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Cosmomycin C inhibits signal transducer and activator of transcription 3 (STAT3) pathways in MDA-MB-468 breast cancer cell

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ABSTRACT

The signal transducer and activator of transcription 3 (STAT3) is constitutively activated in cancer cells. Therefore, blocking the aberrant activity of STAT3 in tumor cells is a validated therapeutic strategy. To discover novel inhibitors of STAT3 activity, we screened against microbial natural products using a dual-luciferase assay. Using the microbial metabolome library, we identified cosmomycin C (CosC), which was isolated from the mycelium extract of *Streptomyces* sp. KCTC19769, as a STAT3 pathway inhibitor. CosC inhibited STAT3 (Tyr705) phosphorylation and subsequent nuclear translocation in MDA-MB-468 breast cancer cells. CosC-mediated inhibition of STAT3 signaling pathway was confirmed by suppressed expression of STAT3 downstream target proteins including cyclin D1, Bcl-xL, survivin, Mcl-1, and VEGF in CosC-treated MDA-MB-468 cells. Flow cytometry showed that CosC caused accumulation in the G_0–G_1 phase of the cell cycle and induced apoptosis via PARP cleavage and caspase-3 activation. Based on these findings, CosC may be a potential candidate for modulation of STAT3 pathway.

1. Introduction

Signal transducer and activator of transcription proteins (STATs) are a family of latent cytoplasmic transcription factors that are activated by extracellular stimuli such as cytokines and growth factors to promote proliferation, survival, and other biological processes.[1,2] Seven different members of the STATs (STAT1, 2, 3, 4, 5a, 5b, and 6) are over-expressed in various cancer cells.[1] STAT3 is activated in human solid and blood tumors. STAT3 activated by upstream regulators such as cytokine receptors and growth factors receptors, and Janus kinases (JAKs) family proteins contributes to carcinogenesis and tumor progression.[2,3] In many human tumors, aberrant STAT3 activation is required for their growth and survival. Once the cytokine or growth factor is bound to its receptor on the cell surface, the receptors are phosphorylated at the critical tyrosine residue Tyr705 by JAKs, Src-family kinases, and Rac1. STAT3 is then recruited through its SH2 domain.[4,5] Previous studies have described that STAT3 tyrosine phosphorylation in breast cancer cells is mediated by the interleukin (IL)-6/gp130/JAK pathway.[6] Tyrosine phosphorylation of STAT3 causes homo- or hetero-dimerization of STAT3 via phosphotyrosine-SH2 interaction followed by translocation to the nucleus and binding to DNA.[3] Whereas Tyr705 phosphorylation of STAT3 regulates dimerization, translocation, and DNA binding, phosphorylation of STAT3 on Ser727 enhances its transcriptional regulatory activities.[7] STAT3 activation contributes to oncogenesis by regulating specific target genes such as antiapoptotic genes (Bcl-xL, Bcl-2, and Mcl-1), proliferation regulatory genes (cyclin D1, survivin), and inducer gene of angiogenesis (VEGF).[8–10] Constitutively activated STAT3 is implicated in breast cancer progression or prognosis.[11,12]

Because STAT3 plays a crucial role in cancer cell survival and inhibition of STAT3 expression or STAT3 pathways induces tumor cell apoptosis, STAT3 is a potential molecular target for cancer treatment.[13,14] During a search for STAT3 signaling pathway modulators from natural sources, we previously reported that cryptotanshinone specifically inhibits STAT3 activity by blocking the formation of STAT3 dimers.[15] In the current study, we screened approximately 1000 microbial methanol extracts of *Streptomyces* sp., myxobacteria, and bacillus to identify STAT3 modulators using a dual-luciferase assay of STAT3 target. We found that cosmomycin derivatives, which were isolated from novel marine *Streptomyces* sp. KCTC19769, inhibited the phosphorylation of STAT3. Many natural products that are produced by streptomyces are a significant source of anticancer chemotherapeutic agents.[16] Anthracyclines such as daunorubicin, idarubicin, and doxorubicin significantly decrease hypoxia-induced luciferase activity and inhibit HIF-1

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transcriptional activity in human prostate cancer xenografts and tumor growth.[16,17] A recent investigation has shown that mythramycin, which bind to the minor groove of DNA, has anti-cancer effects by inhibiting mRNA and protein expression of Ewing sarcoma breakpoint region 1 and Friend leukemia virus integration 1 (EWS-FLI1) downstream targets. Mythramycin also specifically inhibit the growth of Ewing sarcoma family of tumor (ESFT) cells in vitro and in vivo.[18] Anthracyclines contain one or more sugar moieties, which play a critical role in the biological activity of anthracycline drugs.[19] Cosmomycins are one type of anthracyclines that are isolated from the extracts of *Streptomyces cosmosus*. Cosmomycins are interesting compounds because they have one of the most complex glycosylation patterns that are found in anthracyclines.[20,21] CosC has two trisaccharide chains that are attached at the 7- and 10-position of the aglycone. Cosmomycins possess DNA-binding properties and cytotoxicity against MCF-7 breast cancer cells.[22–24] However, little is known regarding the mode of actions that cosmomycins could modulate signaling pathways in tumor cells. In this report, we studied the effects of CosC on STAT3 signaling pathways in MDA-MB-468 breast cancer cells.

2. Results and discussion

The goal of this study was to identify inhibitors of the STAT3 signaling pathway. We conducted a primary screen of 1000 microbial extracts from the MICROBANK in Korea (www.microbank.re.kr) using a dual-luciferase assay system to measure changes in STAT3 activity.[15]

We investigated the effect of CosC on constitutive STAT3 activation in breast cancer cells. We also evaluated the effect of CosC on various mediators of cellular proliferation, cell survival, and apoptosis.

2.1. Inhibition of STAT3-dependent luciferase activity by CosC

We found that the methanol extracts from *Steptomyces* sp. KCTC19769 cultures strongly inhibited STAT3-dependent luciferase activities. The active compound was isolated based on the activity guided fractionations. The compound was identified as CosC by NMR and mass spectroscopy (Fig. 1A).[20] CosC inhibited STAT3 luciferase activity in a dose dependent manner (Fig. 1B). Previous studies have shown that the deoxysugars that are attached to the aglycone regulate the biological activities of cosmomycins because they bind to DNA through interactions between sugar moieties and the DNA minor groove.[23,24] The deoxysugars also play a key role in STAT3-dependent luciferase activities. CosC strongly inhibited the STAT3-dependent dual-luciferase activity. However, CosB with one trisaccharide chain at the 10-position of aglycone (Fig. 1A) and mithramycin with two saccharide chains did not inhibit STAT3 activity (data not shown). CosB was used as a negative control in this study.

2.2. Inhibition of STAT3-activated cancer cells growth by CosC

To examine whether the antitumor effects of cosmomycins mediate STAT3 activity, the expression level and activation status (phosphorylation of STAT3 Tyr705 and Ser727) of STAT3 were evaluated in various human cancer cell lines such as colon (HCT-116), prostate (DU145), and breast (MCF-7, MDA-MB-231, and MDA-MB-468) cancer cells. Among these cancer cell lines, STAT3 protein was highly activated in DU145 and MDA-MB-468 cancer cells, which was confirmed by increased phosphorylation levels of STAT Tyr705 and Ser727 (Fig. 2A). We previously showed that the DU145, MDA-MB-468, MDA-MB-231, and HeLa cell lines display high levels of phosphorylated STAT3.[15] To evaluate the relationship between the antitumor effects of cosmomycins and STAT3 activity in colon, prostate, and breast cancer cell lines, we treated a panel of cell lines for 24 h with concentrations of cosmomycins that ranged from 10 to 500 nM and measured cell viability. As shown in Figure 2B, CosC is a potent cytotoxic agent in a panel of STAT3 activated cell lines. CosC exhibited a dose-dependent inhibition of DU145 and MDA-MB-468 cell growth, and MDA-MB-468 cells were more sensitive to CosC than DU145 cells. The IC_{50} value of CosC in MDA-MB-468 cells was 210 nM (Fig. 2B). MCF7 and HCT116 cells, which lack STAT3 activity, were less sensitive to CosC treatment. However, CosB, which did not affect the STAT3-dependent luciferase activity, did not inhibit the growth of the tumor cells (data not shown). It is notable that MDA-MB-468 breast and DU145 prostate cancer cells with constitutively activated STAT3 were sensitive to CosC. And it was already reported that STA-21, STAT3 inhibitor, strongly induced apoptosis in MDA-MB-468 cells.[13] However, when siRNA of STAT3 was treated in MDA-MB-468 cells, the growth of the cells very mildly inhibited (data not shown). These results suggest that the antitumor effects of CosC against MDA-MB-468 cells are due to the modulation of STAT3 signaling pathways and/or regulation of the activity of other target molecules. Therefore, additional experiments are needed to test this hypothesis.

2.3. CosC inhibits STAT3 phosphorylation on Tyr705 in MDA-MB-468 cells

The ability of CosC to modulate constitutive STAT3 activation in MDA-MB-468 cells was investigated. The breast cancer cells were incubated with different concentrations of CosC for 24 h. Whole cell extracts were prepared and the phosphorylation of STAT3 was examined using Western blot analysis with antibodies that recognize STAT3 phosphorylation at Tyr 705 and Ser 727. As shown in Figure 3A and B, CosC selectively inhibited the phosphorylation of STAT3 Tyr705 in MDA-MB-468 cells in a dose-dependent manner. When MDA-MB-468 cells were treated with 500 nM of CosC for 24 h, STAT3 Tyr705 phosphorylation was completely inhibited. However, phosphorylation of STAT3 Ser727 was slightly decreased (Fig. 3C). The total amount of STAT3 protein remained unchanged during this experiment. As we expected, CosB did not diminished STAT3 phosphorylation and STAT3 expression levels. These data suggested that CosC inhibited the growth of MDA-MB-468 breast cancer cells via specific inhibition of the STAT3 Tyr705 phosphorylation. These results suggested that CosC may be a potent antitumor agent for the treatment of human cancer with constitutively activated STAT3. To our knowledge, this is the first report of CosC inhibiting the activity of cellular signaling molecule in human tumor cells.

STAT3 phosphorylation plays a critical role in tumor cell proliferation and survival of constitutively activated STATs have been detected in a wide variety of human cancers including head and neck cancer.[25–27] The suppression of constitutively activated STAT3 in breast cancer cells raises the possibility that CosC might inhibit constitutively activated STAT3 in other types of cancer cells.

2.4. CosC controls localization of STAT3 and suppresses STAT3 target genes

Activation of STAT3 is mediated by kinase-dependent phosphorylation of tyrosine 705 and/or serine 727. Phosphorylated STAT3 dimerizes and enters the nucleus by interacting with nuclear import proteins.[28] To visualize CosC inhibition of STAT3 localization in intact cells, immunofluorescence assays were performed in MDA-MB-468 cells using a confocal microscope. The cells were seeded onto coverslips and treated with CosC for 24 h. The cells

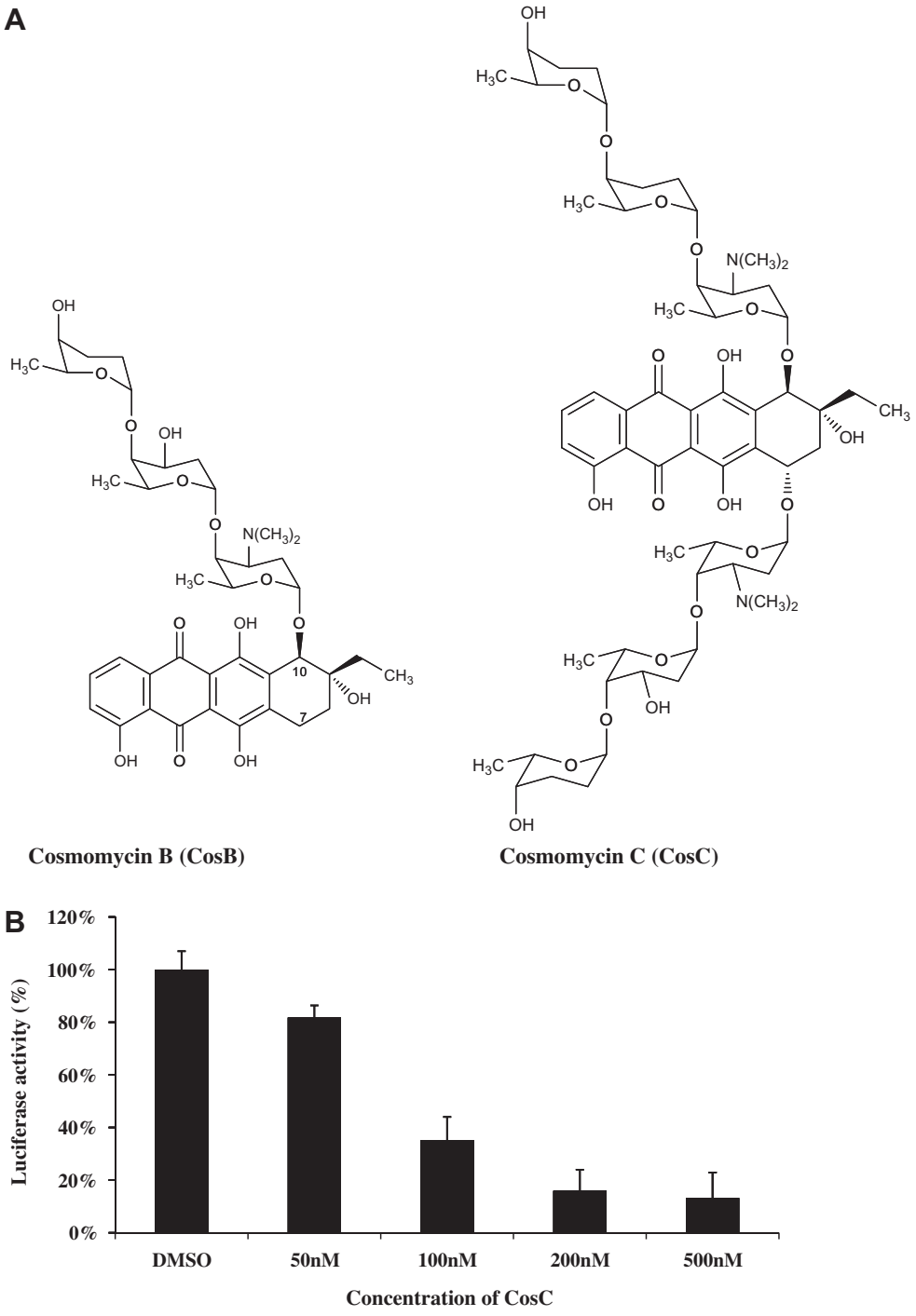

Figure 1. The structure of cosmomycin derivatives and CosC's effects on STAT3-dependent luciferase activities. (A) Molecular structure of cosmomycin derivatives. (B) HCT-116 cells were analyzed using a dual-luciferase assay. The luciferase assay was performed as described in Section 4.

were fixed, stained, and observed under the microscope. As shown in Figure 4A, STAT3 was located to the nucleus. However, STAT3 was mainly located to the cytoplasm in CosC-treated cells. Therefore, CosC blocked the translocation of STAT3 into the nucleus by inhibiting STAT3 Try705 phosphorylation.

Once in the nucleus, STAT3 activates the transcription of its target genes that participate in oncogenesis, such as apoptosis inhibitors (Bcl-xL, Mcl-1, and survivin), cell-cycle regulator (cyclin D1), and angiogenic factor (VEGF).[29] We determined whether CosC regulated the expression of target genes such as Bcl-xL, cyclin D1, VEGF, survivin, and Mcl-1 in MDA-MB-468 cells. As shown in Figure 4B, CosC suppressed STAT3 target genes in a dose-dependent manner. We showed that 200 nM CosC completely inhibited Bcl-xL and Mcl-1expresion.

Because activated STAT3 translocate to the nucleus and induces the expression of its specific target genes, we determined whether CosC suppressed nuclear translocation of STAT3. Figure 4A demonstrates that CosC inhibited the translocation of STAT3 to the nucleus in MDA-MB-468 cells. We showed that CosC suppressed the expression of several STAT3-regulated genes such as Bcl-xL,

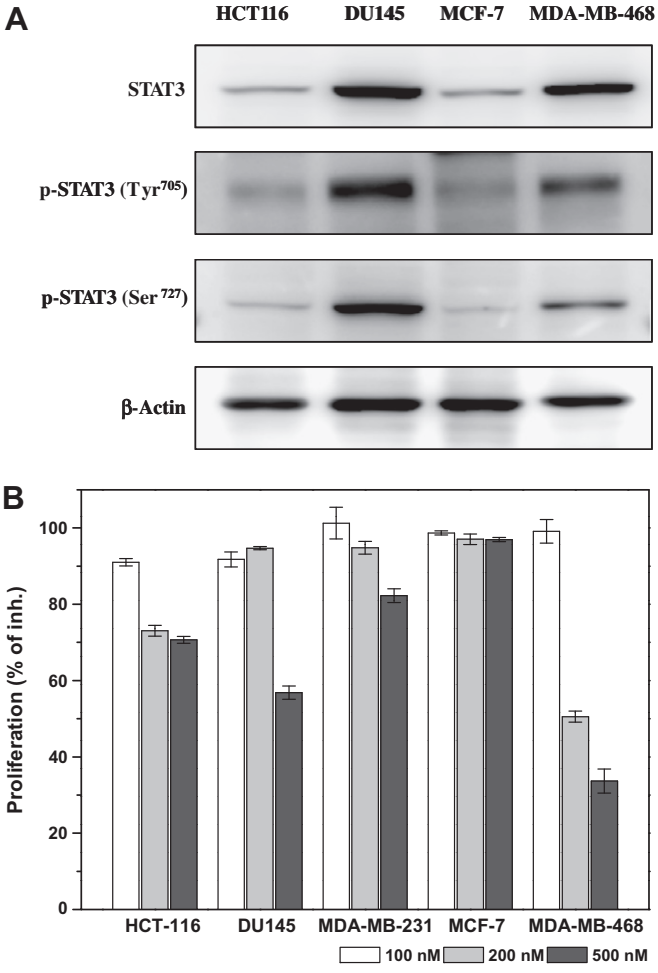

Figure 2. Inhibitory effect of cosmomycin derivatives on cell proliferation. (A) The level of STAT3, p-STAT3 Tyr705, and p-STAT3 Ser727 in various human cancer cell lines were detected by using immunoblotting with specific antibodies. (B) Proliferation of HCT-116, DU145, MCF-7, MDA-MB-231, and MDA-MB-468 cell lines were measured using the WST-1 assay kit at 24 h after CosC treatment.

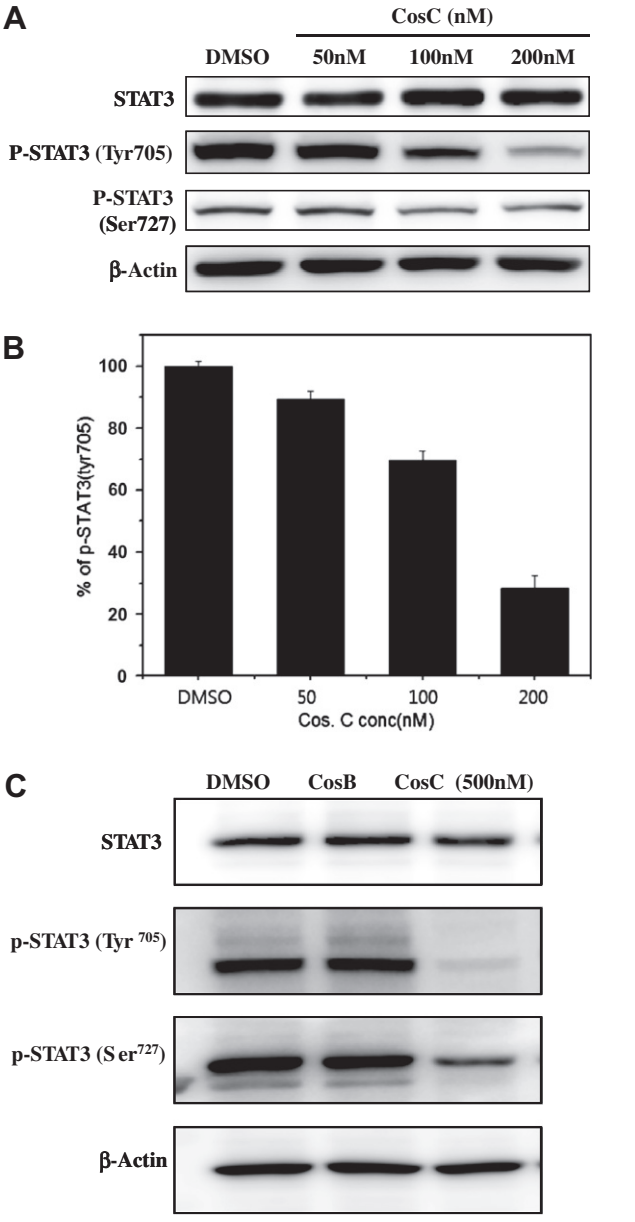

Figure 3. Effects of CosC on STAT3 phosphorylation levels in MDA-MB-468 cells. The lysates from MDA-MB-468 cells treated with CosB or CosC at indicated concentrations for 24 h and processed for immunoblotting with specific antibodies. (A and B) CosC inhibited the phosphorylation of STAT3 Tyr705 in dose dependent manner. (C) The cells were treated with 500 nM of CosB or CosC. CosC completely inhibited the phosphorylation of STAT3 Tyr705 and slightly suppressed the phosphorylation of STAT3 Ser727. CosB did not inhibit the phosphorylation of STAT3 Tyr705 and Ser727.

survivin, Mcl-1, cyclin D1, and VEGF. Tumor cells with highly expressed Mcl-1, survivin, and Bcl-xL resistant to apoptosis and can block chemotherapy-induced cell death.[30] Therefore, CosC may induce apoptosis in the tumor cells by down-regulating of Bcl-xL, surviving, and Mcl-1expression.

Even though the STAT3 target genes (Bcl-xL, cyclin D1, VEGF, survivin) were down-regulated by CoS, we could not exclude the down-regulation of the genes by other transcription factors such as NF-κB, because the genes could be regulated by different kind of transcription factors.

2.5. CosC causes cell accumulation in the G_0–G_1 phase and induces apoptosis

We observed that the antitumor effects of CosC were mediated by suppressing the expression of STAT3-regulated targets such as cyclin D1, Mcl-1, survivin, and Bcl-xL. D-type cyclins are required for the progression of cells from the G_1 phase of the cell cycle to S phase.[31] Therefore we determined the effect of CosC on cell cycle phase distribution. Our results show that cyclin D1 levels are decreased in CosC-treated cells. As illustrated in Figure 5A, treatment of MBA-MB-468 cells with 200 nM of CosC increased the cell cycle in the G_0/G_1-phase fraction from 49.1% (untreated) to 59.5% at 24 h. We demonstrated that 500 nM of CosC increased the cell population in the sub-G_1 phase, which is indicative of apoptosis. In the breast cancer cells that were treated with CosC, we noted a dose-dependent activation of pro-caspase-3, which led to a cleavage of a 118 kDa PARP into an 85-kDa fragment (Fig. 5B).

Therefore, our results indicate that CosC decrease cell viability in part by G_1 cell cycle arrest after STAT3 inhibition. CosC-induced suppression of cyclin D1 expression correlated with the accumulation of cells in the G_0/G_1 phase of the cell cycle (Fig. 5A).

3. Conclusion

Previous studies have reported that the antitumor effects of anthracycline are associated with mechanisms such as inhibition

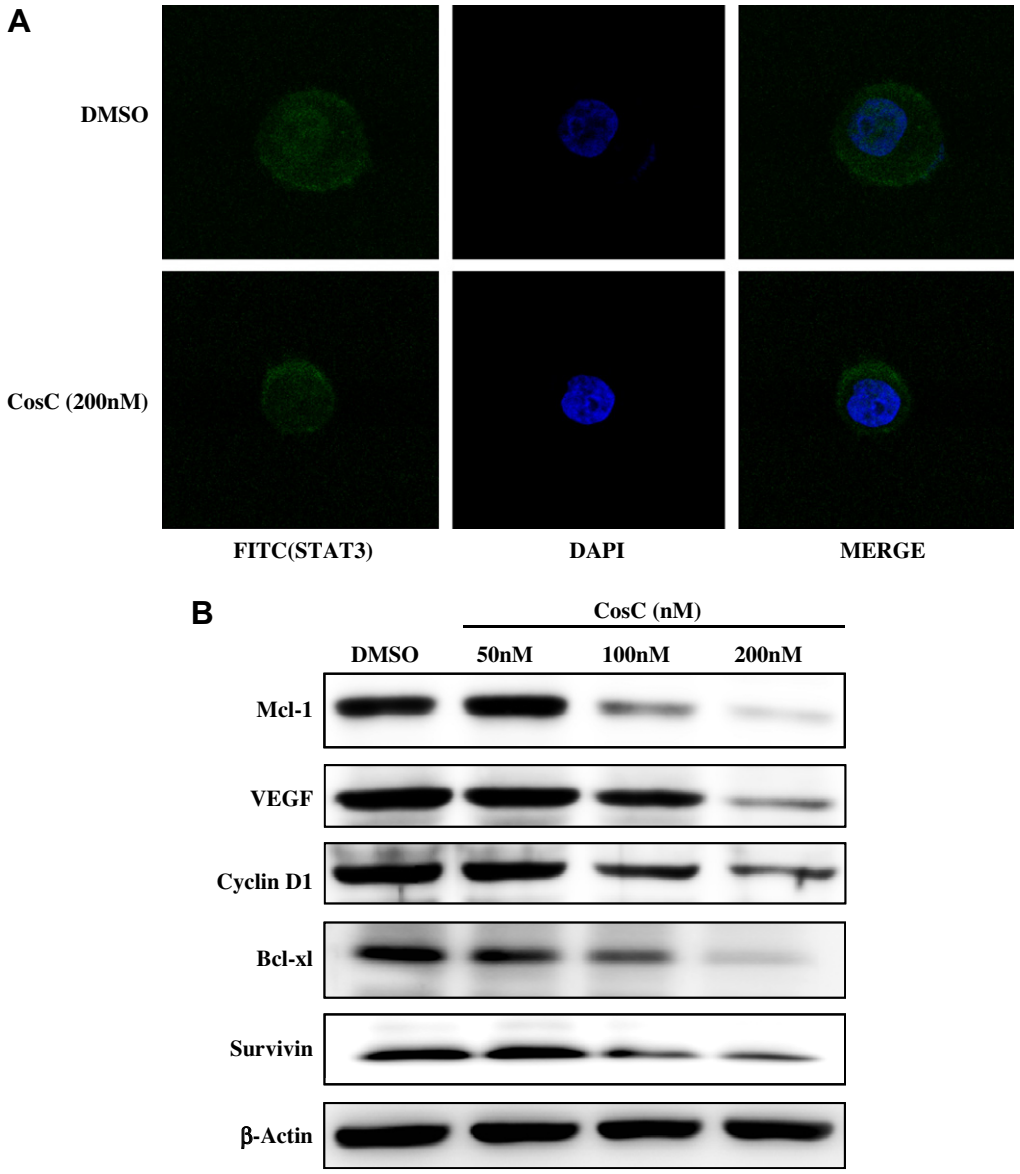

Figure 4. CosC controls localization of STAT3 and suppresses the STAT3 target genes. (A) Localization of STAT3 was measured in MDA-MB-468 cells. The cells were incubated with or without 200 nM CosC. After 24 h, the cells were fixed with 4% paraformaldehyde and stained for STAT3, followed by a FITC-conjugated secondary antibody and propidium iodide. STAT3 and cell nuclei were visualized as green and blue, respectively. (B) CosC suppressed the expression of STAT3 target genes in a dose-dependent manner. Downstream proteins that were regulated by STAT3 activity were subjected to immunoblotting using specific antibodies.

of topoisomerase II, DNA damage due to the formation of reactive oxygen species, and intercalation-induced distortion in the double helix.[17,22–24] The antitumor activities of cosmomycins are due to DNA binding and subsequent suppression of tumor cell growth. In this study, we showed that CosC selectively inhibited the STAT3 phosphorylation on the residue tyrosine 705 and blocked translocation to the nucleus. CosC treatment down-regulated the STAT3 downstream targets such as cyclin D1, survivin, VEGF, Bcl-xL, and Mcl-1, and induced apoptosis in breast cancer cells. These results are consistent with previous reports showing that STAT3 inhibitor such as STA-2 decreases in Bcl-xL, survivin, and Mcl-1 expression in the cells with constitutive Stat3 signaling.[13] CosC inhibited the proliferation and increased the accumulation of cells in the G_0/G_1 phase by down-regulating cyclin D1. However, siRNA of STAT3 minimally inhibits the proliferation of MDA-MB-468 cell, it seems that multiple mechanisms may together be responsible for the apoptosis induced by CosC. Therefore further studies are needed to figure out the exact mechanism of CosC in tumor cells.

Even though the exact mechanism mediating the anticancer effects of CosC has not been investigated yet, our results showed that CosC suppressed constitutive STAT3 activation and then inhibited the expression of the STAT3 downstream targets in MDA-MB-468 cells. Interestingly CosC with two trisaccharide chains inhibited STAT3 pathway, however, CosB with one trisaccharide chain at the 10-position of aglycone and mithramycin with different kind of two saccharide chains did not inhibit STAT3 activity. CosC represents a lead compound that inhibits the constitutively STAT3 signaling in human breast cancer cells.

4. Experimental

4.1. Reagents

RPMI 1640, fetal bovine serum (FBS), and antibiotic/antimycotic solution were purchased from Life Technologies/Bethesda Research Laboratories. Antibodies against STAT3, p-STAT3 Tyr705,

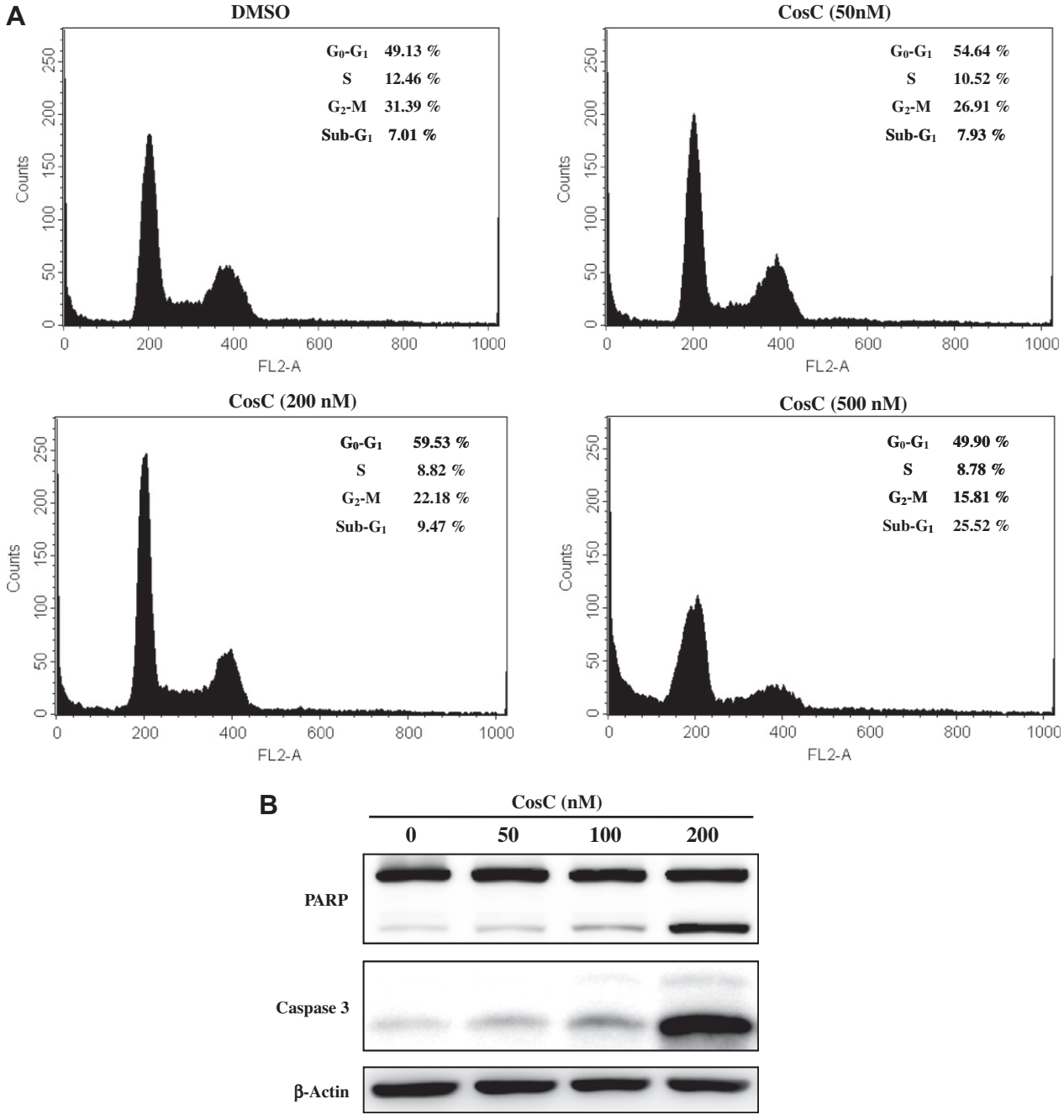

Figure 5. Accumulation of cells at the G_0–G_1 phase of the cell cycle or apoptosis in MDA-MB-468 cells. (A) Increasing CosC concentration slowed cell cycle progression in the G_0–G_1 phase and increased apoptosis of MDA-MB-468 cells. Cells were harvested, fixed, and stained with propidium iodide. Cell cycle distribution was analyzed using FACScalibur Flow Cytometer (Becton Dickinson). The cell lines were treated with CosC at the indicated concentration for 24 h. The cells were harvested and analyzed. (B) MDA-MB-468 cells were treated with CosC for 24 h. Whole-cell extracts were prepared, separated on SDS–PAGE, and subjected to western blotting with the anti-caspase-3 antibody. Whole-cell extracts were subjected to western blotting with the anti-PARP antibody. The same blots were stripped and probed with β-actin antibody to show equal protein loading.

p-STAT3 Ser727, Mcl-1, VEGF, PARP, and caspase 3 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against cyclin D1, Bcl-xL, survivin, and β-actin were purchased from Santa Cruz Biotechnology, Inc. Goat-anti-rabbit and anti-mouse horseradish peroxidase (HRP) conjugates were purchased from Jackson ImmunoResearch Laboratories, Inc. DMSO and chemicals that were used in buffer solutions were purchased from Sigma–Aldrich Chemical Co. Cosmomycins were isolated from *Streptomyces* sp. KCTC19769 and analyzed using a LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., USA) that was equipped with an electrospray ionization (ESI) source. ^{1}H NMR was recorded using a Varian 400 MHz spectrometer in $CDCl_3$.[20] CosC was dissolved in DMSO as a 10 mM stock solution and stored at 4 °C. Further dilution was performed using cell culture medium.

4.2. Cell culture

Cancer cell lines were obtained from the American Type Culture Collection (Manassass, VA). MDA-MB-468, MDA-MB-231, MCF-7 (human breast cancer cell lines), and DU145 (human prostate

cancer cell) cells were maintained in RPMI 1640. HeLa (human cervical adenocarcinoma) cells were maintained in DMEM. HCT-116 (human colon cancer) cells were maintained in McCoy's 5A. All culture media were supplemented with 10% heat-inactivated FBS. Cell cultures were maintained at 37 °C with a humidified atmosphere of 5% CO_2 in an incubator.

4.3. Transient transfection and dual-luciferase assay

HCT-116 cells were seeded at a density of 20×10^5 cells in 100 mm^2 culture plates. On the following day, the cells were transfected with 10 μg of 21pSTAT3-TA-Luc and Ag of pRL-TK, a Renilla luciferase control reporter plasmid (Promega) using x-treme Gene HP DNA Transfection reagent (Roche). After 5 h of transfection, the cells were trypsinized and seeded onto sterile, black-bottom 96-well plates at a density of 2×10^4 cells per well. After incubation in complete medium for 24 h, the cells were treated with either test compounds or 0.1% DMSO for 24 h. After treatment, the cells were harvested in 20 μl of passive lysis buffer. The luciferase activity was evaluated using the Dual Luciferase Reporter Assay kit (Promega) on Wallac Victor2 (Perkin-Elmer, Inc.). The experiments were performed in triplicate and repeated three times. The relative luciferase activity was calculated using the following formula: relative luciferase activity (%) = [(normalized luciferase activity of sample treated with a test compound)/(normalized luciferase activity of sample treated with 0.1% DMSO)] × 100.[15]

4.4. Cell proliferation assay

Briefly, cells were seeded at a density of 5000 cells per well in 96-well plates in RPMI 1640 or McCoy's 5A containing 10% FBS. The cells were replenished with fresh complete medium containing test compounds or 0.1% DMSO. After incubation for 24 h, the cell proliferation reagent WST-1 (Dojindo Laboratories) was added to each well. WST-1 formazan was quantitatively measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Inc.).

4.5. Western blotting

MDA-MB-468 cells (1.5×10^6) were plated in 10 cm^2 dishes and incubated overnight. The medium was aspirated and the cells were incubated with freshly diluted cosmomycins at the times and concentrations specified for each experiment. For detection of proteins and phosphoproteins, cosmomycin-treated whole cell extracts were lysed in radioimmunoprecipitation assay buffer [RIPA; 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% SDS, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 30 mM Na_2HPO_4, 50 mM NaF, and 1 mM Na_3VO_4] containing a protease inhibitor cocktail (Roche Applied Science). Proteins (40 μg) were resolved on 7.5 or 10% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Co.). The membranes were blocked with 5% nonfat dried milk in TBS-T [50 mM TrisHCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20] and probed with primary antibodies for 3 h. The blots were washed with TBS-T and exposed to HRP conjugated goat-anti-rabbit or goat-anti-mouse IgG for 1 h, and examined using chemiluminescence POD reagents (Roche Applied Science).

4.6. Fluorescence-activated cell sorting analyses

The cells were trypsinized at specific times after compound treatment and collected by centrifugation at 300*g* for 5 min at room temperature. The supernatant was discarded and the precipitated cells were washed twice by repeating suspension and precipitation in PBS buffer. Precipitated cells were carefully resuspended in 500 μl PBS buffer and fixed with 4 ml of ice-cold 70% ethanol overnight. Fixed cells were washed twice with PBS. The collected cells were resuspended in PBS (5×10^5 cells/500 μl) and treated with 100 μg/ml of RNase A at 37 °C for 30 min. Propidium iodide (PI; Sigma) was then added at a final concentration of 50 μg/ml for DNA staining. In total, 20,000 fixed cells were analyzed using the FACScalibur (Becton Dickinson, San Jose, CA). The cell cycle distribution was analyzed using the Modifit's program (Becton Dickinson).

4.7. Confocal laser microscopy

Coverslips for cell culture were immersed in 70% ethanol overnight and rinsed with PBS buffer [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.4)]. Coverslips were placed into 6-well plates and the cells were seeded at a density of 2.5×10^5 cells per well. After 24 h of treatment, the cells were rinsed with PBS and fixed for 10 min at room temperature in 4% paraformaldehyde fixative, followed by permeabilization with 0.1% Triton X-100. The cells were blocked with 1.0% bovine serum albumin (BSA) in PBS for 1 h and incubated for 3 h at room temperature with STAT3 antibodies that were diluted in PBS containing 1.0% BSA. After washing three times in PBS buffer, the cells were incubated with FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.). Finally, the cells were washed three times in PBS and treated with 2 μg/ml of DAPI (4′,6′-diamidino-2-phenylindole) (Santa Cruz) in PBS for 5 min to stain chromosomes. Coverslips were washed in PBS and mounted on glass slides. The cells were observed using a confocal laser scanning microscope (Olympus, Inc.).

4.8. Statistical analysis

The data were expressed as the mean ± S.D. the statistical analysis was performed using Student's *t*-test. A *P*-value of 0.05 or less was considered statistically significant.

Conflict of interest

The authors declare that they have no conflict of interest.

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