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GERANYLGERANYLACETONE ATTENUATES CISPLATIN-INDUCED REDUCTIONS IN CELL VIABILITY BY SUPPRESSING THE ELEVATION OF INTRACELLULAR P53 CONTENT WITHOUT HEAT SHOCK PROTEIN INDUCTION

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ABSTRACT

Geranylgeranylacetone (GGA) was originally used as an anti-ulcer drug to protect gastric mucosa from various stresses, and it is also known to induce heat shock proteins (HSPs), especially HSP70. However, it remains unclear how GGA affects cellular functions in the presence of anti-cancer drugs. We investigated the effects of GGA on cellular viability, caspase-3 activation, HSP induction and p53 content in the presence of cisplatin (CDDP). Rat intestinal epithelium-derived IEC-18 cells and human colon cancer-derived CW-2 cells were incubated with GGA in the presence of CDDP, and we observed that GGA attenuated CDDP-induced viability reductions. GGA also suppressed CDDP-induced caspase-3 activation. However, GGA induced neither HSP70 nor GRP78 expression in the presence of CDDP. We found that GGA suppressed the CDDP-induced elevation of intracellular p53 content. In conclusion, GGA attenuates viability reductions and caspase-3 activation in CDDP-treated cells by suppressing the elevation of intracellular p53 content without HSP induction.

Key Words: Geranylgeranylacetone, Heat shock protein 70, p53, Cisplatin

INTRODUCTION

Geranylgeranylacetone (GGA), an isoprenoid compound, has been developed in Japan as an anti-ulcer drug. GGA also increases the synthesis and secretion of gastric mucin[1)] as well as the components of high molecular weight glycoproteins[2)] and surface-active phospholipids.[3-5)] In addition, GGA induces heat shock proteins (HSPs), such as HSP70 and glucose-regulated protein (GRP78), and exerts a protective effect on various tissues, including the gastric mucosa, intestine, liver, kidney, retina, myocardium and central nervous system.[5-13)] HSPs were first discovered in 1962 as a set of highly conserved proteins whose expression was induced by different varieties of stress.[14)] HSP70 is a major stress-inducible molecular chaperone, which plays a key role in

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maintaining the correct folding, assembly and intracellular transport of proteins.[15,16] Moreover, HSP70 plays an important role in cellular protection in many tissues.[17] Heat shock gene expression, represented by the activation of a heat shock factor (HSF1) and binding to heat shock elements, results in the elevated expression of HSPs, such as HSP70.[18,19] GGA causes a rapid activation of HSF1 and expression of HSP70 mRNA in gastric mucosal cells.[5] GRP78 acts as a molecular chaperone in the endoplasmic reticulum, and is regarded as a biomarker for endoplasmic reticulum stress.[20]

HSPs are highly expressed[21] and are correlated with sensitivity to chemotherapeutic agents in malignant tumors, including gastrointestinal cancers.[22] For example, HSP70 is associated with a protective effect against 5-FU-induced cell death.[22] In contrast, over-expression of GRP78 in colon cancer cells is related to increased sensitivity to DNA crosslinking agents, such as cisplatin (CDDP).[23] To our knowledge, it remains unclear whether GGA influences sensitivity to chemotherapeutic agents. To clarify this point, we assessed the effect of GGA on its sensitivity to CDDP in rat intestinal epithelium-derived IEC-18 cells and human colon cancer-derived CW-2 cells. CDDP is an effective chemotherapeutic agent frequently used for the treatment of malignant tumors in testis, ovary, cervix, lung, head and neck, bladder, as well as many other organs and tissues.[24-26] Once inside the cell, CDDP is converted into a reactive molecule that interacts with DNA to form CDDP-DNA adducts, triggering intrastrand and interstrand cross-linking.[25-27] This cross-linking distorts and unwinds the DNA duplex, interfering with DNA replication and transcription, and causing DNA damage that induces cell-cycle arrest and cell death.[25-27]

In the present study, we assessed the effects of GGA on CDDP-induced viability reductions and caspase-3 activation, using rat intestinal epithelium-derived IEC-18 cells and human colon cancer-derived CW-2 cells. We also determined the effects of GGA on the amounts of HSP70, GRP78 and p53 in CDDP-treated cells.

MATERIALS AND METHODS

Reagents

GGA were kindly supplied by Eisai Co., Ltd (Tokyo, Japan). CDDP was purchased from Nippon Kayaku Co., Ltd (Tokyo, Japan). An anti-HSP70 monoclonal antibody was purchased from Stressgen (Victoria, Canada), an anti-GRP78 antibody from AnaSpec Inc. (San Jose, CA), an anti-p53 antibody from Cell Signaling (Beverly, MA), and an anti-actin antibody from Sigma-Aldrich (St. Louis, MO).

Cell culture

IEC-18 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM containing 5% heat-inactivated fetal bovine serum (FBS), 0.1 U/ml insulin and 10 μg/ml gentamycin. IEC-18 cells from passages 15-20 were used in the experiments. CW-2 cells were from RIKEN Cell Bank (Saitama, Japan) and were cultured in DMEM containing 10% heat-inactivated FBS and 10 μg/ml gentamycin.

Cell viability assay

IEC-18 cells or CW-2 cells (1×10^4) were seeded in 96-well plates. One day later, the cells were incubated with CDDP in the absence or presence of GGA for an additional 48 hours. Cell viability was determined by the CellTiter96 cell proliferation assay (Promega, Madison, WI) (which is a colorimetric method), using a spectrophotometer (GE Healthcare, Piscataway, NJ). The results are expressed as the ratio of optical density in the presence versus the absence of drugs.

Caspase-3 activity assay

IEC-18 cells (1×10^4) were seeded in a 96-well plate. One day later, the cells were incubated with CDDP in the absence or presence of 200 μM GGA for an additional 24 hours. Caspase-3 activity in the cell lysates was determined with a caspase-3 assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Cytochrome c release assay

IEC-18 cells (4×10^5) were seeded in 6-cm dishes. One day later, the cells were incubated with CDDP in the absence or presence of 200 μM GGA. Sixteen hours later, the cytosolic fraction was extracted for Western blot analysis using an anti-cytochrome *c* antibody (Biovision).

Western blot analysis

IEC-18 cells or CW-2 cells (8×10^4) were seeded into 12-well plates. One day later, the cells were incubated with 0–200 μM GGA in the presence of CDDP. Two or six hours later, the cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 2 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0; 50 mM NaF; 1% Triton X-100; 1 mM sodium vanadate and 5.8 KIU/ml aprotinin).

After centrifugation, the supernatant was collected. The protein concentration of the supernatant was determined using the BCA protein assay (Pierce, Rockford, IL) to enable an equal loading of the samples in Western blot analysis.

Statistical analysis

We used Student's *t*-test for statistical analysis between two groups. To evaluate the concentration-dependent effects of GGA, one-factor analysis of variance (ANOVA) was performed. We considered a *P*-value of < 0.05 statistically significant.

RESULTS

Effect of GGA on cell viability in the presence of CDDP

IEC-18 cells were incubated with 0–200 μM GGA in the presence of 30 μM CDDP for 48 hours. GGA significantly inhibited reductions in the viability of CDDP-treated IEC-18 cells at relatively high concentrations of 50–200 μM (Fig. 1A). To assess the effect of GGA on the IC_{50} of CDDP, IEC-18 cells were incubated with 0–30 μM CDDP in the absence or presence of 200 μM GGA (Fig. 1B). The IC_{50} of CDDP was higher in the presence of 200 μM GGA (33 μM) than in its absence (8.3 μM). Similarly, GGA inhibited reductions in the viability of CDDP-treated CW-2 cells at a concentration of 200 μM (Fig. 1C).

Effect of GGA on caspase-3 activation in CDDP-treated IEC-18 cells

Caspase-3 plays a key role in the signaling cascade of apoptosis in CDDP-induced viability reductions.[28] To evaluate the effect of GGA on caspase-3 activation, IEC-18 cells were incubated with 30 μM CDDP in the absence or presence of 200 μM GGA for eight, 16 or 24 hours, after which caspase-3 activation was determined. We found that 200 μM GGA inhibited CDDP-induced caspase-3 activation (Fig. 2).

Effect of GGA on p53 content in CDDP-treated IEC-18 cells and CW-2 cells

CDDP activity depends on p53 protein.[29,30] To elucidate the effect of GGA on the p53 content in CDDP-treated cells, IEC-18 cells and CW-2 cells were incubated with 0–200 μM GGA in the

presence of CDDP. We found that GGA suppressed the elevation of p53 content in CDDP-treated cells at a concentration of 200 μM (Fig. 3A and 3B).

Effect of GGA on CDDP-induced cytochrome c release from the mitochondria into the cytosol in IEC-18 cells

Several p53-regulated genes, such as Bax, enhance the release of cytochrome *c*, which interacts with APAF-1 to initiate a protease cascade resulting in the activation of caspase-9 and

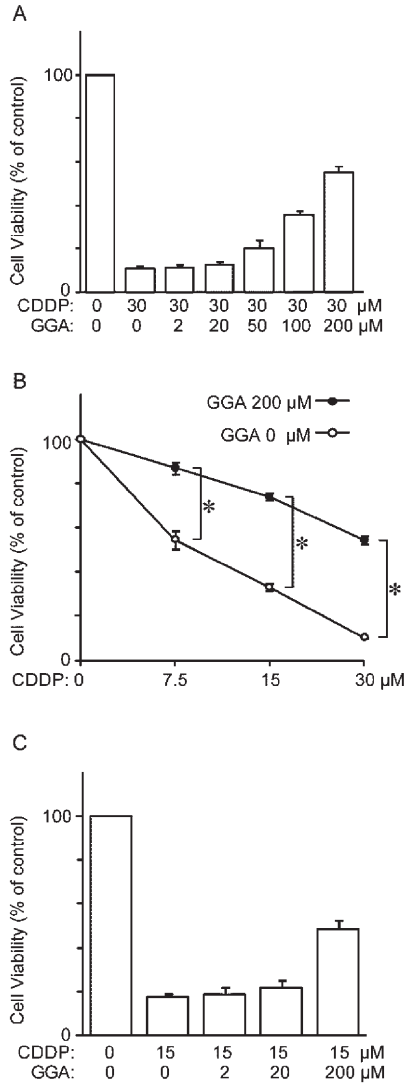

Fig. 1 Effect of GGA on viability of CDDP-treated cells. (A) IEC-18 cells were incubated with 0–200 μM GGA in the presence of 30 μM CDDP for 48 hours, and cell viability was determined. Data are presented as means ± SD. GGA inhibited viability reductions with CDDP in a concentration-dependent manner ($P < 0.05$, one-factor ANOVA, N = 3). (B) IEC-18 cells were incubated with 0-30 μM CDDP in absence or presence of 200 μM GGA for 48 hours, and cell viability was determined. *, $P < 0.05$ (Student's t-test); N = 3 at each concentration of CDDP. (C) CW-2 cells were incubated with 0–200 μM GGA in the presence of 15 μM CDDP for 48 hours, and cell viability was determined.

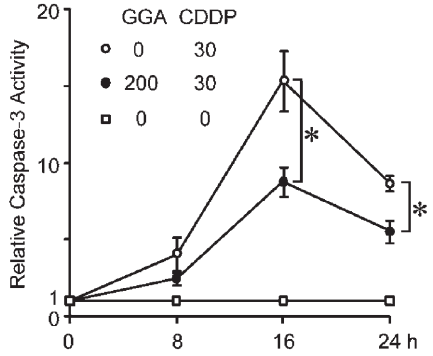

Fig. 2 Effect of GGA on CDDP-induced caspase-3 activation. IEC-18 cells were incubated with 30 μM CDDP in the absence or presence of 200 μM GGA. Caspase-3 activity was determined eight, 16 and 24 hours later. *, $P < 0.05$ (Student's *t*-test); N = 3 at each time point.

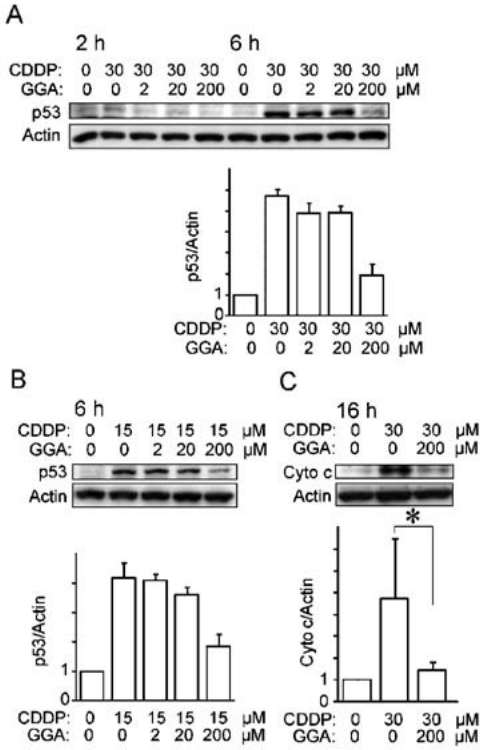

Fig. 3 Effects of GGA on intracellular p53 content and cytochrome c release. (A) IEC-18 cells were incubated with 0–200 μM GGA in the presence of 30 μM CDDP for two or six hours and lysed for Western blot analysis using anti-p53 or anti-actin antibodies. Intensity of p53 bands was normalized to intensity of actin bands. (B) CW-2 cells were incubated with 0–200 μM GGA in the presence of 15 μM CDDP for six hours, and lysed for Western blot analysis. (C) IEC-18 cells were incubated with 30 μM CDDP in the absence or presence of 200 μM GGA for 16 hours, and the cytosolic fraction was obtained to determine cytochrome *c* release from the mitochondria into the cytosol with Western blot analysis. *, $P < 0.05$ (Student's *t*-test); N = 3.

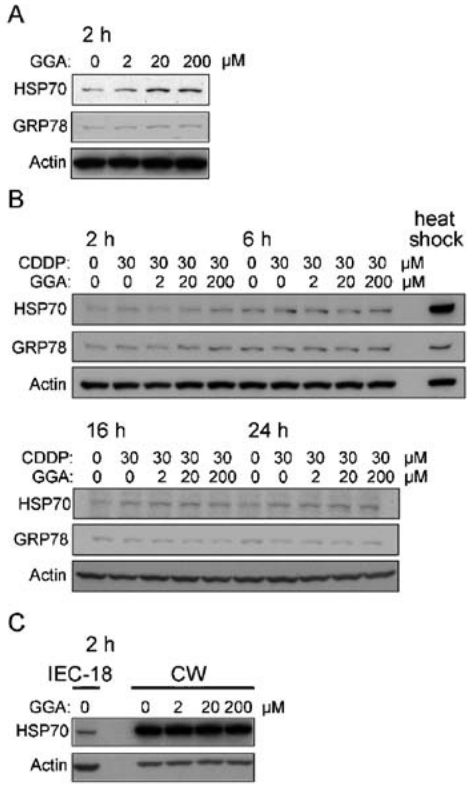

Fig. 4 Effect of GGA on heat shock protein induction in the absence or presence of CDDP. (A) IEC-18 cells were incubated with 0–200 μM GGA in the absence of CDDP for two hours and lysed for Western blot analysis using anti-HSP70, anti-GRP78 or anti-actin antibodies. Three independent experiments showed similar results. (B) IEC-18 cells were incubated with 0–200 μM GGA in the presence of 30 μM CDDP for two, six, 16 or 24 hours and lysed for Western blot analysis using anti-HSP70, anti-GRP78 or anti-actin antibodies. As a positive control, heat shock (42°C for 30 minutes followed by two hours of recovery at 37°C) was applied to the cells. (C) CW-2 cells were incubated with 0–200 μM GGA in the absence of CDDP for two hours and lysed for Western blot analysis.

caspase-3, from the mitochondria into the cytoplasm.[24,31] To evaluate the effect of GGA on the release of cytochrome *c* in CDDP-treated cells, IEC-18 cells were incubated with CDDP in the absence or presence of 200 μM GGA for 16 hours, and the cytosolic fraction was extracted for Western blot analysis using anti-cytochrome *c* antibody. We found that 200 μM GGA suppressed a CDDP-induced cytochrome *c* release (Fig. 3C).

Effect of GGA on HSP70 and GRP78 expression in CDDP-treated or untreated cells

IEC-18 cells were incubated with 0–200 μM GGA in the absence of CDDP for two hours and then lysed for Western blot analysis. GGA induced the expression of HSP70 in a dose-dependent manner as previously reported,[13] but did not affect the expression of GRP78 in IEC-18 cells (Fig. 4A) unlike that in rat mesangial cells.[12] In the presence of CDDP, HSP70 expression was not induced with GGA treatment for 2–24 hours (Fig. 4B). The expression level of HSP70 was much higher in colon cancer-derived CW-2 cells than in IEC-18 cells, and was not affected with GGA treatment even in the absence of CDDP (Fig. 4C).

DISCUSSION

In this study, we found that high concentrations of GGA (50–200 μM) attenuated the CDDP-induced reductions in cell viability by suppressing the elevation of intracellular p53 content without causing HSP70 induction. These findings indicated a novel role of GGA protective action. Previous pharmacokinetic studies in humans have revealed that serum concentrations of GGA reach around 1 μM following the clinical administration of GGA (150 mg per day, approximately 3 mg/kg/day).[32,33] Therefore, we presumed that chemotherapy using CDDP may not be hampered by clinical usage of GGA, and that GGA can be administered to patients in the treatment with CDDP. In contrast, a large amount of GGA (200–500 mg/kg/day) was administered to rats or mice to determine its protective effects on gastrointestinal damage, colitis, hepatic ischemia, renal damage, glaucomatous damage, heart ischemia or viral infection.[5-10,34] Thus, in animal experiments using a high dosage of GGA, the serum GGA concentration should be determined, and its effect on the intracellular content of p53 has to be assessed as well as the HSP induction if GGA concentrations are to reach around 50–200 μM.

CDDP is a DNA crosslinking agent that not only inhibits the transcription from genomic DNA but also reduces cellular viability by inducing apoptosis.[24] Its inhibition of transcription may explain why HSP protein expression is not induced by GGA in the presence of CDDP. CDDP-induced apoptosis is mediated by multiple molecules,[24] and p53 plays a critical role in the pathway leading from CDDP-DNA crosslinking to caspase-3 activation.[24] Under normal conditions, p53 is maintained at a low level through its interaction with murine double minute 2, which targets p53 for degradation in cells.[35,36] In response to DNA damage, the levels of p53 are greatly increased through posttranslational modifications, such as phosphorylation.[35,36] There is a possibility that GGA might suppress the DNA crosslinking activity of CDDP, resulting in a reduced elevation of intracellular p53 content. However, we observed that 200 μM GGA did not affect CDDP-induced p53 phosphorylation at either Ser15 or Ser20 in CW-2 cells (data not shown). This finding suggests that CDDP can damage genomic DNA even in the presence of GGA. Other than Ser15 and Ser20 of p53, Ser37 and Ser46 are phosphorylation sites to increase the intracellular content of p53.[37-39] Moreover, CDDP-induced DNA damage activates p38 MAPK, which phosphorylates p53 at Ser33.[40] In addition, acetylation is another posttranslational modification that increases p53 stability by preventing the ubiquitination of key lysine residues and subsequent proteasomal degradation.[41,42] Further research will be required to elucidate the precise mechanism of GGA action on the regulation of intracellular p53 content in CDDP-treated cells.

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