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ORIGINAL RESEARCH

Andrographolide Reduces Cytokine Release and Cyclooxygenase-2 Expression by Inhibiting the JNK and NF-κB Pathways in Glioblastoma Cells Exposed to Cadmium

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Background: Neuroinflammation is associated with brain cancer and several neurodegenerative diseases. At nontoxic concentrations, the environmental pollutant cadmium is known to increase the secretion of pro-inflammatory cytokines, including interleukin (IL)-6, IL-8, and chemokine (C-C motif) ligand 2 (CCL2) by activating the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) pathways. Andrographolide, a diterpenoid lactone, exhibits anti-inflammatory and antioxidant activity in vitro and in vivo. Hence, in this study, we aimed to determine the effects of andrographolide on cadmium-induced inflammation and the underlying mechanisms in U-87 MG glioblastoma cells.

Methods: U-87 MG cells, obtained from American Type Culture Collection (ATCC), are adherent cells derived from malignant gliomas and express the astrocyte cell marker glial fibrillary acidic protein. Cell viability was measured using the methyl thiazolyl tetrazolium (MTT) assay. Human IL-6, IL-8, and CCL2 levels were measured using enzyme-linked immunosorbent assays (ELISAs). Cyclooxygenase-2 (COX-2) and the proteins involved in the MAPK and NF-κB pathways were detected via Western blotting.

Results: Treating cells with andrographolide or cadmium alone or in combination did not alter cell viability. Andrographolide decreased cadmium-induced IL-6, IL-8, and CCL2 release and downregulated cadmium-induced COX-2 expression. Andrographolide also reduced the levels of cadmium-induced phospho-Jun N-terminal kinase (JNK) and phospho-p65.

Conclusion: In this study, andrographolide exerted an anti-inflammatory effect on cadmium-induced inflammation by inhibiting the JNK and NF-κB pathways. These findings have implications for the development of therapies for cadmium poisoning since the efficacy of current therapeutic approaches is limited.

Keywords: andrographolide, cadmium, inflammation, MAPK, NF-κB

Introduction

Cadmium, a heavy metal commonly used in industrial processes, has harmful biological effects and has been detected in soil, plants, dangerous fumes, and particulate matter (PM 2.5).[1–3] To date, the most prominent instance of cadmium intoxication has been the occurrence of itai itai disease in Japan.[4] Industrial mining in the high mountain areas of the Japanese Alps caused cadmium contamination of the water, soil, and rice fields of the Toyama Prefecture. Daily consumption of rice and agricultural products in the region led to systemic cadmium-related damage, particularly to the bones, kidneys, and other internal organs.[5,6] Cadmium is primarily absorbed through inhalation of airborne particulate in industrial environments and tobacco smoke, while secondary exposure occurs through oral ingestion of contaminated dust, food or water.[7] Field surveys conducted across 30 sites in Japan reported dietary intake of cadmium ranging from 12.5 to 70.5 μg/day, with cadmium levels in blood and urine ranging from 0.46 to 9.38 μg/L and 1.16 to 11.02 μg/g

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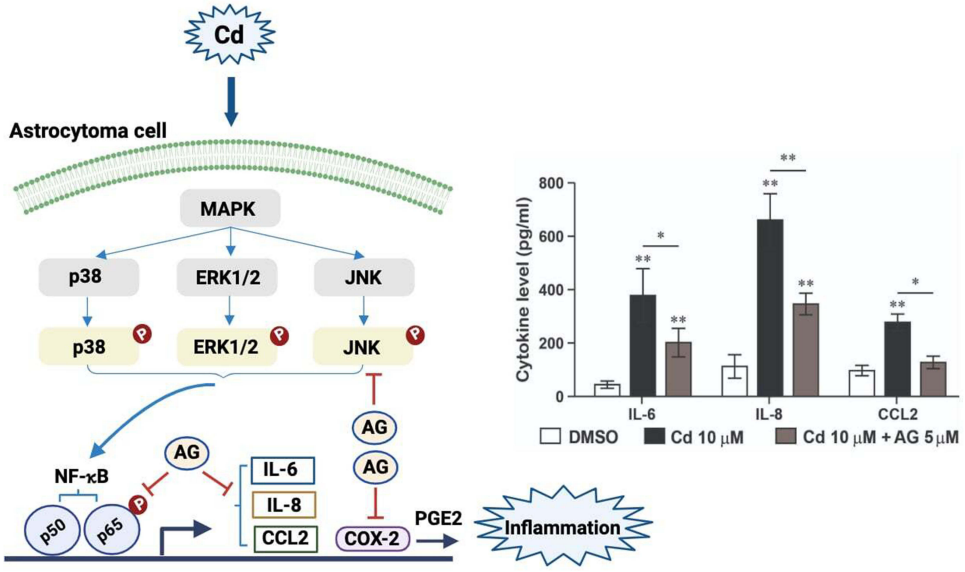
Graphical Abstract



creatinine, respectively.[8] While the mean concentrations for cadmium in whole blood and serum of residents from mining communities in Ghana were 303 ±117 μg/L and 134 ± 12 μg/L, respectively.[9]

After being absorbed into the bloodstream, cadmium predominantly forms complexes with albumin in the blood and accumulates in the liver. Over time, cadmium accumulates in the liver, kidneys, bone, lung, central nervous system, and other organs.[10] Cadmium enters the brain via the nasal mucosa or olfactory pathways into the central nervous system. Cadmium disrupts the blood-brain barrier permeability in human and animals, leading to its accumulation in the brain.[11,12] The average and highest levels of cadmium detected in the brain tissue of patients with brain tumors were 2.02 ug/g and 72.79 ug/g wet tissue, respectively.[13] Due to its low rate of excretion from the body, cadmium has a very long biological half-life in the human body, ranging from 16 to 30 years,[7] Only 0.01–0.02% of the total cadmium burden is excreted daily through urine and feces.[14] The current acceptable biological limit of blood cadmium in human (threshold limit value) is 5 μg/L according to the American Conference of Governmental Industrial Hygienists.[10] Regarding the treatment options for cadmium poisoning, there is limited information available on the current therapies used to treat cadmium intoxication.[15] Treatment with the commonly used chelator ethylenediaminetetraacetic acid (EDTA) is not recommended due to the risk of kidney damage, and the efficacy of activated charcoal (as a universal absorbent) remains unknown.[16]

Increased levels of cytokines and chemokines, including interleukin (IL)-6, IL-8, and chemokine (C-C motif) ligand 2 (CCL2), have been linked to neurodegenerative disorders, psychiatric disorders, and gliomagenesis.[17–20] It has been suggested that astrocytes, which are specialized glial cells, are a major source of IL-6, IL-8, and CCL2, as well as cyclooxygenase-2 (COX-2).[18,21] Furthermore, epidemiological studies have shown that cadmium exposure is a risk factor for brain cancers and Alzheimer's disease.[22,23] In previous studies, we showed that treating human astrocytes with nontoxic concentrations of cadmium (1 and 10 uM) led to the release of IL-6, IL-8, and CCL2 via activation of the mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), phosphoinositide-3-kinase, and protein kinase C signaling pathways.[24,25] In addition, others have reported that cadmium increased COX-2 levels in C6 rat glioma and mouse neuronal cells.[26,27]

Andrographolide, a diterpenoid lactone extracted from the plant *Andrographis paniculata*, is recognized for its antioxidant and anti-inflammatory effects.[28] It has a molecular weight of 350.4 g/mol, an XlogP value of 2.2, a hydrogen bond donor count of 3, and a hydrogen bond acceptor count of 5.[29,30] A pharmacokinetic study revealed

that andrographolide accumulated in brain tissues after oral administration.[31] It was also found to suppress microglial activation and IL-6 production in a rat brain-injury model[32] and tumor necrosis factor (TNF)-α-stimulated IL-8 expression in human colorectal cancer.[33] In another study, orally administered andrographolide attenuated lipopolysaccharide (LPS)-induced CCL2 expression in rodent cortex and primary astrocytes.[34] Andrographolide has also been shown to inhibit amyloid-beta 42-induced COX-2 expression in microglia.[35,36] Given that andrographolide has antioxidant and anti-inflammatory properties and satisfies Lipinski's "rule of five" for development as an oral drug candidate,[37] it is worth examining this compound as a therapeutic agent for cadmium poisoning.

Therefore, in this study, we aimed to investigate the anti-inflammatory effect of andrographolide on cadmium-induced cytokine secretion and COX-2 expression in human astrocytes. Given that 20 μM cadmium has been shown to induce apoptosis in human astrocytes,[38] we capped the concentration at 10 μM in this study. It is anticipated that the findings of this study will be used in future explorations of common medicinal plants as therapeutic agents for cadmium poisoning.

Material and Methods

Cell Line and Chemicals

Cells from the U-87 MG astrocyte cell line were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin–streptomycin (Gibco, Grand Island, New York, USA) at 37 °C in a humidified chamber with 5% CO_2. Cells were used from the fifth to the twelfth passage. Cadmium chloride ($CdCl_2$) (Sigma, Saint Louis, USA) was dissolved in sterile water at 1 M and stored at 20 °C until use. Andrographolide (Sigma, Saint Louis, USA) was dissolved in dimethyl sulfoxide (DMSO) at 20 mm and stored at 20 °C until use.

Measurement of Cell Viability

A previous study in primary rat astrocytes demonstrated that andrographolide was not cytotoxic at concentration up to 5 μM.[39] Therefore, we selected andrographolide in the concentration range of 1–20 μM to observe the anti-inflammatory effect. Cells were seeded in 96-well plates and treated with andrographolide (up to 20 μM) with or without $CdCl_2$ (10 μM) for 24 h. Methyl thiazolyl tetrazolium (MTT) solution was added (final concentration: 0.5 mg/mL) for 2 h at 37 °C. The formazan crystals that formed in the cells were dissolved in DMSO, and the absorbance at 562 nm was measured using a microplate reader (Biotek, Santa Clara, California, USA). The cell viability of the treated cells was calculated as the percentage of the cell viability of untreated cells.

Measurement of IL-6, IL-8, and CCL2 secretion

Cells were cultured in 12-well plates and treated with $CdCl_2$ (10 μM) with or without andrographolide at 1 to 20 μM for 24 h. The supernatant was collected, centrifuged at 5000 rpm for 5 min to remove residual cells, aliquoted, and stored at −80 °C until tested. IL-6, IL-8, and CCL2 levels were measured using specific enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Carlsbad, California, USA). Microtiter plates were coated with antibodies against IL-6, IL-8, or CCL2 overnight at 4 °C. Following a blocking step, samples and standards at various dilutions were added in duplicate and incubated for 2 h. The bound cytokines were detected by adding biotin-conjugated anti human cytokine (IL-6, IL-8, CCL2) antibody for 1 h followed by the addition of streptavidin bound to horseradish peroxidase for 30 min. After washing, tetramethylbenzidine (TMB) substrate solution was added and the enzymatic reaction was stopped with 2N sulfuric acid within 10 min. The absorbance was measured at 450 nm using a microplate reader (BioTek, USA). The cytokine levels were quantified using standard curves (Supplementary Figure 1) and reported in pg/mL.

Detection of Protein Expression via Western Blotting

Cells were grown in 60 mm dishes and treated with $CdCl_2$ (10 μM) with or without andrographolide (5 μM) for 24 h. Cells were lysed in ice-cold lysis buffer [20 mm Tris, 1% NP-40, 50 mm NaCl and Protease Inhibitor Cocktail (PIC) Set III (1:1000, Calbiochem, La Jolla, CA)]. Proteins were harvested using cell scrapers, then centrifuged at 12000 rpm for 15 min at 4 °C. Cell supernatant was collected, and total protein levels were measured colorimetrically using the Bradford Protein Assay (Bio-Rad).

Twenty-five micrograms of protein were loaded onto 10% SDS-polyacrylamide gel, separated by electrophoresis, and transferred onto nitrocellulose membranes (Bio-Rad). Subsequently, membranes were blocked with 5% non-fat dry milk (Bio-Rad) in 0.5% TBS-T (Tris-Buffered Saline with Tween 20) at 4 °C overnight and probed with primary antibodies (Cell Signaling Technology, Danvers, Massachusetts, USA) against phospho-p42/44 MAPK (Extracellular signal-regulated kinase isoform 1 and 2, ERK1/2; cat. no. 4370), p42/44 MAPK (cat. no 4695), phospho-JNK (cat. no. 4668), JNK (cat. no. 9252), phospho-p38 (cat. no. 9215), p38 MAPK (cat. no. 9212), phospho-p65 (cat. no. 3033), p65 (cat. no. 8242), and COX-2 (cat. no. 12282) for 2 h at room temperature (Cell Signaling Technology, Danvers, Massachusetts, USA). Beta-actin used as an internal loading control was detected by anti-beta-actin antibody (Cell Signaling Technology, Danvers, Massachusetts, USA, cat. no. 3700) for 45 min at room temperature. After that, membranes were incubated with anti-rabbit conjugated with horseradish peroxidase enzymes for 1 h at room temperature. Chemiluminescent signal was visualized using Clarity Western ECL substrate (Bio-Rad) and band intensities were examined using ImageJ software (version 1.54; National Institutes of Health and the Laboratory for Optical and Computational Instrumentation). The expression of the proteins of interest was normalized using untreated cells.

Statistical Analysis

All results are reported as mean ± standard error mean (SEM). Data were subjected to a one-way analysis of variance (ANOVA) using Prism software (GraphPad, USA). A post hoc analysis was conducted using Tukey's test, and differences were considered statistically significant when p was < 0.05.

Results

Effect of $CdCl_2$ and Andrographolide on Cell Viability

We first determined the concentration at which andrographolide exhibited a cytotoxic effect. More than 80% of the U-87 MG cells remained viable after exposure to andrographolide at concentrations of up to 20 μM. Within this same concentration range (up to 20 μM), andrographolide did not impact the cytotoxic effect that $CdCl_2$ had on the U-87 MG cells at 24 h (Figure 1).

Inhibition of $CdCl_2$-Induced IL-6, IL-8, and CCL2 Release by Andrographolide

The supernatant collected from the cells treated with $CdCl_2$ showed higher levels of IL-6, IL-8, and CCL2 compared with that from the DMSO-treated cells. As shown in Figure 2, exposure to andrographolide at 5 μM or a higher concentration resulted in less $CdCl_2$-induced IL-6, IL-8, and CCL2 secretion. Therefore, we selected a concentration of 5 μM for further studies.

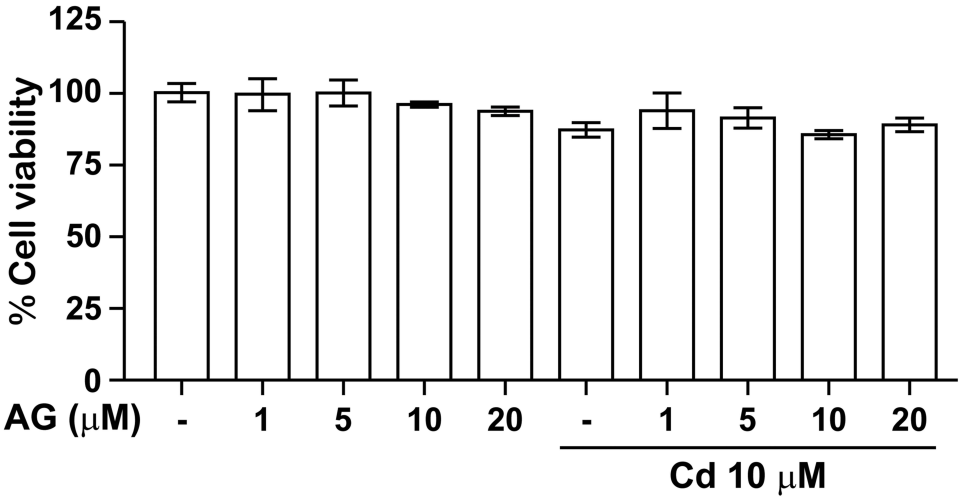


Figure 1 Andrographolide (AG) was not toxic to U-87 MG cells. Cells were treated with andrographolide at concentrations of up to 20 μM, and cell viability was evaluated via MTT assay. Co-treating the cells with andrographolide and $CdCl_2$ (Cd) did not reduce their viability. The results are representative of four independent experiments and expressed as mean ± SEM.

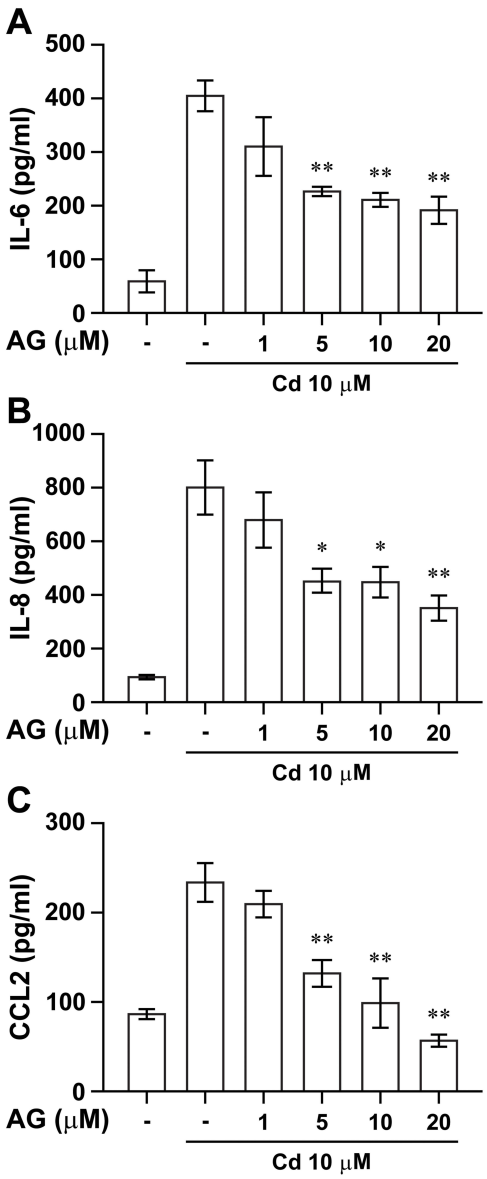


Figure 2 Andrographolide (AG) inhibited $CdCl_2$-mediated cytokine secretion in U-87 MG cells. (**A**) The IL-6, (**B**) IL-8, and (**C**) CCL2 present in the supernatant were analyzed via ELISA after 24 h. The cells in the andrographolide-treated group showed lower levels of cytokines compared to the cells in the $CdCl_2$-treated group. The results are representative of four independent experiments and expressed as mean ± SEM. A one-way ANOVA was performed; * indicates $p < 0.05$, and ** indicates $p < 0.01$.

Inhibition of $CdCl_2$-Induced COX-2 Expression by Andrographolide

Exposure to 10 μM $CdCl_2$ stimulated COX-2 expression at 24 h, and exposure to andrographolide had no effect on COX-2 expression. Co-treating cells with $CdCl_2$ and andrographolide resulted in lower levels of COX-2 than treatment with $CdCl_2$ only (Figure 3).

Reduction of $CdCl_2$-Induced JNK and NF-κB Activation by Andrographolide

The activation of the MAPK and NF-κB pathways was determined by measuring the levels of associated phosphorylated proteins. It was found that andrographolide suppressed $CdCl_2$-induced phosphorylation of JNK and p65 NF-κB; however, it did not alter the levels of phospho-ERK1/2 and phospho-p38 MAPK (Figures 4 and 5).

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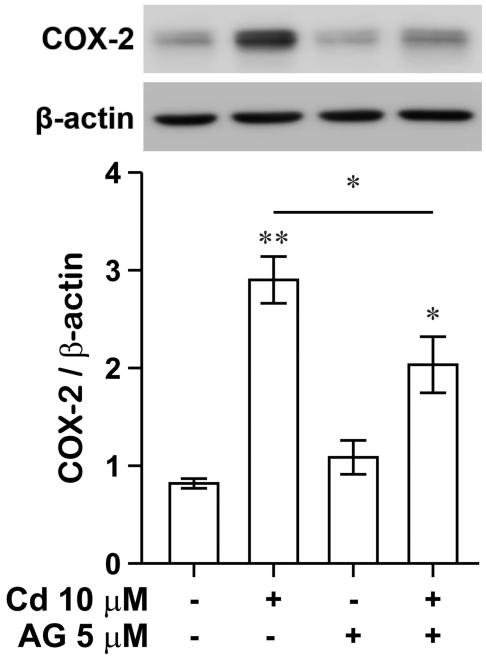

Figure 3 Andrographolide (AG) inhibited $CdCl_2$-induced COX-2 expression in U-87 MG cells. COX-2 was detected via Western blotting. The results are representative of four independent experiments. One-way ANOVA was used for statistical analysis; * indicates $p < 0.05$, and ** indicates $p < 0.01$.

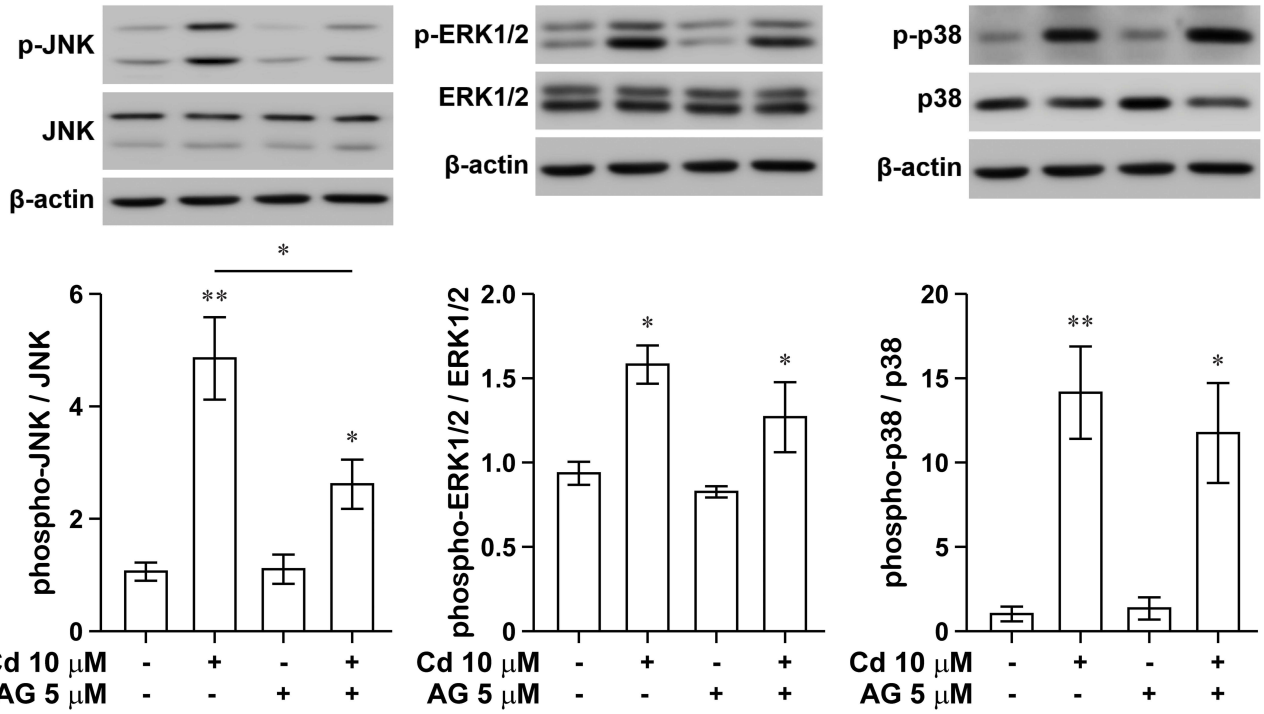

Figure 4 Andrographolide (AG) reduced $CdCl_2$-induced activation of JNK in U-87 MG cells. (**A**) The level of phospho-JNK in the cells treated with $CdCl_2$ and andrographolide was lower than in the cells treated with $CdCl_2$ only. (**B** and **C**) The levels of phospho-ERK1/2 and phospho-p38 in the cells treated with $CdCl_2$ and/or AG were similar. The results are representative of four independent experiments. One-way ANOVA was used for statistical analysis; * indicates $p < 0.05$, and ** indicates $p < 0.01$.

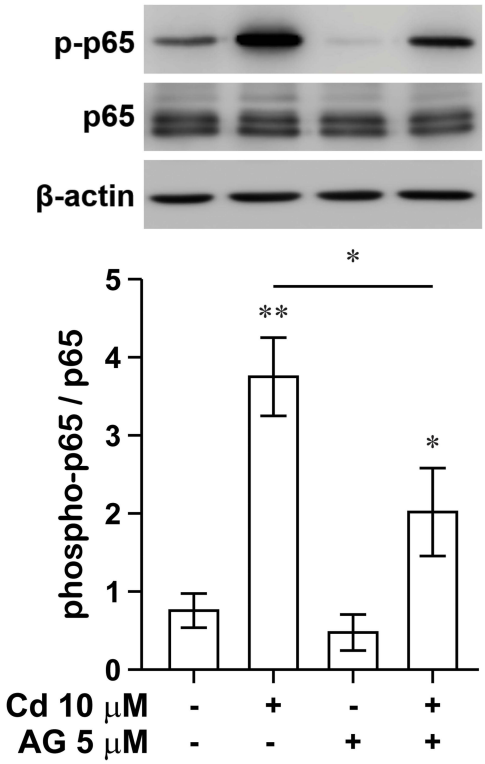

Figure 5 Andrographolide (AG) reduced $CdCl_2$-induced NF-κB activation in U-87 MG cells. $CdCl_2$ triggered an increase in phosphor-p65 levels. Co-treatment with $CdCl_2$ and andrographolide resulted in lower phosphor-p65 levels. The results are representative of four independent experiments. One-way ANOVA was used for statistical analysis; * indicates $p < 0.05$, and ** indicates $p < 0.01$.

Discussion

The uncontrollable inflammation mediated by cadmium could contribute to the pathogenesis of neurodegenerative diseases and brain cancers. The results of this study suggest that the pro-inflammatory effect of cadmium was at least partially negated by the anti-inflammatory effect of andrographolide in U-87 MG cells. In this study, nontoxic concentrations of andrographolide reduced the secretion of cytokines and chemokines stimulated by $CdCl_2$, including IL-6, IL-8, and CCL2. Moreover, the expression of $CdCl_2$-induced COX-2, phospho-JNK, and phospho-p65 was decreased in cells treated with andrographolide. The andrographolide dose used in the present study is in line with doses used in previous studies. For example, andrographolide at 10 μM has been shown to reduce IL-6, prostaglandin E2 (PGE_2), and COX-2 levels in the microglial cell line BV-2.[35] In addition, andrographolide at 30 μM significantly attenuated LPS-mediated C-C and C-X-C chemokines, including CCL2, in cultured primary astrocytes from mice.[34] It is also worth noting that andrographolide can penetrate the blood–brain barrier (BBB) and mediate a protective effect in the brain, especially during active neuroinflammation when BBB permeability is disrupted by cytokines and chemokines.[31,34,40] A previous study reported that andrographolide can reduce BBB disruption and cerebral edema after traumatic brain injury.[32] These findings provide further support for the development of andrographolide as a therapeutic agent for cadmium poisoning, and particularly for treating cadmium-induced inflammation in astrocytes.

We previously showed that inhibiting the MAPK and NF-κB pathways reduces cadmium-induced IL-6, IL-8, and CCL2 release in U-87 MG cells.[24,25] Andrographolide has been shown to act as an NF-κB inhibitor by blocking components of the signal transduction pathway that leads to NF-κB activation, such as MAPK, or inhibiting NF-κB translocation into the nucleus and its binding to its target genes. For example, in a rat traumatic brain injury model, andrographolide (1 mg/kg) inhibited microglial activation and the expression of pro-inflammatory cytokines, including IL-6, by inhibiting MAPK and NF-κB activation.[32] Andrographolide reportedly reduced the levels of pro-inflammatory mediators by downregulating phosphorylated-NF-κB in the nucleus of BV-2 cells,[35] and by downregulating JNK and NF-κB activation in primary astrocytes.[34] Andrographolide at 5 μM reduced IκBα phosphorylation and nuclear translocation

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via upregulation of miR-210, leading to the inhibition of IL-6 and IL-8 secretion in human alveolar epithelial A549 cells.[41] In another study, andrographolide at 10 μM inhibited LPS-induced PGE_2 and IL-6 in human keratinocyte cells by preventing IκBα degradation.[42] At a higher concentration (50 μM), andrographolide inhibited NF-κB p65 nuclear translocation through activation of protein phosphatase 2A, leading to dephosphorylation of the NF-κB p65 subunit at Ser536 in vascular smooth muscle cells.[43] Herein, andrographolide reduced JNK and p65 phosphorylation in U-87 MG cells treated with $CdCl_2$ and had no effect on ERK1/2 and p38 MAPK phosphorylation. These results suggest that andrographolide reduced $CdCl_2$-induced cytokine and chemokine release by inhibiting the JNK and NF-κB pathways.

COX-2 expression is regulated by several transcription factors (including NF-κB), and the COX-2 promoter region contains a putative NF-κB binding site.[44] Andrographolide at 10 μM has been found to directly interfere with NF-κB binding to DNA in HL-60 cells and thus reduce COX-2 expression.[45] At the same concentration, andrographolide also inhibited the expression of COX-2 and its downstream product PGE_2 in microglial BV-2 cells and LPS-stimulated RAW264.7 cells.[35,46] At a dose of 0.1 mg/kg (i.p)., andrographolide decreased the production of PGE_2 through the inhibition of the NF-κB pathway in rats with cerebral ischemia.[47] Furthermore, molecular dynamic simulation studies have indicated that andrographolide is a potent COX-2 inhibitor.[48] Our results align with these findings and further imply that andrographolide has potential as a therapeutic agent against cadmium poisoning.

Andrographolide is a major bioactive component of well-known medicinal plants utilized in Southeast Asia countries.[49] *A. paniculata* is documented in World Health Organization monographs on selected medicinal plants as active against upper respiratory tract infections.[50] In a recent study, we showed that an *A. paniculata* extract could reduce IL-6 and systemic inflammation induced by SARS-CoV-2 in hamster models compared with a vehicle control.[51] In addition, the *A. paniculata* extract showed minimal toxicity in the SARS-CoV-2-infected hamsters up to a dosage of 1000 mg/kg/day for 7 days. Reducing inflammation is a key goal in the treatment of heavy metal poisoning, and the anti-inflammatory activity of andrographolide demonstrated in vitro shows its promise as a therapeutic agent. However, it is essential to conduct further in vivo experiments to confirm the beneficial properties of andrographolide and its activity against cadmium poisoning. Given that environmental contamination with cadmium is rising due to its use in multiple industries,[3] there is an urgent need to develop effective therapies for cadmium poisoning, and andrographolide is a promising candidate.

Currently, there is little evidence for andrographolide in clinical research. For example, oral administration of andrographolide 5 mg/kg body weight for 3 weeks, escalating to 10 mg/kg body weight for the next 3 weeks, and to 20 mg/kg body weight for a final 3 weeks increased the $CD4^+$ lymphocyte count in HIV patients in a Phase I trial.[52] A systemic review of safety and efficacy in the treatment of upper respiratory tract infections reported that oral administration of *A. paniculata* containing andrographolide 60–360 mg/day in duration of 3–8 days is superior to placebo in alleviating the subjective symptoms of uncomplicated upper respiratory tract infection and adverse events were generally mild and infrequent.[53] Another phase I pilot study, patients with relapsing-remitting multiple sclerosis orally treated with one tablet containing 170 mg of *A. paniculata* purified extract (total andrographolides: 85 mg per tablet) every 12 h for 12 consecutive months showed a 44% reduction in fatigue compared to placebo and patients were well tolerated.[54] The use of *A. paniculata* extract in COVID-19 patients (pilot study) with dose containing andrographolide 180 mg/day or 360 mg/day is expected to be effective for use in the co-treatment of the early symptoms of COVID-19.[55,56] Therefore, taking oral andrographolide 60–360 mg/day in short period 3–8 days and 170 mg/day for 12 months could be safe for the patients according to clinical research. However, the recommended dosage may vary for different patients with certain diseases. Large-scale randomized controlled trials are required to confirm whether andrographolide is effective and safe in the treatment of related diseases in clinical practice.

Conclusions

Epidemiological studies have shown that cadmium exposure is a risk factor for brain cancers (standardized incidence ratio 1.26)[57] and Alzheimer's disease (hazard ratio 3.83).[58] In this study, andrographolide reduced cadmium-induced cytokine release and COX-2 expression in human astrocytes by attenuating the JNK and NF-κB pathways. Our findings suggest that andrographolide has therapeutic potential for the treatment of cadmium-induced inflammation. Further in vivo experiments are required to confirm the efficacy of andrographolide against cadmium poisoning in animal models.

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Disclosure

The authors have no conflicting interests.

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