Chikusetsusaponin IVa Butyl Ester (CS-IVa-Be), a Novel IL6R Antagonist, Inhibits IL6/STAT3 Signaling Pathway and Induces Cancer Cell Apoptosis

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Abstract

The activation of IL6/STAT3 signaling is associated with the pathogenesis of many cancers. Agents that suppress IL6/STAT3 signaling have cancer-therapeutic potential. In this study, we found that chikusetsusaponin IVa butyl ester (CS-IVa-Be), a triterpenoid saponin extracted from Acanthopanx gracilistylus W.W.Smith, induced cancer cell apoptosis. CS-IVa-Be inhibited constitutive and IL6-induced STAT3 activation, repressed STAT3 DNA-binding activity, STAT3 nuclear translocation, IL6-induced STAT3 luciferase reporter activity. IL6-induced STAT3-regulated antiapoptosis gene expression in MDA-MB-231 cells, and IL6-induced TF-1 cell proliferation. Surprisingly, CS-IVa-Be inhibited IL6 family cytokines rather than other cytokines induced STAT3 activation. Further studies indicated that CS-IVa-Be is an antagonist of IL6 receptor via directly binding to the IL6R with a $K_d$ of 663 ± 74 nmol/L and the GP130 (IL6R) with a $K_d$ of 1,660 ± 243 nmol/L, interfering with the binding of IL6 to IL6R (IL6R$^b$ and GP130) in vitro and in cancer cells. The inhibitory effect of CS-IVa-Be on the IL6–IL6R–GP130 interaction was relatively specific as CS-IVa-Be showed higher affinity to IL6R than to LIFR ($K_d$: 4,910 ± 1,240 nmol/L) and LeptinR ($K_d$: 4,990 ± 915 nmol/L). We next demonstrated that CS-IVa-Be not only directly induced cancer cell apoptosis but also sensitized MDA-MB-231 cells to TRAIL-induced apoptosis via upregulating DR5. Our findings suggest that CS-IVa-Be as a novel IL6R antagonist inhibits IL6/STAT3 signaling pathway and sensitizes the MDA-MB-231 cells to TRAIL-induced cell death. Mol Cancer Ther; 15(6); 1190–200. ©2016 AACR.

Introduction

IL6 is a pleiotropic cytokine-mediating cancer associated chronic inflammation and inflammatory response, plays important roles in the tumorigenesis (1). IL6 possesses three topologically distinct receptor binding sites: site 1 for binding to the 80 kDa chain IL6R, and sites 2 and 3 for interacting with the two subunits of the signaling chain glycoprotein 130 (GP130) (GP130). The IL6 signaling transduces after the homodimerization of GP130, which becomes associated with IL6-binding chain (IL6Rb) in the presence of IL6. (2). As a common receptor, GP130 also transduces signals delivered by the leukemia inhibitory factor (LIF), the IL11, the oncostatin M (OSM), and the others (3).

STAT3, the key factor mediating the IL6-induced signaling becomes tyrosine phosphorylated by IL6-induced cytokine receptor-associated kinases, the Janus kinase (JAK) family proteins (4). The IL6/STAT3 signaling pathway mediates tumor immune suppression (5), tumor cell survival, premalignant niche formation, and chemotherpay resistance (6).

Breast cancer is the most common malignancy and the primary cause of cancer-related death in women worldwide (7). IL6/JAK/STAT3 signaling plays a critical and pharmacologically targetable role in orchestrating the composition of the breast tumor microenvironment that promotes cancer cell growth, invasion, and metastasis (8, 9). Increasing clinical data have indicated that the circulating levels of IL6 in breast cancer patients correlate with clinical tumor stage and poor prognosis. IL6 plays two distinct roles in breast cancer: the systemic IL6, reflecting whole body metabolic and inflammatory status, is correlated with poor prognosis, advanced disease, and metastasis; the paracrine and autocrine IL6 signaling controls cancer cell growth, cancer stem cell renewal, and metastasis (10, 11). In summary, the IL6-activated excessive STAT3 activation plays important roles in breast cancer.

Inhibitors of the IL6/STAT3 signaling pathway should be effective against the cancers with high levels of STAT3 activation. As a matter of fact, JAK3 inhibitor tofacitinib (12, 13) and IL6R mAb tocilizumab (14) have been used in clinical trial. However, no natural IL6/STAT3 inhibitory compound has been
used in cancer clinical trial. Identifying novel compounds that suppress the JAKs signaling or antagonize IL6R is a pressing issue to prevent and treat the cancers with aberrant activated IL6/STAT3 signaling.

TRAIL is a promising target for cancer therapy because it preferentially induces cancer cell apoptosis with little or no effects on normal cells (15). However, some highly malignant cancers, including breast cancer (16), are resistant to TRAIL-induced apoptosis. The resistance of cancer cells to TRAIL occur at various points of the TRAIL signaling pathways, dysfunctions of the death receptors DR4 and DR5, overexpression of cellular FLICE-like inhibitory protein (c-FLIP), B-cell lymphoma extra-large (Bcl-xL), myeloid cell leukemia 1 (Mcl-1), and the inhibitor of apoptosis proteins (IAP), all lead to TRAIL resistance (17). TRAIL resistance can occur through activating STAT3 in cancer cells (18). Therefore, a combination of TRAIL with STAT3 inhibitor may prove to be a sound strategy to overcome the resistance. Identifying novel agents that inhibit STAT3 activation not only can directly induce apoptosis but also sensitize cancer cells to TRAIL-mediated apoptosis.

CS-IVA-Be (Fig. 1A) is a triterpenoid saponin extracted from a traditional Chinese medicine (TCM) herb Acanthopanax graciifolius W.W.Smith that exhibits immunomodulation (19) and antithrombotic effects (20). The pharmacologic effects of CS-IVA-Be have not been reported yet. In this study, we demonstrated that as an IL6R antagonist, CS-IVA-Be inhibited IL6/STAT3 signaling, repressed cancer cell growth, and synergized with TRAIL to induce breast cancer cell apoptosis.

Materials and Methods

Cell lines and cultures

Human breast cancer MDA-MB-231 and MCF-7 cells, human hepatocellular cancer HepG2 and MHCC97L cells, cervical cancer HeLa cells, leukemic U937 and TF-1 cells, and gastric cancer SGC-7901 cells were purchased from Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology in June 2013. Human fetal liver cell line LO2 was purchased from Kunming Institute of Zoology, Chinese Academy of Sciences in June 2013. All cell lines had been authenticated by the provider through short tandem repeat (STR) profiling. All cell lines were preserved in liquid N2 on March 6, 2021. © 2016 American Association for Cancer Research. mct.aacrjournals.org Downloaded from

Agents and antibodies

CS-IVA-Be was identified and purified from the fruit of the Acanthopanax graciifolius W.W.Smith. The purity of CS-IVA-Be is 98.8 %, which was determined by high-performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) method. A 20 mmol/L solution of CS-IVA-Be was prepared in DMSO, stored as small aliquots at 4°C. STAT3, phospho-STAT3 (Tyro5), phospho-STAT1(Tyro1), STAT1, phosphor-STAT5(Tyro9), STAT5, phosphor-ERK1/2 (Thr202/Tyr204), ERK1/2, phosphor-AKT(Ser473), AKT, phosphor-IAK1(Tyro1022/1023), phosphor-JAK2(Tyro1007/1008), phosphor-Src(Tyro116), Bcl-xL, Mcl-1, Survivin, XIAP, caspase-3, caspase-8, caspase-9, PARP, DR5, DR4, c-FLIP, IL6R, GAPDH antibodies were purchased from Cell Signaling Technology. Anti-IL6 and anti-gP130 antibodies were purchased from Abcam. IL6 ELISA Kit was purchased from R&D Systems. His-probe, NE-PER Nuclear and Cytoplasmic Extraction, and LightShift Chemiluminescent EMSA Kit were obtained from Santa Cruz Biotechnology. Methyl thiazolyl tetrazolium (MTT), N-acetyl-c-cysteine (NAC), SP600125, PD98059, SB203580, and Z-VAD-FMK were purchased from Sigma-Aldrich. Recombinant human TNFR1, IL6, EGF, IFNγ were obtained from Tocris Bioscience. Recombinant human IL6Rx, Gp130, LIFR, and Leptin receptor were purchased from Sino Biological Inc. Pierce Classic Magnetic IP/Co-IP Kit were purchased from Thermo Fisher Scientific.

Immuno blot assay

Cells were plated in 6-well plate at a density of 5 $\times$ 10^4 cells/mL overnight before being treated with CS-IVA-Be. The total cell lysates were prepared as described previously (21). The model amounts of proteins from each sample were subjected to SDS-PAGE followed by transfer to polyvinylidene difluoride (PVDF) membranes. After being blocked with 1% (w/v) BSA in TBST for 2 hours, the membranes were incubated with primary antibody overnight at 4°C then washed and incubated with secondary antibody conjugated with IgG DyLight for 1 hour at room temperature. Afterward, membranes were washed and scanned with an Odyssey infrared fluorescent scanner (LI-COR) and analyzed with Odyssey software version 3.

Cytotoxicity assay

LO2, HepG2, MHCC97L, SGC-7901, TF-1, U937, MDA-MB-231, and MCF-7 cells were plated in triplicate in a 96-well plate at a density of 1 $\times$ 10^4 cells with 100 μl culture medium per well in the absence or presence of indicated concentrations of CS-IVA-Be for 24 hours. Cell viability was then determined by the MTT assay as described previously (22).

Apoptosis assay

Apoptotic cells were determined by using an FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s protocol. The cells were washed and incubated with binding buffer containing Annexin V-FITC and PI in the dark at room temperature for 15 minutes. Afterward, the degree of apoptosis was analyzed by FACS Calibur, Becton Dickinson. The data were analyzed using the software CELLQuest.

JC-1 staining

Mitochondrial membrane potential was examined via JC-1 staining (Beyotime Institute of Biotechnology, China). MDA-MB-231 cells were incubated with JC-1 working solution at 37°C in the dark for 20 minutes and observed by fluorescence microscopy. In healthy cells, JC-1 forms complexes of J-aggregates showing punctate red fluorescence at 590 nm emission
wavelength; however, in apoptotic cells, JC-1 remains in the monomeric form showing diffused green fluorescence at 530 nm emission wavelength.

**Immunofluorescence staining**

MDA-MB-231 cells were seeded at the density of 70% to 80% confluence per well into 24-well chamber slides. After being treated with test drugs for the indicated times, cells were fixed with cold 4% (w/v) paraformaldehyde for 20 minutes, rehydrated in PBS for 15 minutes, and permeabilized in 0.1% (w/v) TritonX-100 at room temperature for 10 minutes. After being washed with PBS, the cells were blocked with 3% BSA for 1 hour and then incubated with primary antibody at 4°C overnight followed by FITC-conjugated secondary antibody for 1 hour at room temperature. The images of green fluorescence–stained pSTAT3 were captured using a fluorescence microscope.

**Luciferase reporter gene activity assay**

We used the luciferase reporter assay (the Dual-Luciferase Reporter Assay System; Promega) to investigate the IL6-induced transcripational activity of STAT3. Transient transfection was performed in 96-well plates at a cell density of 50% to 70% confluence per well. The STAT3 luciferase reporter plasmid was cotransfected with the pRL-TK plasmid (which encodes Renilla luciferase as an internal control for transfection efficiency) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The transfected cells were treated with CS-Iva-Be for various periods of time, and the cell lysates were prepared for assessment of luciferase activity. Firefly and Renilla luciferase activities were measured using a luminometer (Centro XS3 LB960) according to the manufacturer’s instructions. Relative firefly luciferase activity normalized by Renilla luciferase activity was expressed as fold induction after treatment with CS-Iva-Be compared with vehicle control (DMSO).

**Electrophoretic mobility shift assay**

STAT3–DNA-binding activity was analyzed by electrophoretic mobility shift assay (EMSA) using biotin-labeled, double-stranded STAT3 consensus-binding motif probe. STAT3 consensus oligo primers were as follows: forward, 5’-GATCCCTTCTGGAGATTCCTAGATC-3’; reverse, 3’-CTAGGAAACCCCTAGATCCTAG-5’. Nuclear protein extracts were prepared from CS-Iva-Be–treated MDA-MB-231 cells and incubated with biotin-labeled probe. STAT3–DNA complex was separated from free oligonucleotide on 5% nondenaturing polyacrylamide gels, then transferred to nylon membranes, and cross-linked for 15 minutes under a handheld UV lamp. Cross-linked, biotin-labeled DNA was detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology, Inc.).

**IL6 inhibitory bioassay**

After being starved in media without GM-CSF for 24 hours, various concentrations of CS-Iva-Be (2.5, 5, 7.5, 10, 12.5) μmol/L in the presence of IL6 (25 ng/mL) were added to the TF-1 cells and incubated for 24 or 48 hours; cell viability was then determined by the MTT assay.

**In vitro kinase assay**

In vitro JAKs kinase (JAK2, JAK3, TYK2) assay for a series of CS-Iva-Be concentrations was performed by BPS Bioscience Service using Kinase-Glo Plus Luminescence Kinase Assay Kit (Promega). In vitro panel kinases (JAK1, JAK2, JAK3, TYK2, EGFR, IGF1R, PDGFRα, PDGFRβ) assay with two concentrations (25 and 50 μmol/L) of CS-Iva-Be was performed by Eurolab’s Kinase Profiler service according to Cerep’s validation Standard Operating Procedure.

**IL6 receptor binding assay in cell level**

The cell-based IL6-receptor binding was performed using the kit of Fluorokine Biotinylated Human Interleukin 6 (R&D Systems) followed by the manufacturer’s protocol. First, IL937, MDA-MB-231, HeLa, and HepG2 cells were treated with the test compound for various periods of time and the cells were harvested and washed twice with PBS to remove any residual growth factors that may be present in the culture media. Next, the cells were resuspended in PBS to a final concentration of 4 × 10^5 cells/mL, 10 μL of biotinylated IL6 was added to a 25 μL aliquot of the cell suspension for a total reaction volume of 35 μL. As a negative staining control, an aliquot of cells were stained with 10 μL of negative control reagent (a soybean trypsin inhibitor protein biotinylated as the IL6). The cells were then incubated for 30 minutes at 4°C followed by the addition of 10 μL Avidin-FITC reagent and another 30-minute incubation at 4°C in the dark before being analyzed by flow cytometry.

**Analysis of IL6Re, DR4, and DR5 surface expression**

Cells were treated with CS-Iva-Be for various periods of times before being detached with trypsin containing EDTA and washed twice with staining buffer. Cells were then incubated with mouse monoclonal anti-human DR5, DR4, or IL6R antibodies for 45 minutes at 4°C, washed twice, incubated with FITC-conjugated secondary antibody for 30 minutes at 4°C and washed again, then resuspended in staining buffer for flow cytometric analysis.

**IL6 receptor binding assay in vitro**

IL6 receptor binding was performed following the method described previously (23). Briefly, 96-well plates (Nunc) were coated with 500 ng/mL of human recombinant soluble IL6 receptor (sIL6R), washed, and blocked. The sIL6R-coated 96-well plates were incubated with 6.2 nmol/L of recombinant human IL6, or 6.2 nmol/L of IL6 plus one of six concentrations of CS-Iva-Be, in triplicates, and washed to remove unbound IL6. The wells then were incubated with a monoclonal rabbit anti-human IL6 antibody (200 ng/mL), washed, and incubated with goat anti-rabbit IgG-HRP (40 ng/mL; Santa Cruz Biotechnology). Sample wells were washed and developed with tetramethylbenzidine (100 μL/well, 10 minutes at room temperature and stopped with 2 mL/L H2SO4). Optical density in each well was determined using a microplate reader (Multiskan Ascent, Thermo Scientific) 450 nm with a correction wavelength of 630 nm. Nonspecific binding of IL6 is determined by the reading of wells without sIL6R. Data were analyzed by nonlinear regression analysis using GraphPad Prism4 (GraphPad). Maximum binding of IL6 (100% bound) is defined as the absorbance in the absence of CS-Iva-Be. The absorbance in the presence of various concentrations of CS-Iva-Be is calculated as percent of maximum binding of IL6.
Microscale thermophoresis assay

Binding assay of labeled recombinant human IL6Rα, GP130, LIFR, and LeptinR to CS-IVa-Be was performed using the Microtherm NT.115 Kit (NanoTemper Technologies). Briefly, 10 μmol/L recombinant human IL6Rα, GP130, LIFR, and LeptinR were labeled in PBS buffer using the Protein Labeling Kit RED-NHS followed by the manufacturer’s protocol (NanoTemper Technologies). A 16-point titration series of CS-IVa-Be were added to the labeled protein (200 nmol/L) in PBST buffer (PBS + 0.05% Tween-20), the total volume of mixed sample was 20 μL and the volume ratio of CS-IVa-Be to labeled protein is 1:1, the final DMSO concentration for each protein-compound sample was 2%. The protein-compound samples were equilibrated for 5 minutes at room temperature, loaded into the MST Premium Coated capillaries (NanoTemper Technologies) and assayed by microscale thermophoresis (MST). The thermophoresis data were analyzed using NT Analysis 1.5.41 (NanoTemper Technologies) to compute $K_D$ value according to the law of mass action. The experiment was performed in triplicate, and the fits are represented as mean ± SD. Data normalization and curve fitting were performed using GraphPad Prism 5.

Coimmunoprecipitation assay

The interaction of IL6Rα with GP130 in the presence of IL6 was assayed by coimmunoprecipitation (co-IP) assay according to the manufacturer’s protocol of Pierce Classic Magnetic IP/Co-IP Kit. At room temperature, 10 μg of recombinant human IL6Rα was preincubated with 0, 5, or 10 μmol/L of CS-IVa-Be for 2 hours in IP wash buffer, 10 μg of recombinant human IL6 was added and incubated with GP130 for another 2 hours, and then 2 μg of His antibody was added and incubated for 2 hours to form immune complex. Twenty-five microliters of prewashed Pierce Protein A/G Magnetic Beads were placed into the above immune complex and incubated for 1 hour with mixing. Then the beads were washed and the target antigen was eluted with Alternative Elution, and the target antigen and the binding proteins were immunoblotted with the indicated antibodies by Western blot assay.

siRNA-mediated gene silencing

DR5-specific siRNA was obtained from RiboBio. Transient transfection was performed using Lipofectamine 2000. Briefly, cells were transfected with indicated concentration of siRNAs for
24 hours, followed by treatment with CS-IVa-Be for indicated time, and the cells were then harvested for flow cytometry analysis.

Statistical analysis
The experimental results presented in the figures are representative of three or more independent experiments. The data are presented as the mean values ± SD. Statistical comparisons between the groups were done using one-way ANOVA. Values of P < 0.05 were considered to be statistically significant.

Results
CS-IVa-Be induces cancer cell apoptosis
CS-IVa-Be (Fig. 1A), a triterpenoid saponin extracted from Acanthopanax gracilistylus W.W.Smith, was identified as a cell death inducer. We investigated the effects of CS-IVa-Be on the viability of cancer cells and found that CS-IVa-Be inhibits the viability of various kinds of cancer cells (including hepatocellular, gastric, leukemia, and breast cancers). Breast cancer MDA-MB-231 and MCF-7 cells were more sensitive to the CS-IVa-Be–induced cell viability attenuation than other cancer cells (Fig. 1B). We next investigated whether the CS-IVa-Be induced breast cancer cell inhibition due to apoptosis. We then found that CS-IVa-Be induced pro-caspase-3, -8, and -9 cleavage (Fig. 1C). Furthermore, ZVAD-FMK, a pan-caspase inhibitor, significantly offsets the CS-IVa-Be–induced MDA-MB-231 cell viability attenuation and apoptosis (Fig. 1D and E). In addition, CS-IVa-Be induced a decrease of mitochondrial membrane potential (Fig. 1F). The results indicate that CS-IVa-Be promotes breast cancer cell apoptosis.

CS-IVa-Be selectively inhibits constitutive and IL6 family member–induced STAT3 activation in MDA-MB-231 cells
To identify the signaling pathways that were involved in the CS-IVa-Be–induced cell apoptosis, we investigated the effects of CS-IVa-Be on STAT3, NF-κB, MAPK, and AKT signaling. CS-IVa-Be was found to inhibit the constitutive and IL6-induced STAT3 and JAK1, JAK2, Src activation rather than constitutive and IL6-induced ERK1/2 and AKT activation (Fig. 2A and B). Other IL6 family cytokines (IL11, LIF, and OSM, which share the same GP130 receptor to transduce signal) inducing STAT3 activation were also inhibited by

![Figure 2.](image-url)

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**Figure 2.** CS-IVa-Be inhibits IL6/STAT3 activation in MDA-MB-231 cells. A, cell lysates from MDA-MB-231 cells treated with various concentrations (0, 5, 7.5, 10 μmol/L) of CS-IVa-Be for 24 hours were analyzed for pSTAT3 (Tyr705), STAT3, pSTAT1 (Tyr701), STAT1, pSTAT5 (Tyr694), STAT5, pAKT, AKT, pERK1/2, and ERK1/2, with GAPDH as a loading control. B, cell lysates from MDA-MB-231 cells treated with various concentrations (0, 2.5, 5, 7.5, 10 μmol/L) of CS-IVa-Be for 8 hours, followed by 15-minute IL6 (25 ng/mL) stimulation were analyzed for pSTAT3 (Tyr705), STAT3, pJAK1, pJAK2, pSrc, pAKT, AKT, pERK1/2, and ERK1/2. GAPDH was used as a loading control. C, cell lysates from MDA-MB-231 cells treated with 10 μmol/L of CS-IVa-Be for 8 hours, followed by 15-minute stimulation of 25 ng/mL IL11, LIF, OSM, respectively, were analyzed for pSTAT3 (Tyr705), STAT3, pJAK1, pJAK2, pSrc, pAKT, AKT, pERK1/2, and ERK1/2. GAPDH was used as a loading control. D, cell lysates from MDA-MB-231 cells treated with 10 μmol/L of CS-IVa-Be for 8 hours, followed by 15-minute stimulation of 100 ng/mL EGF and IFNγ, respectively, were analyzed for pSTAT3 (Tyr705), STAT3, pSTAT1 (Tyr701), STAT1, pSTAT5 (Tyr694), and STAT5. GAPDH was used as a loading control. This is a representative result of three repetitive experiments with similar results.
CS-IVa-Be (Fig. 2C). But interestingly, constitutive and EGF-induced STAT3, STAT5 activation, or IFNγ-induced STAT3, STAT1 activation were not inhibited by CS-IVa-Be (Fig. 2A and D). In addition, ROS production was observed upon CS-IVa-Be treatment (Supplementary Fig. S1A), but treatment with ROS, JNK1/2/3, ERK1/2, or p38-specific inhibitor did not reverse the CS-IVa-Be-induced cell viability attenuation in MDA-MB-231 cells (Supplementary Fig. S1B). CS-IVa-Be had no inhibitory effects on the TNFα-induced NF-κB transcription activity (Supplementary Fig. S1C) and NF-κB DNA-binding activity (Supplementary Fig. S1D).

CS-IVa-Be abrogates IL6/STAT3 downstream signaling

We further investigated the effects of CS-IVa-Be on constitutive and IL6-induced STAT3 downstream signaling in MDA-MB-231 cells. CS-IVa-Be was found to inhibit the pSTAT3 nuclear translocation (Fig. 3A), the STAT3–DNA binding activity (Fig. 3B), and IL6-induced STAT3 luciferase activity (Fig. 3C) in MDA-MB-231 cells. CS-IVa-Be also downregulated the constitutive and IL6-induced Survivin, XIAP, Bcl-xL, and Mcl-1 expression (Fig. 3D and E). IL6-induced TF-1 cell proliferation was also inhibited by CS-IVa-Be (Fig. 3F). These findings suggest that CS-IVa-Be inhibit IL6/STAT3 downstream signaling.

Figure 3.
CS-IVa-Be inhibits IL6/STAT3 downstream signaling. A, immunofluorescence staining of pSTAT3 (Tyr705). MDA-MB-231 cells were treated with 5 μmol/L CS-IVa-Be for 8 hours and then pSTAT3(Tyr705) was labeled through immunofluorescence staining; scale bar, 20 μm. B, MDA-MB-231 cells were treated with various concentrations (0, 2.5, 5, 7.5, 10 μmol/L) of CS-IVa-Be for 24 hours, after which nuclear proteins were extracted and subjected to EMSA with STAT3 probe. C, MDA-MB-231 cells were transfected with STAT3 luciferase reporter vector for 24 hours, and then treated with various concentrations (0, 2.5, 5, 7.5, 10 μmol/L) of CS-IVa-Be for 8 hours. The STAT3 luciferase activity was measured following stimulation with IL6 (25 ng/mL) for 8 hours. D, cell lysates from MDA-MB-231 cells treated with various concentrations (0, 5, 7.5, 10 μmol/L) of CS-IVa-Be for 24 hours were analyzed for Survivin, c-FLIP, XIAP, Mcl-1, and Bcl-xL. GAPDH was used as a loading control. E, cell lysates from MDA-MB-231 cells treated with various concentrations (0, 2.5, 5, 7.5, 10 μmol/L) of CS-IVa-Be for 24 hours, followed by IL6 (25 ng/mL) stimulation for 15 minutes, were analyzed for Survivin, c-FLIP, XIAP, Mcl-1, and Bcl-xL. GAPDH was used as a loading control. F, inhibition of IL6-induced proliferation of TF-1 cells by CS-IVa-Be. TF-1 cells were incubated with IL6 (25 ng/mL) in the presence of various concentrations (0, 2.5, 5, 7.5, 10, 12.5 μmol/L) of CS-IVa-Be for 24 or 48 hours, and cell viability was measured by MTT assay. This is a representative result of three repetitive experiments with similar results and error bars mark SDs (*, P < 0.05).
CS-IVa-Be does not inhibit the enzyme activity of nonreceptor or receptor tyrosine kinases of STAT3 in vitro

As STAT3 phosphorylation was mediated by several non-receptor tyrosine kinases (24) and receptor kinases (25), we investigated whether CS-IVa-Be impact the activity of upstream kinases. CS-IVa-Be has showed inhibitory effects on the IL6-induced phosphorylation of JAK1, JAK2, and Src (Fig. 2B) in MDA-MB-231 cells. The inhibitory effect of CS-IVa-Be on IL6-induced STAT3 activation might be through its repression on JAK1, JAK2, and Src kinase activity. However, by in vitro kinase assay, we found no inhibitory effect of CS-IVa-Be on the enzyme activity of tyrosine kinases such as JAKs family kinases (JAK1, JAK2, JAK3, and TYK2; Fig. 4A and B), Src, EGFR, insulin-like growth factor 1 receptor (IGF1R), platelet-derived growth factor receptor α (PDGFRα), and fibroblast growth factor receptor 1 (FGFR1; Fig. 4B), even at 50 μmol/L, the dose is much higher than the IC50 of CS-IVa-Be in MDA-MB-231 cells. Therefore, we suggest that CS-IVa-Be is not a tyrosine kinase inhibitor.

CS-IVa-Be blocks IL6 binding to its cell surface receptor

We had shown that CS-IVa-Be inhibited IL6-induced JAKs and STAT3 activation in breast cancer cells, without affecting JAKs enzyme activity in vitro. These findings made us speculate that CS-IVa-Be might disrupt the upstream signaling such as the interaction of IL6 to its receptor. To test this hypothesis, we investigated whether CS-IVa-Be affect the binding of IL6 to cell surface IL6R in cancer cells using biotin-labeled IL6 by flow cytometry analysis. To rule out the possibility that CS-IVa-Be might downregulate IL6R protein levels, we also measured the cell surface IL6R expression upon CS-IVa-Be treatment. The results demonstrated that CS-IVa-Be indeed lowered biotin-labeled IL6 binding to IL6R in various cancer cells tested (U937, MDA-MB-231, Hela, HepG2) in a dose-dependent manner, without decreasing IL6R cell surface expression (Fig. 5A and B and Supplementary Fig. S2).

To further confirm our hypothesis that CS-IVa-Be inhibits IL6–IL6R binding, we investigated the effects of CS-IVa-Be on IL6–IL6R interaction using an in vitro ELISA assay. Our results showed that CS-IVa-Be significantly inhibited the IL6–IL6R interaction with maximal inhibition rate less than 20% (Fig. 5C). As CS-IVa-Be cannot antagonize IL6–IL6R interaction completely, we considered CS-IVa-Be could be a non-competitive IL6R antagonist.

Biochemical characterization of CS-IVa-Be/IL6R interaction

As our results have illustrated that CS-IVa-Be inhibited the IL6–IL6R interaction, we next studied the binding specificity
of CS-IVa-Be to IL6 or IL6R. We first measured the binding affinity (Kd: 48 ± 2 nmol/L) of IL6 to IL6Rx (Supplementary Fig. S3A) using MST analysis, which allows a sensitive detection of small-molecule binding to a protein target. Next, we measured the binding characteristics of CS-IVa-Be to IL6 or IL6R and found that CS-IVa-Be can bind to IL6Rx (Kd: 663 ± 74 nmol/L) and IL6Rβ (GP130; Kd: 1,660 ± 243 nmol/L; Fig. 5D and E) but had no binding affinity to IL6 (Supplementary Fig. S3B). The affinity of CS-IVa-Be to IL6Rx was relatively higher than other IL6 family receptors such as LeptinR (Kd: 4,990 ± 915 nmol/L) and LIFR (Kd: 4,910 ± 1,240 nmol/L; Fig. 5E and Supplementary Fig. S3D). IL6Rx pretreatment with CS-IVa-Be also inhibited IL6Rx/GP130 interaction in the presence of IL6 (Fig. 5F). The expression of IL6R, IL6, and pSTAT3 in different cancer cell lines were compared (Supplementary Fig. S4A–S4C). As MDA-MB-231 cells secrete the highest levels of IL6 (Supplementary Fig. S4B), we also considered whether CS-IVa-Be could inhibit IL6 secretion; however, CS-IVa-Be exhibited little inhibitory effect on IL6 secretion (Supplementary Fig. S4D). Overall, these results suggest that CS-IVa-Be binds to IL6R (IL6Rx and GP130) and disrupts IL6/IL6Rx/GP130 interaction.

CS-IVa-Be synergized with TRAIL to induce apoptosis in breast cancer cells

Resistance to TRAIL-induced apoptosis in many cancers limits its clinical use as an anticancer agent (26), and aberrant STAT3 activation has been suggested to be a part of the causes (27). As we have proved that CS-IVa-Be is an effective IL6/STAT3 inhibitor, we next determined whether CS-IVa-Be can synergize with TRAIL to induce apoptosis in breast cancer cells. We treated the breast cancer MDA-MB-231 cells with CS-IVa-Be alone or combined with TRAIL and analyzed cell apoptosis. The cells treated by TRAIL/CS-IVa-Be combination demonstrated obvious apoptotic morphology (Fig. 6A) and increased cell apoptosis (Fig. 6B and C).

CS-IVa-Be upregulates DR5 synergized with TRAIL

With our data showing CS-IVa-Be activating caspase-8, we hypothesized that the death receptor pathways could be involved in the CS-IVa-Be–induced apoptosis. We then investigated the effects of CS-IVa-Be on the expression of death receptors and found that CS-IVa-Be increased DR5 protein levels in breast cancer MDA-MB-231 and MCF-7 cells (Fig. 6D). We next analyzed the cell surface DR5 expression levels in CS-IVa-Be–treated MDA-MB-231 cells, which were also increased upon CS-IVa-Be treatment. In contrast, the levels of DR4 did not increase by CS-IVa-Be (Fig. 6D). It has previously been suggested that the upregulation of DR5 synergizes with TRAIL to induce apoptosis. We then examined whether CS-IVa-Be induced upregulation of DR5 synergized with TRAIL. Transfection of MDA-MB-231 cells with DR5-specific siRNA resulted in a marked inhibition of DR5 surface expression (data not shown). After the combined treatment with CS-IVa-Be and TRAIL, the apoptosis rate in the DR5 knocked down cells was significantly reduced compared with the control (Fig. 6E). We suggest that the synergized induction of apoptosis by CS-IVa-Be

Figure 5.

CS-IVa-Be disrupts IL6/IL6Rx/GP130 interaction. Cytometry analysis of the inhibitory effect of CS-IVa-Be on IL6–IL6Rx interaction in U937 cells. Biotinylated IL6 was used to label cell surface IL6Rx. A, the levels of cell surface IL6Rx in cells with CS-IVa-Be treatment or not (left). B, cell surface IL6–IL6R binding levels in cells with CS-IVa-Be treatment or not (right). C, ELISA analysis of the inhibitory effect of CS-IVa-Be on IL6–IL6Rx binding in vitro. The maximum binding of IL6 (100% bound) is defined as without CS-IVa-Be, whereas the effects of various concentrations of CS-IVa-Be were calculated as the percentages of maximum binding of IL6. MST analysis of CS-IVa-Be binding to IL6Rx, GP130, LeptinR, and LIFR. D, CS-IVa-Be–binding affinity to IL6Rx. E, CS-IVa-Be–binding affinity to GP130, LeptinR, and LIFR, respectively. F, recombinant human IL6Rx with His tag was incubated with 0, 5, or 10 μmol/L CS-IVa-Be for 2 hours, then mixed with recombinant human GP130 and IL6, and immunoprecipitated with anti-His antibody. The precipitates were washed, suspended in reducing sample buffer, and immunoblotted with anti-His antibody. These are representative results of three repetitive experiments with similar results.
and TRAIL was dependent on the DR5 upregulation, at least partially.

**Discussion**

CS-IVa-Be, a triterpenoid saponin from TCM *Acanthopanasa gracilistylus* W.W.Smith, has previously been shown to possess cytotoxic activity in many cancer cells (28), although the mechanism remains elusive. Herein, we uncovered that CS-IVa-Be decrease cell viability in various cancer cells by inducing apoptosis. Numerous signaling pathways, such as IL6/STAT3, ROS/MAPK, TNFα/NF-κB, and AKT, are involved in regulating cell proliferation and survival (29, 30). Although CS-IVa-Be treatment induced ROS production, we demonstrated that oxidative stress is not the primary cause for CS-IVa-Be-induced cell apoptosis. TNFα/NF-κB and AKT are also not involved in CS-IVa-Be-
mediated cell apoptosis. We next discovered CS-IVa-Be inhibited IL6 family cytokines induced STAT3 activation. Especially, CS-IVa-Be showed more potential inhibitory effect on IL6-induced STAT3 activation than other family cytokines (Fig. 2B and C). Interestingly, CS-IVa-Be showed no inhibitory effect on EGF or IFNγ-induced STAT3, STAT5, or STAT1 activation as well as constitutive STAT5 or STAT1 activation.

Tyrosine kinases (JAKs, Src, EGFR, FGFR1, IGF1R, and PDGFRα) are not involved in the mechanism of CS-IVa-Be-mediated IL6/STAT3 inhibition because CS-IVa-Be showed no inhibitory effect on the enzyme activity of any of the kinases. We further found that CS-IVa-Be directly binds to IL6Rα (IL6Rα and GP130) and disrupts the interaction of IL6/IL6Rα/GP130 in vitro and in cancer cells. As CS-IVa-Be showed higher affinity to IL6Rα than other IL6 family receptors (ILFRα and LeptinR), we suggest that CS-IVa-Be is a relatively specific IL6R antagonist, interfering IL6-induced STAT3 activation. In view of the fact that GP130 is a common subunit of IL6 family receptors and CS-IVa-Be can directly bind GP130, CS-IVa-Be exhibits inhibitory effect on other IL6 family cytokines (IL11, LIF, and OSM) inducing STAT3 activation. The evidence that U937 cells express relatively high levels of cell surface IL6Rα than other cancer cells (Fig. 5A and Supplementary Fig. S4A) and IL6Rα knockdown partly reversed the CS-IVa-Be–induced U937 cell apoptosis (Supplementary Fig. S4E and S4F) also demonstrate the key role of IL6Rα in mediating CS-IVa-Be–induced cancer cell apoptosis.

Many kinds of natural compounds have been reported to abolish IL6/STAT3 signaling through different mechanisms (31) and targeting IL6Rα (32) or GP130 (33), directly inhibiting JAKs (34), inducing PTPase expression (35), inhibiting STAT3 translocation (36), and blocking JAK–STAT3 interaction (37). The natural antagonist of IL6R is rarely reported except for the ERBF, which is isolated from bufadienolide and sensitizes breast cancer cells to TRAIL-induced apoptosis via inhibition of STAT3/Mcl-1 pathway (38). In accordance with their results, our data suggest CS-IVa-Be is a novel natural IL6R antagonist and shows antitumor activity.

To further confirm the inhibitory effect of CS-IVa-Be on cancer cells was via IL6/STAT3 inhibition, we investigated the GP130, pSTAT3, STAT3, IL6 secretion levels, and cell surface IL6Rα expression in different cancer cells (Supplementary Fig. S4A–S4C) and compared the sensitivity of different cancer cells to CS-IVa-Be. MDA-MB-231 cells express the highest levels of GP130, pSTAT3, and IL6 than other cancer cells determined MDA-MB-231 cell line is sensitive to CS-IVa-Be. We found that MCE-7 cell line expresses low level of IL6 and pSTAT3, but it is also sensitive to CS-IVa-Be. It has been reported that low level of IL6 is indispensable to maintain the growth of MCE-7 breast cancer cells (39). In addition, CS-IVa-Be also can bind GP130, and the expression of GP130 in MCE-7 cells is higher than other cancer cells except MDA-MB-231. We speculate that MCE-7 cells are more dependent on GP130-mediated other IL6 family cytokine–induced signaling to survive, and antagonizing GP130 by CS-IVa-Be can effectively inhibit MCF-7 viability.

TRAIL resistance in many cancers limits its therapeutic use in clinical scenarios. The STAT3-trans-regulated prosurvival genes Survivin, c-FLIP, XIAP, Bcl-xl, and Mcl-1 are involved in regulating cell apoptosis and TRAIL sensitivity to cancer cells (40). The expression levels of these genes were attenuated by CS-IVa-Be, which exhibits enhanced apoptosis in breast cancer cells via upregulating DR5 expression, which is consistent with the report that upregulation of the cell surface DR5 was dependent on the suppression of STAT3 activation (41). These studies suggest that DR5 is negatively regulated by STAT3, and the inhibition of STAT3 phosphorylation by CS-IVa-Be accounts for DR5 induction and the apoptosis enhancing synergy with TRAIL.

In summary, we characterized CS-IVa-Be as a novel natural IL6R antagonist, which inhibits IL6/STAT3 signaling, induces cancer cell apoptosis, and synergizes with TRAIL in breast cancer cells. Therefore, synergistic combination of CS-IVa-Be with TRAIL has potential to be a more effective strategy to treat cancers with aberrant IL6/STAT3 activation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Development of methodology: Y. Yang, Q. Yu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Yang, X. Cai
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Yang, S. Qian, X. Sun, Q. Yu, S.P. Gao
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