Original Article

Docetaxel Enhances the Expression of STING Protein in PC3 Cells, and cGAMP Attenuates this Effect

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Abstract

Background: The stimulator of interferon genes (STING) agonist (cGAMP) kills the cancer cells through the activation of the innate immune system. PC3 cells are high in BTK and low in STING. In this study, the effect of adding STING agonist, cGAMP, to docetaxel investigated.

Materials and Methods: PC3 cells were treated with docetaxel, cGAMP, and a combination of the docetaxel and cGAMP. Cell toxicity was evaluated by MTT assay, and changes of *STING*, *IRF3*, *BTK*, and *DDX41* genes' expression were quantified by the real-time PCR. STING protein was also detected by Western blotting.

Results: The IC_{50} of docetaxel was 31.1 nM, and cGAMP did not change it significantly but decreased docetaxel toxicity about 30%. Docetaxel increased *IRF3*, *BTK*, and *DDX41* gene expression significantly, and STING protein about 5 folds. By adding cGAMP to docetaxel *STING*, *IRF3*, and *BTK*, expression decreased several folds.

Conclusion: In this in vitro study, cGAMP potentiated docetaxel's effects and alleviated it.

Keywords: Docetaxel; cGAMP; PC3; Interferon; STING; Prostate Cancer

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Introduction

Since 2004, docetaxel has considered as first-line drug that use for treatment of metastatic castration-resistant prostate cancer (MCRPC). It is effective but little increases the survival. The disease relapses after docetaxel chemotherapy was seen and more treatment is required[1, 2].

DNA leakage from the host cell nucleus or mitochondria into the cytoplasm in pathological conditions like cancer activates the innate immune system. This activation occurs by binding to aberrant double-strand DNA (dsDNA) leaked from the host into the cytosol, cyclic GMP-AMP synthase (cGAS) makes a molecule called cyclic GMP-AMP (cGAMP)[3, 4]. By attaching a cytosolic protein called the stimulator of interferon genes (STING), cGAMP starts an intrinsic immune response. STING, which is based in the cytosol, is an important receptor in the pathway for sensing cytosolic DNA[5, 6]. STING detects cGAMP and activates cytosolic kinase TKB1. Activated TKB1 binds to IRF3 and phosphorylates this transcription factor. This transcription factor enters the nucleus and produces type I interferons[7, 8]. As a cytosol-based protein, STING plays a key role in stimulating the secretion of interferon. Therefore, the use of this protein

agonist (cGAMP) can activate immune response against cancer cells[9-19].

Many clinical trials have been conducted to improve the effectiveness of docetaxel in the treatment of MCRPC. Docetaxel has been tested in combination with various anticancer drugs, such as tyrosine kinase inhibitors (dasatinib), zebras (Zoledronic acid), and some experimental drugs such as atrasentan. However, clinical trials for the combination of docetaxel with other drugs failed[20-22]. Due to the lack of effective treatment for castration-resistant prostate cancer (CRPC), there is a pressing need for a new and effective therapeutic approach.

There are many studies that used the combination of cGAMP with other anticancer drugs or cancer immunotherapy, and they found that these combinations potentiate the anticancer therapy[23, 24]. In a study by *Tiejun Li* on mice, it was observed that cGAMP improved the anti-tumor activity and clearly increased the in vivo toxicity of 5-FU. These results indicated that cGAMP is a new anti-tumor agent and has potential implications for cancer immunotherapy[24]. There has been no report on the effects of docetaxel with cGAMP on prostate cancer, and the current study was the first that may illustrate the synergistic effect of both drugs. Therefore, the importance of cGAMP in inhibiting the growth of cancer cells and the role of this agonist as an enhancer of the anti-cancer effect of docetaxel have been addressed in this in vitro study.

Methods

Cell culture:

The PC3 cell line was purchased from the Iranian cell bank based at Iran's Pasteur Institute. Cells were grown in RPMI containing 10% FBS in the presence of one cc penicillin/streptomycin (100 µg/ml) in a humidified incubator with 5% CO2 at 37 °C. When the cells reached the confluence, they were seeded and used for experiments in their growth phase.

Cytotoxicity assay:

Cell toxicity was measured using the method of MTT (3- [4,5 -dimethyl-2-thiazolyl] -2,5-diphenyl tetrazolium bromide), a yellow water-soluble salt. A total of 10^4 cells per well were seeded in a 96-well microplate. After 24 hours of incubation, cells were exposed to docetaxel for 48 hours at concentrations of 7.5, 15, 25, 37 and 75 nM, cGAMP at concentrations of 2.2, 4.4, 8.75, 17.5 and 35 µM, or the combination of 35 µM of cGAMP and the above-mentioned docetaxel concentrations. After 48h incubation, the cell suspension was washed with PBS. Then, the prepared MTT solution (5mg MTT/1ml PBS) was added to each well. After 4 hours of incubation, formazan crystals were formed at 37 °C and 200 µl DMSO was added to each well in order to dissolve the purple crystals. After 20 minutes of shaking, ELISA reader at 570 and 630 nm, respectively measured the absorbance of the samples. The Graph Pad Prism software calculated the IC_{50} and the IC_5 was selected in order to use the nontoxic concentrations of these two compounds to study the signaling pathway without interfering with the cell numbers.

Gene expression study:

PC3's mRNAs were extracted using a Biobasic extraction kit after the cells were treated with docetaxel 3.5 nm (IC_5), cGAMP 1. 1 µM (IC_5), or a combination of the two for 48 hours. The total concentration of mRNA and its quality were measured by the absorption ratio at 280/290 nm using a Nanodrop spectrophotometer (Allshang). Subsequently, the cDNA of the extracted RNA was synthesized using a kit (Takara Bio Inc., Japan) and 1 µg total RNA. Then, the cDNA was amplified by a real-time quantitative polymerase chain reaction (RT qPCR) using the SYBR® Green PCR (Ampliqon) master mix and primers designed at the NCBI site and synthesized by Bioneer Co, South Korea. *β-actin* primers as housekeeping genes were: (Forward: CACACAGGAGAGGTGATAGCAAGT) (Reverse: GACCAAAAGCCTTCATACATCTCA); *STING*: (Forward: TGTCATCTGCAGGTTCCTGGT) (Reverse: GCCATGTCACAATACAGTCAAGC); *DDX41*(Forward: GCCCTCAGTCCAACGTCAG) (Reverse: CACTGACATCAATGCTCGGC), *BTK*: (Forward: GCTCAAAAACGTAATCCGGTACA) (Reverse: GTCTTCCGGTGAGAACTCCC), and *IRF3*: (Forward: CGACCTTCCATCGTAGGAGTT) (Reverse: TGGGGCCAACACCATGTTA).

The temperature profile in the real-time PCR reaction was an initial denaturation at 95 °C for 10 minutes and then included a three-step program for 40 cycles, which included 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 25 seconds.

Western blot:
Cells were lysed on ice by RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% v/v Triton X100, 0.5% v/v sodium deoxycholate, 1 mM EDTA, 0.1% v/v sodium azide, 50 mM NaF, 0.1% sodium dodecyl sulfate [SDS], and 1 mM PMSF)[21]. The concentration of extracted proteins was measured by the Bradford method. Proteins were mixed with an equal volume of loading buffer (0.125 M Tris-HCl with pH 6.8, 4% of SDS, 20% glycine, and 10% of 2-mercaptoethanol). The mixture was heated to 96 °C for 5 minutes. The denatured proteins were separated on 10% SDS-polyacrylamide gel by a Bio-Rad electrophoresis apparatus. The proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane by a Bio-Rad Mini Trans-Blot device. PVDF was placed overnight in a blocking solution (skim milk powder 5% w/v in TBS-Tween buffer containing 10 mM Tris, 100 mM NaCl, and 0.1 mM Tween-20 pH 7.4) at 4 °C. The membrane was then washed with a TBS-Tween buffer solution 3 times for 10 minutes. Then it was incubated for 3 hours in the original antibody (TMEM 173) or in beta-actin as housekeeping. Then, the membrane was washed for 10 minutes 3 times with TBS-Tween buffer solution. Subsequently, it was incubated for 2 hours at room temperature with anti-rabbit IgG secondary antibody. After washing the membrane 3 times with TBS-Tween buffer solution, the bands were detected in the darkroom by ECL. The density of the spots darkness was analyzed by ImageJ software so we reported the quantitative protein expression.
Statistical analysis was performed by Graph pad Prism software for IC_5 & IC_{50} calculations and REST software for gene expression analysis.

Results

Cytotoxicity study:
The PC3 cells were treated with 2.2, 4.4, 8.75, 17.5, and 35 µM cGAMP for 48 hours, and their IC_{50} was 11.46 µM (CI 95%: 7.0 – 18.8) (Figure 1B). The cells were treated with docetaxel at 7.5, 15, 25, 37, and 75 nM for 48 hours, and the IC_{50} value was 31.1 nM (CI 95%: 20.8 – 46.6) (Figure 1A). The IC_{50} of docetaxel at the above-mentioned concentrations in the presence of 35 µM cGAMP did not change significantly [33.8 nM (CI 95%: 10.7 – 107.3)] (Figure 1A). The addition of cGAMP to the docetaxel caused an increase in the toxicity of the lower concentrations of docetaxel and reduced the toxicity of the higher concentrations of docetaxel, and the ceiling of the toxicity effects of the highest docetaxel concentration dropped from about 90% to about 60% (Figure 1A).

Gene expression:
The PC3 cell line was treated with the IC_5 concentrations of docetaxel (3.75 nM), cGAMP (1.1 µm), or their combination (cGAMP+docetaxel). All treatments reduced *STING* gene expression significantly, and in combination, they showed an additive effect (Figure 2). Docetaxel increased *IRF3* expression, but cGAMP decreased it significantly so cGAMP+docetaxel did not change it (Figure 2). *DDX41* expression was increased by docetaxel treatment significantly, but cGAMP and cGAMP+docetaxel did not change it (Figure2). While cGAMP caused a significant reduction in *BTK* gene expression, other treatments did not show any change (Figure2).

STING protein expression:
In the western blot analysis, the beta-actin protein was used as a housekeeping protein, and STING protein expression with a molecular weight of 43 kDa was investigated by anti-STING antibody TMEM173. The results showed that STING protein expression increased in docetaxel treated cells (Figure 3).

Discussion

In this study, the effects of cGAMP, docetaxel and their combination on the growth of the prostate cancer cell line (PC3) investigated. While the IC_{50} of docetaxel was 31.1 nM, the addition of cGAMP did not change it (33.8 nM) significantly. While cGAMP reduced the effect of the highest toxic concentration of docetaxel by about 30%, it potentiated the toxicity of its lower concentrations. While all treatments reduced the *STING* gene expression, its protein was higher than control in docetaxel treated cells. Except for *STING*, docetaxel treated cells showed an increase in *IRF3*, *BTK*, and *DDX41* gene expression. In addition to *STING*, *IRF3* and *BTK* gene expression was suppressed several folds in cells treated by cGAMP. Therefore, docetaxel in

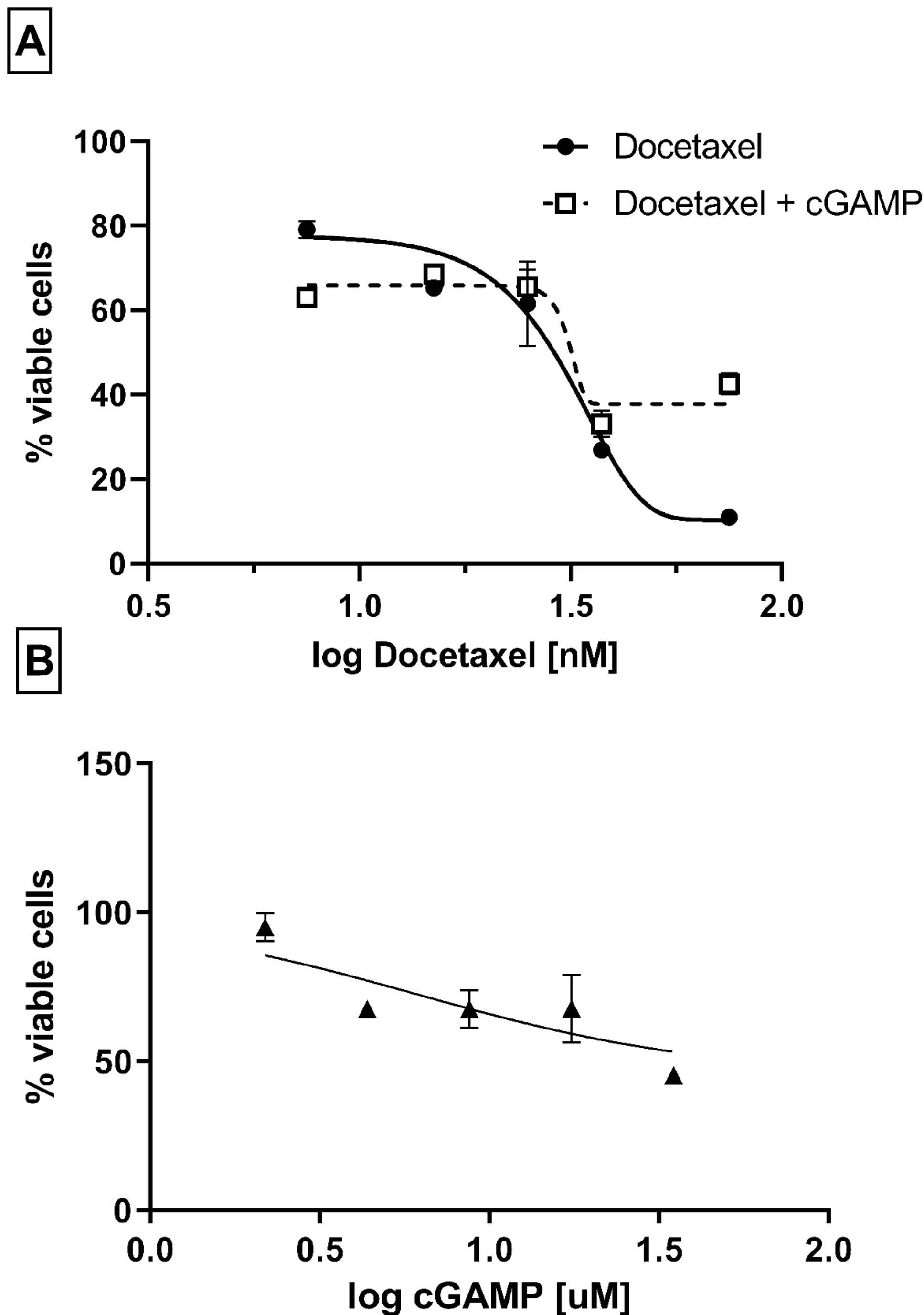

Figure 1. Cell survival curve after treatment by 48 hours with 7.5, 15, 25, 37, and 75 nM docetaxel (A), 35, 17.5, 8.75, 4.375, and 2.187 µM cGAMP (B), and 7.5, 15, 25, 37, and 75 nM docetaxel in the presence of 35 µM cGAMP (A).

combination with cGAMP lost its positive inducible effect on *IRF3*, *BTK*, and *DDX41* gene expression.
STING is a transmembrane protein that attaches to the endoplasmic reticulum system and has 379 amino acids. It is also one of the most important DNA sensor that is directly stimulated by the cyclic di-nucleotides (CDNs) produced by DNA viruses and bacteria[25-27]. STING is actually a cytosolic DNA sensor. STING is activated by cGAMP produced by cGAS or by other DNA sensors DAI, IFI16, DDX41 after detection of

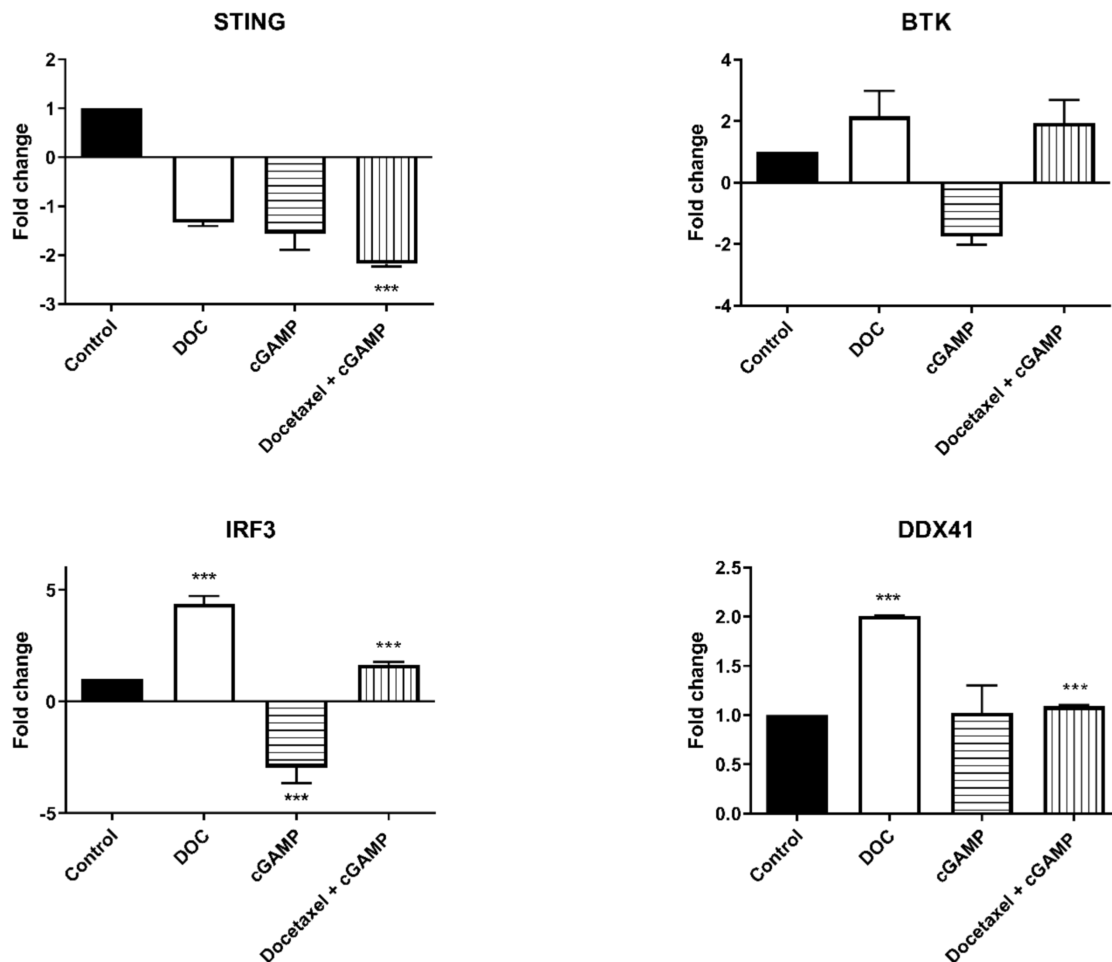

Figure 2. STING, IRF3, BTK, and DDX41 gene expression in docetaxel, cGAMP, and docetaxel + cGAMP treated PC3 cell line. Gene expression related to the beta actin housekeeping gene was calculated by the REST software program. Statistical significance (* $p < 0.05$, ** $p < 0.01$) was calculated to the control group.

DNA in the cytoplasm[28]. The activation of STING triggers the production of INF1 and inflammatory cytokines. Interferons can directly activate the apoptosis in cancerous cell lines in vitro[29]. It is well-known that dimerization, translocation, phosphorylation, and degradation are all STING activation and signaling hallmarks[30, 31].

Low concentration of c-di- GMP (0.01 nmol) provided strong effects as adjuvant therapy in 4T1 cancer cells and at high concentrations (15-150 nmol) activate caspase 3 and killed tumor cells directly [32]. In their study, Xiangshi et al. found that cGAMP injections into node mice, which had been subcutaneously implanted with human tumor cells line (MNK-45 human stomach, A549 human lung adenocarcinoma, luv colorectal carcinoma, SMV-7721 hepatocellular carcinoma, PC3 prostate carcinoma, and SW1990 pancreatic carcinoma), had an acute toxicity effect, and it can be used as an anti-tumor drug. In that study, a serum concentration of 4 mg/ml was achieved with the administration of 10 mg/kg and 40 mg/kg cGAMP [33]. High concentrations of c-di-GMP alone can reduce tumor growth, partly due to the expression of Caspase 3. Activating STING directly induces cell apoptosis and autophagy in cancer cells[34, 35]. In our study 2- 35 µM concentrations of cGAMP showed cytotoxicity, and its IC_{50} (11.46 µM) was about 500 fold weaker than Xiangshi's study, and boosted lower concentrations docetaxel cytotoxicity, but reduced high concentration docetaxel cytotoxicity effect (MTT

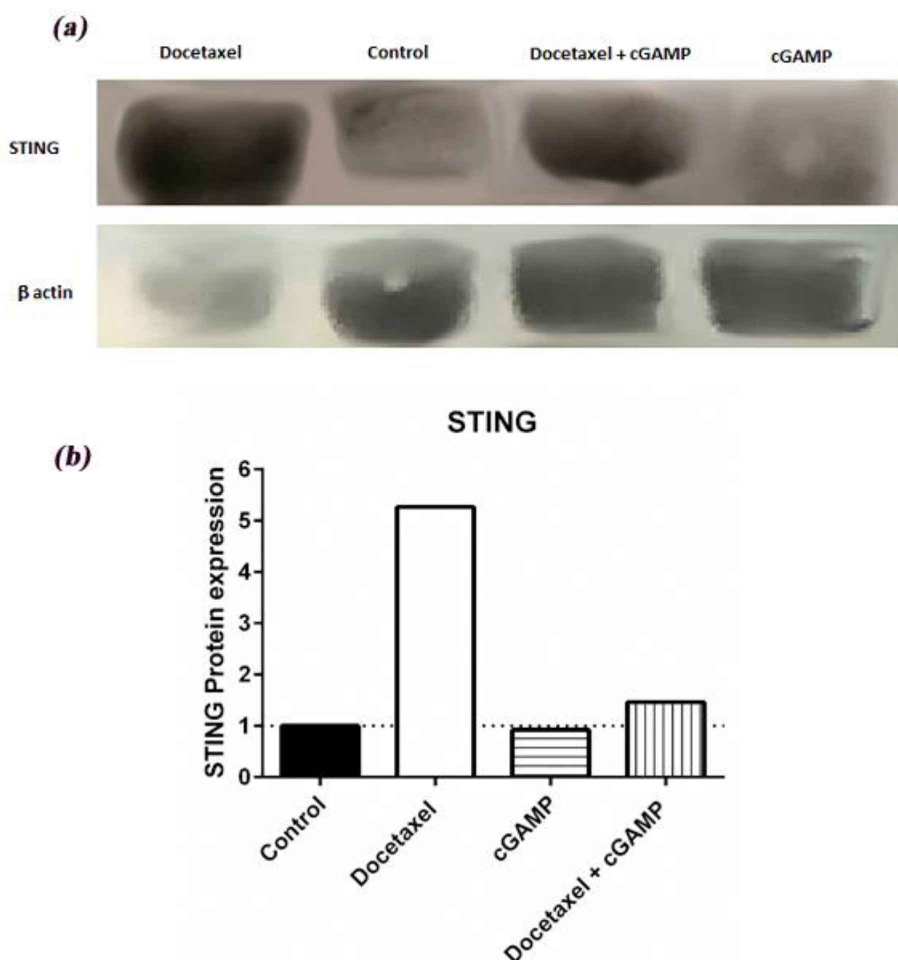

Figure 3. (a) Photograph of STING protein assayed by Western blot method. (b) The chart represents STING protein expression compared to housekeeping beta-actin protein.

assay).

CDNs increase the efficacy of several cancer treatments, including vaccines, CD47 blockage, and 5-FU [36]. Chemotherapy agents and radiotherapy can activate the cGAS-STING pathway and enhance the antitumor response of the immune system by inducing DNA damage. For example, cisplatin and etoposide cause cGAS STING induction through DNA damage and its cytosolic leakage [37].

In a review article, Ng stated that there is strong evidence that DNA damage and leakage to the cytosol have profound effects on the innate immune response through the activation of the STING pathway. In vivo studies on mice have shown that STING agonists can potentiate anti-tumor treatment [38]. In this study, we showed that STING protein translation, and *IRF3*, *BTK*, and *DDX41* gene expression was significantly abolished after combining cGAMP to docetaxel.

BTK, which mainly reported in the hematopoietic cells and B cell malignancies, is aberrantly highly expressed in the PC3 cell line and there is a correlation between its expression and prostate cancer grade[39]. Indeed, the kinase domain of BTK binds to DDX41 at the DEAD-box domain, and phosphorylate it at Tyr414 boosting dsDNA attachment to DDX41 and this complex recruited and binds to the transmembrane region of STING and activates TBK/IRF3 pathway[40]. Our results showed that BTK, which is not involved in the cGAS-STING pathway, was significantly reduced by cGAMP treatment. While DDX41 was not affected.

The inhibitory effect of STING activation on tumorigenesis of prostate, colorectal, and melanoma

cancers has been proven[11, 14, 17]. STING expression has been reduced in CRC, melanoma, hepatic carcinoma (HCC), breast cancer, and gastric cancer [41]. In a clinical study conducted by *He et al.*, the expression of the STING protein was lower in prostate cancer cells than in the control group [41]. An increase in *STING* expression in MCF-7 breast cancer was shown to reduce cell survival. Increasing the expression of *STING* in the MCF-7 cell line decreased survival due to increased NF-kB induction and caspase-8-mediated cell death [42]. A defect in STING can help tumors escape from the immune system, and mutation or deletion in the STING coding genome can indicate a defect upstream of the STING pathway. In the study by *He et al.*, the expression of *STING* in hepatocyte carcinoma was decreased compared with the control group. Lower *STING* expression is associated with higher disease stages and less patient survival. In a study by *Song* et al., *STING* expression was significantly lower in tumor tissue than in healthy tissue, and the severity of its decline was directly correlated to tumor mass, tumor penetration depth, lymphoid metastasis, TNM, and loss of survival [43]. In *in vitro* studies, it has been observed that STING destruction results in the proliferation, invasion, and migration of cancer cells. Finally, STING can be considered as a potential factor in gastric cancer immunotherapy [43]. In our study docetaxel increased STING level by 5 fold. Although its gene expression was decreased. STING turnover is controlled by numerous mechanisms, and this effect may be due to STING decay prevention.

A study by *Bose* indicated that the STING/cGAMP pathway acts like a 2-edged sword in cancer; knowing the molecular details and adjusting the time and place in this pathway is very important. STING activators play a key role in this area. While the optimal cDNA concentration contributes to the diagnosis of the immune response to the tumor, the high level of cDNA after uncontrolled activation of STING can itself cause inflammation and carcinogenesis. Therefore, determining the optimal concentration of STING agonist in cancer has an effective therapeutic effect[44]. Several studies have suggested that STING activation can induce some tumor types[45, 46]. The complex role of STING may be related to the type of cell and the intensity of the downstream signals and the over-activation of STING may give the reverse result[41].

The regulation of *STING* expression has been studied. Although cGAS and STING expression is low in the basal state, their type I IFN product increases the expression of these two proteins greatly by a positive feedback[47-49]. In contrast, treatment with dsDNA and cGAMP has been reported to reduce STING protein expression in gastric cancer cells[43], probably due to the STING negative feedback control[50]. Moreover, 2 ', 3'-cGAMP affects AMPK and induces negative feedback on STING production through ULK1[50]. ULK1 kinase, which phosphorylates STING in S366, as well as an E3 ubiquitin ligase, TRIM30a, and RNF5, degrade STING and are considered as negative regulators of STING[50-52]. The poly ubiquitination of STING attached to K48 by RNF5[52] and TRIM30a[51] degrades them by negative feedback control. Not only is STING stimulated by 2', 3'-cGAMP, but it also plays a key role in facilitating clearance of 2', 3'-cGAMP [53]. We reported in this study that, cGAMP not only reduced STING gene expression, but also reduced IRF3 and BTK gene expression too.

Conclusion

According to the current *in vitro* study, the addition of cGAMP to docetaxel deteriorated docetaxel cytotoxicity effects and reduced its effect on the STING activation pathway, which may be caused by the cGAMP negative feedback on STING pathway components.

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