumor immunity causing immunosuppressive tumor microenvironment [29,30]. Moreover, a recent study indicates the involvement of STAT3 signaling in the develop-

ment of resistance to the available therapies for renal cancer [31]. Thus, STAT3 is regarded as an interesting target for the effective therapy of renal cancer. STAT3 activation leads to the formation of dimer and movement to the nucleus where it plays a pivotal role in the transcription of various genes priming cell survival and proliferation [9]. The downregulation of STAT3 phosphorylation and expression of cyclins and survivin by domperidone indicate its potential to halt the growth and survival of renal cancer cells. Besides STAT3 inhibition, we found that domperidone suppressed the phosphorylation of various other oncogenic targets including JNK, extracellular signal-regulated kinase, p38 MAP kinase, Akt, EGFR, and phosphatase and tensin homolog in Caki-2 cells which uncovers the multifaceted roles of domperidone in mediating its anticancer effects (Figure S1).

One of the major concerns for effective therapy of renal cancer is metastasis. Metastatic renal cancer is often associated with poor prognosis and offers a great deal of challenges for successful clinical outcomes [32]. Matrix metalloproteinase 9 (MMP 9) is the major driver of extracellular matrix degradation and progression of metastasis [33,34]. Herein, domperidone profoundly suppressed the expression of MMP 9 and its activity (Figure S2). In addition, we observed marked inhibition of migration of Caki-2 cells as indicated by migration and wound healing assay (Figure S2).

In conclusion, our study demonstrates the antitumor property of domperidone against renal cancer Caki-2 cells. These findings underscore the potential of domperidone as a repurposed anticancer agent for the treatment of renal cancer which requires further research work.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.24.032.

ORCID

Geumi Park, https://orcid.org/0009-0000-8993-2725
Manoj Kumar Baniya, https://orcid.org/0009-0004-9316-9434
Eun-Jeong Cha, https://orcid.org/0009-0003-2024-3069
So Jin Sim, https://orcid.org/0009-0006-2413-8153
Joon-Seok Choi, https://orcid.org/0000-0002-9569-8055
Kyung-Soo Chun, https://orcid.org/0000-0001-9933-2897

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