RESEARCH ARTICLE

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Plumbagin Inhibits Cadmium-Induced Interleukin-6/STAT3 Signaling in the Triple-Negative Breast Cancer Cell Line

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Abstract

Introduction: Plumbagin has been found to reduce proinflammatory cytokine expression in activated macrophages and carrageenan-induced paw edema. Cadmium triggers the release of interleukin-6 (*IL-6*), a key mediator of inflammation and carcinogenesis in many cell types. The effects of plumbagin on cadmium-induced inflammation in triple-negative breast cancer cells are unknown. **Method:** We investigated the effects of plumbagin on cadmium-induced *IL-6* expression and signal transducer and activator of transcription 3 (STAT3) activation in MDA-MB-231, a triple-negative breast cancer cell line, using real-time PCR, ELISA, and Western blotting. **Result:** Non-cytotoxic concentrations of cadmium chloride at 1 and 10 μM upregulated the IL-6 mRNA expression after 3 h of exposure and increased the *IL-6* release after 24 h. Plumbagin at 4 μM or more was toxic to cells after 24 h. Plumbagin at 1 μM co-treated with cadmium reduced the expression and secretion of *IL-6*. At 24-h post-exposure, plumbagin decreased the levels of phosphorylated STAT3 induced by cadmium. **Conclusion:** Plumbagin inhibits cadmium-induced IL-6/STAT3 signaling in a triple-negative breast cancer cell and further in vivo studies are required to elucidate the potential use of plumbagin on cancer progression.

Keywords: Plumbagin- cadmium- interleukin-6- STAT3- TNBC

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Introduction

Cadmium exposure has been associated with the development of breast cancers [1, 2]. There are reports that mean levels of cadmium in mammary tumor tissues are higher than those in adjacent tissues [3, 4]. Cadmium at 1 μM promotes cell growth and migration of MDA-MB-231 triple-negative breast cancer (TNBC) cells [5]. The median inhibitory concentration (IC50) of cadmium chloride ($CdCl_2$) was 53.52 μM in MDA-MB-231 at 24 h after exposure [6]. High blood levels of cadmium are a risk factor for distant metastasis in breast cancer patients [7]. An analysis of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 beta (IL-1β), and *IL-6* in serum showed elevated *IL-6* levels in breast cancer patients with metastatic sites. Moreover, high *IL-6* levels are associated with poor response to chemo-endocrine therapy and survival [8]. Cadmium at 1 to 60 μM increases the *IL-6* production in various cell types including A549 lung adenocarcinoma cells [9], Calu-3 bronchial epithelial cells [10], THP-1 monocytic cells [11], HepG2 hepatocyte cells [12], and U-87 MG astrocytoma cells [13]. *IL-6* triggers cell proliferation, survival, differentiation, migration, invasion, metastasis, angiogenesis, inflammation, and metabolism [14]. The accumulation of IL-6 in breast cancer activated the phosphorylation of signal transducer and activator of transcription 3 (STAT3), resulting in breast cancer progression [15].

Plumbagin, 5-hydroxy-2-methyl-1,4-napthoquinone, is isolated from the root of the medical herb Plumbago zeylanica. Plumbagin at 2 μM induces autophagy and cell cycle arrest in MCF-7 and MDA-MB-231 breast cancer cells by inhibiting phosphatidylinositol 3-kinase/AKT signaling pathway [16]. It also induces paraptosis by triggering the dilation of the endoplasmic reticulum (ER) in MDA-MB-231 cells [17]. Plumbagin inhibits STAT3 phosphorylation in breast and prostate cancers [18, 19]. Plumbagin decreases the expression of pro-inflammatory cytokines and chemokines including IL-1, *IL-6*, chemokine (C-C motif) ligand 2 (CCL_2), and TNF-α by the inhibition of nuclear factor-kappa B and mitogen-activated protein kinase signaling pathways in mice and a bone-seeking clone of MDA-MB-231 cells [18, 20, 21]. The present study aims to determine the effect of plumbagin on cadmium-induced *IL-6* expression in a

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triple-negative MDA-MB-231 breast cancer cell line.

Materials and Methods

Cell culture and chemicals

Human MDA-MB-231 cells (American Type Culture Collection, ATCC, Virginia, USA), a TNBC cell line, were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, New York, USA) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Merck, Massachusetts, USA). Cells were maintained at 37 °C in a 5% CO2 humidified atmosphere. Mycoplasma contamination was checked by MycoStrip® (InvivoGen, California, USA).

Cadmium chloride ($CdCl_2$, Cat. 202908, Sigma, Missouri, USA) was dissolved in sterile water at 1000 mM and plumbagin ($CdCl_2$, Cat. P7262, Sigma) was dissolved in dimethyl sulfoxide (DMSO, Cat. D2438, Sigma) at 100 mM and stored at -20°C. Cells were treated with the non-toxic concentrations of $CdCl_2$ at 1 or 10 μM and plumbagin at 1 μM for RNA extraction, ELISA, and western blotting. DMSO-treated cells were used as the solvent control group.

MTT assays

MDA-MB-231 cells were treated with plumbagin at concentrations of 1 to 8 μM or co-treated with cadmium at 1 and 10 μM for 24 h. At 2 h before the indicated incubation time, MTT solution was added to each well to a final concentration of 0.5 mg/ml. The formazan crystals were then dissolved in DMSO and the optical density (OD) at 562 nm was measured using a microplate reader (Varioskan Flash Microplate Reader, Thermo Fisher Scientific, Massachusetts, USA). The percentage of cell viability was calculated as a percentage in comparison to untreated cells.

Real-time polymerase chain reaction (PCR) assays

At 3 h after treatment with cadmium and plumbagin, RNA was extracted by Total RNA Mini Kit according to the manufacturer's protocol (Cat. RB300; Geneaid). Complimentary DNA (cDNA) was synthesized using superscript III reverse transcriptase (Cat. 18080-044, Invitrogen, Massachusetts, USA) and Hexanucleotide Mix (Cat. 11277081001, Sigma). Quantitative real-time PCR was performed using SensiFAST SYBR LO-ROX (Cat. BIO-94005, Bioline, London, UK) on an Applied Biosystems Real-time PCR 7500 system (ABI 7500, Applied Biosystems, Massachusetts, USA). The thermal cycling condition was 50 °C for 1 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min with a melting curve stage of 95 °C for 10 s and 60 °C for 1 min. The cDNA product was amplified with forward and reverse primers shown in Table 1. The threshold cycle value (Ct) of each target gene was normalized to the expression of *GAPDH*. The expression of genes is shown as relative fold change (RFC) calculated against untreated cells based on $2^{-\Delta\Delta Ct}$ method. The RFC value of untreated cells was defined as 1.

ELISA

After 24 h, cell culture supernatant was collected according to Phuagkhaopong S., 2017. The levels of *IL-6* were determined using a*IL-6* human ELISA kit (Thermo Fisher Scientific) following the manufacturer's instruction.

Western blotting

After 24 h, cells were washed with cold PBS and lysed by lysis buffer with protease inhibitor cocktail as previously described [22]. The protein concentrations were measured by Bradford protein assays. The protein samples were loaded into sodium dodecyl sulfate-polyacrylamide gels and were transferred into nitrocellulose membranes. Membranes were blocked by 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T). Proteins were probed with primary antibodies including phospho-STAT3 (Cat. 9145, 1:2000 dilution, Cell Signaling Technology, Massachusetts, USA), STAT3 (Cat. 9139, 1:3000 dilution, Cell Signaling Technology) and β-actin (Cat. 4970, 1:8000 dilution, Cell signaling Technology) followed by anti-rabbit horseradish peroxidase-linked secondary antibodies (1:4000, Jackson ImmunoResearch, Pennsylvania, USA). Bands were visualized by incubation with enhanced chemiluminescence substrate (Bio-Rad, California, USA). The band density of the protein of interest was determined and normalized with β-actin as an internal loading control using ImageJ software. The normalized ratio of phospho-STAT3 to STAT3 of untreated cells was defined as 1.

Statistical analysis.

Data are presented as the mean±SEM of four independent replicates. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's post-hoc test using Prism, version 5.0 (GraphPad Software Inc., San Diego, CA).

Results

Plumbagin decreased cell viability in MDA-MB-231 TNBC cells

Cells were exposed to plumbagin at concentrations of 1 to 8 μM for 24 h. The increased concentrations of plumbagin decreased cell viability in a dose-dependent manner (Figure 1A). The cell viability of DMSO-treated cells were similar to untreated cells. We selected the non-cytotoxic concentration at 1 μM to determine the anti-inflammatory effects of plumbagin. To avoid the cytotoxic effect, the maximum concentration of $CdCl_2$ used in this study was 10 μM. The cell viability of cells treated with the combination of 1 μM plumbagin and 1 or 10 μM of $CdCl_2$ was greater than 80% compared to DMSO-treated cells at 24 h (Figure 1B). Non-toxic concentrations of plumbagin and cadmium had no effect of the expression of two proliferating markers: *Ki-67* and proliferating cell nuclear antigen (*PCNA*) (Figure 2).

Plumbagin reduced IL-6 expression and secretion induced by cadmium

Cells treated with $CdCl_2$ at 1 and 10 μM showed 10.7 and 12.2-fold increase in *IL-6* mRNA levels compared

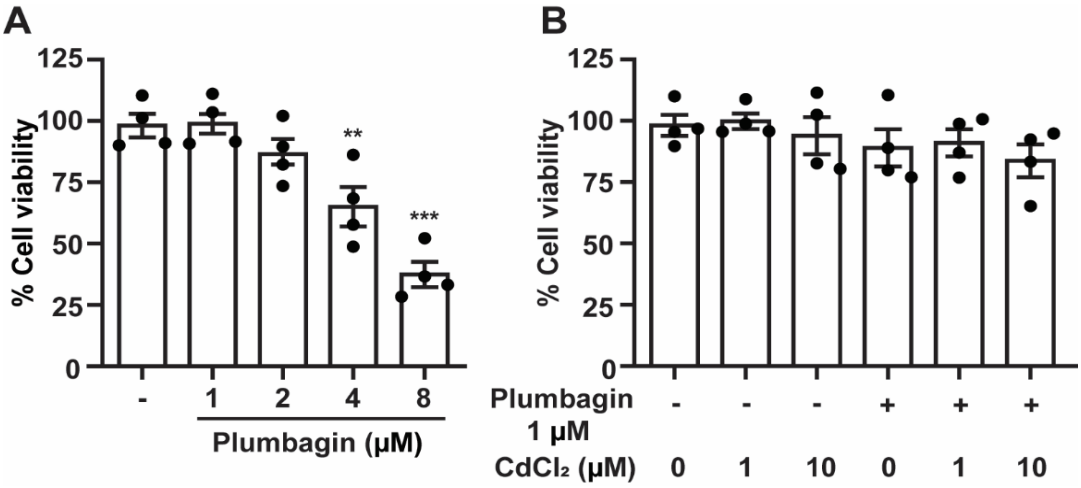

Figure 1. Effects of Plumbagin on MDA-MB-231 TNBC cell viability using MTT assays. (A) Plumbagin at 4 μM or highersignificantly decreased cell viability at 24 h. (B) The non-toxic concentrations at 1 and 10 μM of $CdCl_2$ were co-treated with plumbagin at 1 μM for 24 h. The combination treatment did not affect TNBC cell viability. Data represents the mean±SEM from 4 independent experiments. Each dot represents an individual sample. One-way ANOVA was used for statistical analysis. ** ($p < 0.01$) and *** ($p < 0.001$) compared with DMSO-treated cells.

Table 1. List of Primers

Target gene	Sequence
Ki-67	Forward: 5'-CTTTGGGTGCGACTTGACG-3'
	Reverse: 5'-GTCGACCCCGCTCCTTTT-3'
PCNA	Forward: 5'-GGCCGAAGATAACGCGGATAC-3'
	Reverse: 5'-GGCATATACGTGCAAATTCACCA-3'
IL-6	Forward: 5'-ACCCCTGACCCAACCACAAAT-3'
	Reverse: 5'-AGCTGCGCAGAATGAGATGAG-3'
CCL_2	Forward: 5'-GCGAGCTATAGAAGAATCACC-3'
	Reverse: 5'-ATAAAACAGGGTGTCTGGGG-3'
MMP-2	Forward: 5'-TTGACGGTAAGGACGGACT-3'
	Reverse: 5'-CTTGCAGTACTCCCCATCG-3'
MMP-9	Forward: 5'-TTGACAGCGACAAGAAGTGG-3'
	Reverse: 5'-CCCTCAGTGAAGCGGTACAT-3'
GAPDH	Forward: 5'AGCCTTCTCCATGGTGGTGAAGAC-3'
	Reverse: 5'-CGGAGTCAACGGATTTGGTCG-3'

with DMSO-treated MDA-MB-231 TNBC cells at 3 h (Figure 3A). Plumbagin at 1 μM did not decrease *IL-6* mRNA levels, while co-treatment of plumbagin and cadmium reduced *IL-6* mRNA levels compared with cells treated with cadmium. Similarly, $CdCl_2$ at 1 and 10 μM increased *IL-6* secretion by 4.1 and 4.0 folds, and plumbagin at 1 μM reduced cadmium-induced *IL-6* secretion at 24 h (Figure 3B). Cadmium did not induce the expression of *CCL_2* and MMP9 (Figure 4). DMSO did not alter levels of *IL-6*, *CCL_2*, and MMP9 mRNA expression.

Plumbagin decreased the phosphorylation of STAT3 in cadmium-treated MDA-MB-231 TNBC cells

MDA-MB-231 TNBC cells were treated with $CdCl_2$ at 1 and 10 μM and plumbagin at 1 μM for 24 h. $CdCl_2$ at 10 μM increased the ratio of phospho-STAT3/STAT3. Plumbagin decreased the ratio of phospho-STAT3/STAT3 in cadmium-induced cells at 1 and 10 μM compared with

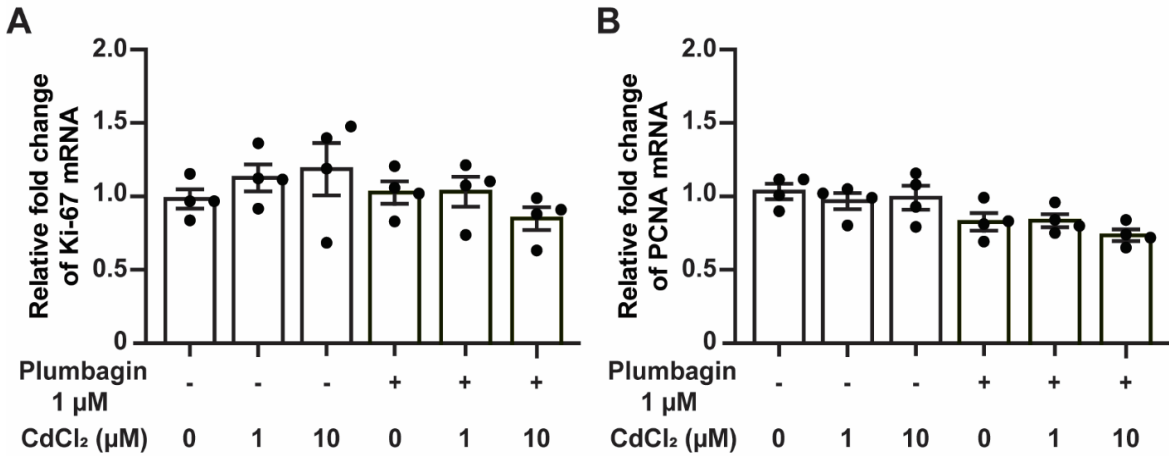

Figure 2. Effects of Plumbagin on Cell Proliferation of MDA-MB-231 TNBC Cells. The non-toxic concentrations at 1 and 10 μM of $CdCl_2$ were co-treated with plumbagin at 1 μM for 3 h and mRNA levels were determined by real-time PCR. $CdCl_2$ and plumbagin did not alter the expression of proliferating markers: (A) Ki-67 and (B) Proliferating cell nuclear antigen (PCNA). Data represents the mean±SEM from 4 independent experiments. Each dot represents an individual sample. One-way ANOVA was used for statistical analysis.

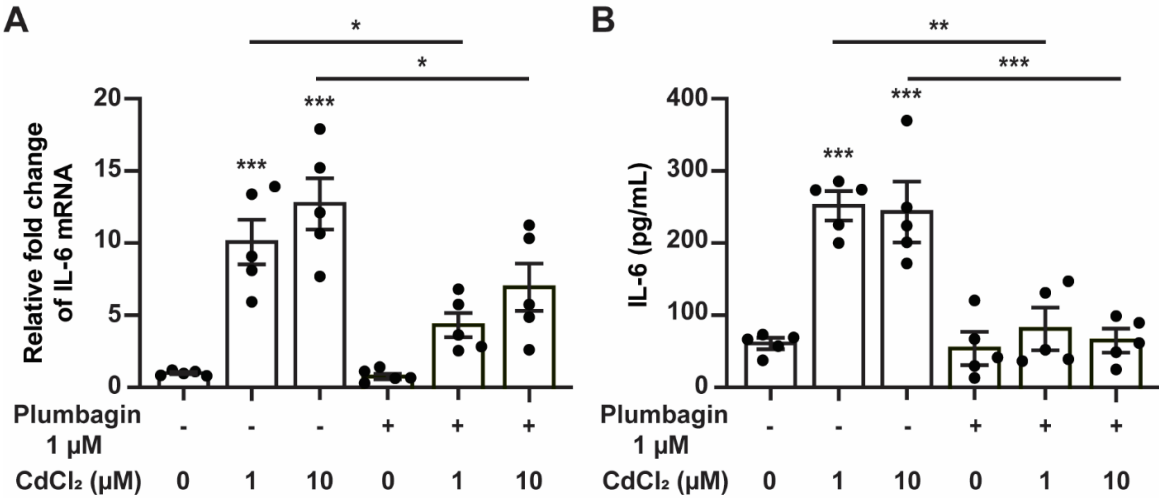

Figure 3. Plumbagin-reduced IL-6 Expression and Secretion in MDA-MB-231 TNBC Cells Exposed to Cadmium. Levels of IL-6 mRNA and protein in MDA-MB-231 TNBC cells were determined by real-time PCR and ELISA, respectively. (A) Plumbagin at 1 μM reduced cadmium-induced IL-6 mRNA transcripts at 3 h post-exposure. (B) Plumbagin at 1 μM decreased cadmium-induced IL-6 at 24 hours after treatment. Each bar represents the mean±SEM of 5 independent experiments. Each dot represents an individual sample. One-way ANOVA was used for statistical analysis. * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

$CdCl_2$-treated cells (Figure 5). DMSO did not alter levels of STAT3 phosphorylation.

Discussion

The present study showed that low levels of cadmium trigger the release of *IL-6* and STAT3 phosphorylation in MDA-MB-231 cells. The upregulation of *IL-6* and phospho-STAT3 by cadmium in A549 lung cancer is associated with inflammation and cell proliferation [9]. The dose of cadmium in this study is comparable to the previous study in A549 lung adenocarcinoma cells [9]. The activation of STAT3 by *IL-6* plays a role in breast cancer progression and metastasis by increasing the levels of cyclin D1, Bcl-2, Bcl-xL, vascular endothelial growth factor, and matrix metalloproteinases [23, 24]. However, the low doses of cadmium in the present study did not increase proliferation. STAT3 also induces the expression of *IL-6*, creating an autocrine and paracrine positive feedback loop. Therefore, the reduction of *IL-6* expression and secretion could be caused by the inhibition of STAT3 activation. TNBC is aggressive and shows resistance to chemotherapy. The current targeted therapy is ineffective for TNBC due to the lack of estrogen receptors, progesterone receptors, and human epidermal growth factor receptors. Inhibitors of *IL-6*, *IL-6* receptors, and STAT3 are currently been investigated for cancers, especially TNBC.

Plumbagin at 2 μM or more is cytotoxic and inhibits cell proliferation of MDA-MB-231 [16, 17, 25]. Herein, plumbagin at 1 μM did not decrease the proliferation of MDA-MB-231 similar to a previous experiment [25].

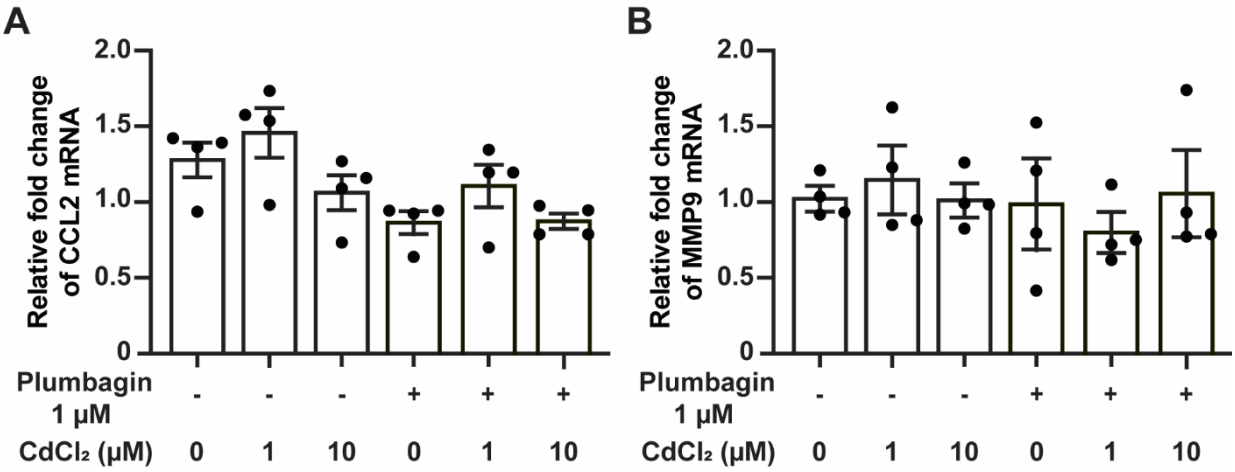

Figure 4. No induction of CCL_2 and MMP9 by cadmium in MDA-MB-231 TNBC cells. Levels of CCL_2 (A) and MMP9 (B) mRNA were determined by real-time PCR. Each bar represents the mean±SEM of 4 independent experiments. Each dot represents an individual sample. One-way ANOVA was used for statistical analysis.

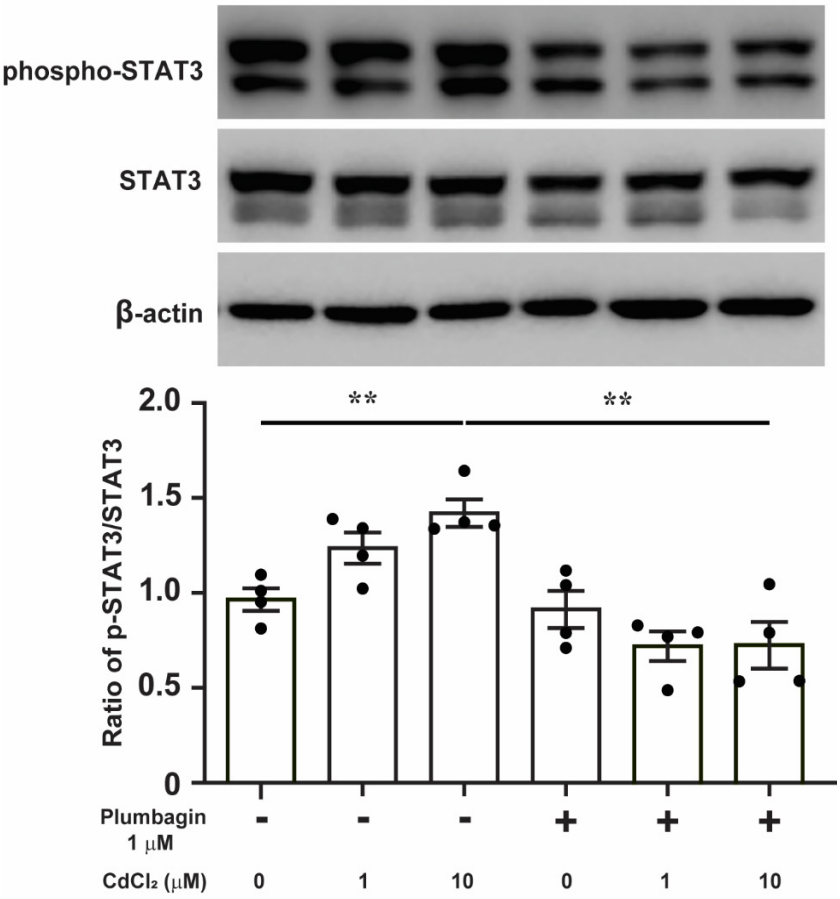

Figure 5. Plumbagin-Reduced Phosphorylation of STAT3 Expression in MDA-MB-231 TNBC Cells Exposed to Cadmium. MDA-MB-231 cells were treated with 1 and 10 μM CdCl2 and 1 μM of plumbagin. Levels of phospho-STAT3 and STAT3 were determined using Western blotting. CdCl2 at 10 μM increased the ratio of phospho-STAT3/STAT3, while similar levels of total-STAT3 or β-actin were detected at 24 h post-exposure. Each bar represented the mean±SEM of 4 independent experiments. Each dot represents an individual sample. One-way ANOVA was used for statistical analysis ** ($p < 0.01$).

Plumbagin at 4 μM reduced CCL_2 release from MDA-MB-231 cells induced by TNFα. We did not observe the inhibition of CCL_2 upregulation likely due to minimal effects of cadmium on CCL_2 production in MDA-MB-231 cells. Plumbagin at 5 μM or more decreased the migration and p-STAT3 expression in a bone-seeking clone of MDA-MB-231 cells [18]. In the present study, cadmium did not induce MMP9 expression, and plumbagin showed no effect on the expression of MMP9. The difference from the previous result by Yan and colleagues [18] may be caused by the difference between cells obtained from ATCC and a clone selected for bone metastasis. Plumbagin at less than 3 μM is less toxic to normal breast epithelial cell MCF-10A. According to a guide to in vitro pharmacological studies from EORTC-PAMM, the suggested concentrations range from 10 nM to 100 μM, given that the concentrations used in the present study is not cytotoxic; therefore, plumbagin could be further developed to reduce inflammation in cancer tissues.

In conclusion, the dual ability of plumbagin to reduce *IL-6* release and inhibit STAT3 activation could disrupt the *IL-6*/STAT3 signaling pathway. Plumbagin could be developed as an inhibitor of the *IL-6*/STAT3 signaling pathway; however, further in vitro and in vivo studies in other types of cancers are required.

Author Contribution Statement

TT was involved in conceptualization, methodology, data analysis, and writing the original draft. RS was involved in methodology, data analysis, and writing the original draft. TK was involved in conceptualization and manuscript editing. PV was involved in conceptualization, supervision, and manuscript writing. TT and RS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

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Data Availability Statement

All data generated or analyzed during this study are

included in this article. Further inquiries can be directed to the corresponding author.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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