

**Geranylgeranylacetone induced mTOR Activity has anti-hepatitis C virus activity**

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**Abbreviations used in this paper:**

IFN: interferon

HCV: hepatitis C virus

STAT: signal transducers and activators of transcription

ISGF-3: IFN-stimulated gene factor 3

ISRE: IFN-stimulated regulatory element

PKR: double-stranded RNA-dependent protein kinase

Rapa: Rapamycin

PI3-K : Phosphatidylinositol 3-kinase

mTOR: mammalian target of rapamycin

GGA: geranylgeranylacetone

siRNA: small interfering RNA

**Key words:**

mTOR

STAT-1

Interferon

HCV

GGA

**ABSTRACT**

**Object.** Geranylgeranylacetone (GGA), an isoprenoid compound, which includes retinoids, has been used orally as an anti-ulcer drug in Japan. GGA acts as a potent inducer of anti-viral gene expression by stimulating the ISGF3 formation in human hepatoma cells. The drug that has few side effects and reinforces the effect of IFN when administered in combination with peg-IFN and ribavirin. This study verified the anti-HCV activity of GGA in a replicon system. In addition, mechanisms of anti-HCV activity were examined in the replicon cells.

**Result.** OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of IFN. The results showed that GGA had the anti-HCV activity. GGA induced anti-HCV replicon activity in a time- and dose-dependent manner. GGA did not activate the tyrosine 701 and serine 727 on STAT-1 and did not induce HSP-70 in OR6 cells. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity and PKR. An additive effect was observed with a combination of IFN and GGA.

**Conclusion.** GGA has mTOR dependent anti-HCV activity. There is a possibility that the GGA anti-HCV activity can be complimented by IFN. It will be necessary to examine the clinical effectiveness of the combination of GGA and IFN for HCV patients in the future.

## INTRODUCTION

Currently, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide (1). Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. The treatment of HCV with a combination of pegylated interferon (IFN) and ribavirin is effective in 80% of HCV genotype 2 or 3 cases, but less than 50% of genotype 1 cases. New anti-HCV agents have been developed to inhibit the life cycle of HCV and are used in combination with IFN- $\alpha$  to ameliorate the salvage rate of HCV infection (2). It is necessary to improve the salvage rate of HCV infection by clarifying the efficacy of IFN treatment since IFN- $\alpha$  is the most basic agent for HCV treatment. Any agents that can support IFN activity will improve the therapeutic effect for HCV infected patients.

Geranylgeranylacetone (GGA), an isoprenoid compound, which includes retinoids, has been used orally as an anti-ulcer drug developed in Japan(3). GGA protects the gastric mucosa from various types of stress without affecting gastric acid secretion (4,5). Moreover, GGA suppresses cell growth and induces differentiation or apoptosis in several human leukemia cells (6,7).

3,7,11,15-Tetramethyl-2,4,6,-10,14-hexadecapentaenoic acid, another isoprenoid compound which is designated as an acyclic retinoid because of it has the ability to interact with nuclear retinoid receptors (8) causes apoptosis in certain human hepatoma cells (9). GGA acts as a potent inducer of anti-viral gene expression by stimulating the ISGF3 formation in human hepatoma cells (10). GGA induces the expression of antiviral proteins such as 2'5'-oligoadenylate synthetase (2'5'-OAS) and double-stranded RNA-dependent protein kinase (PKR) in hepatoma cell lines. GGA stimulates

2'5'-OAS and PKR gene expression at the transcriptional level through the formation of interferon-stimulated gene factor 3 (ISGF-3), which regulates the transcription of both genes. GGA induces the expression of signal transducers and activators of transcription 1, 2 (STAT-1, STAT-2) and p48 proteins, components of ISGF3, together with the phosphorylation of STAT1 (10).

However, no anti-HCV activity was observed.

A cell culture HCV replicon system has been developed as a useful tool for the study of HCV replication and mass screening for anti-HCV reagents. OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE (11), were used to examine the influence of the anti-HCV effect of IFN. The luciferase activity in cell lysate of OR6 was correlated with the HCV-RNA concentration, and the IC<sub>50</sub> of IFN- $\alpha$  was less than 10 IU/ml (11). The OR6 system is a useful and sensitive cell culture replicon system.

This study verified the anti-HCV activity of GGA in the OR6 system. In addition, the mechanisms of anti-HCV activity were examined in OR6 cells.

## MATERIALS AND METHODS

### *Reagents*

GGA was a generous gift from Eisai Co. (Tokyo, Japan). Recombinant human IFN- $\alpha$ 2a was purchased from Nippon Rosche Co. (Tokyo, Japan). Wortmannin, LY294002, Akt inhibitor and rapamycin were purchased from Calbiochem (La Jolla, CA, USA).

### *HCV replicon system*

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen) supplemented with 10% fetal bovine serum, penicillin and streptomycin and maintained in the presence of G418 (300 mg/L; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the Renilla luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After the treatment, the cells were harvested with Renilla lysis reagent (Promega, Madison, WI, USA) and then they were subjected to a luciferase assay according to the manufacturer's protocol.

### *Cell viability assay*

The cells were measured using the colorimetric cell viability assay method. Cell viability was determined by the colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). After 2 days of 100 IU/mL of IFN- $\alpha$  and 1000

nmol/L of rapamycin treatment, Cell viability is expressed as a percentage of the viability in standard media without IFN- $\alpha$  and rapamycin. Data were expressed as the mean  $\pm$  standard deviation (SD). Statistical significance was assessed using Student's *t*-test. Statistical difference was defined as  $P < 0.05$ . All numerical results were reported as the mean of four independent experiments.

### ***Reporter gene assay***

The OR6 cells were grown in 24-well. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers and GGA, and the luciferase activities in the cells were determined using a luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative luciferase activity.

### ***Western blotting and antibodies***

Western blotting with anti-STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-serine-727 phosphorylated STAT-1, anti-serine-2448 phosphorylated mTOR, anti-mTOR (Cell Signaling, Beverly, MA, USA), anti-HSP70 (Stressmarq Biosciences Inc.) were performed as described previously(10). Briefly, OR6 cells were lysed by the addition of a lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mg/mL each of aprotinin, leupeptin and pepstatin, 1 mmol/L sodium o-vanadate and 1 mmol/L NaF). The samples were separated by electrophoresis on 8–12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each

antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England).

#### ***siRNA transfection assay***

mTOR gene knock down was performed using siRNA (Cell Signaling). OR6 cells were transfected with 100 nmol/L mTOR specific and non-targeted siRNA as a control in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 50  $\mu$ mol/L GGA.

#### ***mTOR kinase activity assay***

The cells were washed two times with TBS and lysed by addition of lysis buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 50 mM  $\beta$ -glycerophosphate, 10% glycerol (w/v), 1% Tween-20 detergent (w/v), 1 mM EDTA, 20 nM microcystin-LR, 25 mM NaF, and a cocktail of protease inhibitors). The insoluble materials were removed by centrifugation at 10,000 rpm for 15 min at 4°C, and the supernatants were collected and subjected to analysis of the mTOR kinase activity using a commercially available kit (Calbiochem) according to the manufacturer's instructions.

## RESULTS

### ***GGA with or without IFN had anti- HCV activity***

OR6 cells, the full-length HCV replication system, were used to examine the effect of GGA. The cells were treated with 1-100  $\mu\text{mol/L}$  of GGA for 24 hours and the amount of HCV replicon was measured by the *Renilla* luciferase assay (**Fig.1A**). The relative *Renilla* luciferase activity decreased in a dose-dependent manner. Furthermore, GGA induced anti-HCV replicon activity was time dependent (**Fig.1B**). GGA was combined with IFN- $\alpha$  to examine the additive effect (**Fig.1C**). One or 10  $\mu\text{mol/L}$  of GGA combined with IFN- $\alpha$  decreased the relative *Renilla* luciferase activity slightly (**Fig. 1C**). However, 50 or 100  $\mu\text{mol/l}$  of GGA combined with IFN- $\alpha$  decreased the relative *Renilla* luciferase activity with statistical difference. GGA treatment did not have any statistically significant effect on cell viability from 1-100  $\mu\text{mol/L}$  of GGA for 24 hours (data not shown).

### ***GGA did not activate the tyrosine 701 and serine 727 on STAT-1 and did not induce PKR and HSP-70 in OR6 cell***

GGA mediated phosphorylation of STAT-1 at the tyrosine-701 and serine-727 residues was investigated using antibodies to phospho-specific STAT-1 on OR6 cells, GGA induces phosphorylation of STAT-1 in HuH-7. No phosphorylation of the tyrosine 701 and serine 727 on STAT-1 was detected in OR6 cells (**Fig.2A**). IFN induce anti-viral protein, PKR, and STAT-1 has an interferon stimulating responsive element (ISRE) in the promoter region (12). Neither protein

was detected by Western blotting in this study (data not shown). Next, the role of HSP in the mechanism of GGA activity was examined because GGA is an inducer of the HSP. The HSP-70 expression was increased by pre-exposure to heat shock (**Fig. 2B** lane 2 and 4), but it did not increase due to the effects of GGA (**Fig.2B** lanes 3 and 4).

***Rapamycin and mTOR specific siRNA, but not PI3-K inhibitor and Akt inhibitor, was able to cancel the GGA induced anti-HCV activity***

The role of the PI3-K-Akt-mTOR pathway the anti-HCV activity of GGA was examined in OR6 cells. The cells were treated with GGA after 3 hours in the presence or absence of rapamycin as an mTOR inhibitor, Akt inhibitor or wortmannin as a PI3-K inhibitor (**Fig.3**). Pretreatment with rapamycin attenuated the anti-HCV replication effect in comparison to GGA alone (**Fig.3**, lanes 14-17), whereas pretreatment with wortmannin and Akt inhibitor did not increase the *Renilla* luciferase activity (**Fig.3**, lanes 6-13). siRNA transfection was used for mTOR knockdown to explore role of mTOR in the anti-HCV activity (**Fig. 4**). Although the transfection efficiency of siRNA was barely 10%, GGA-induced anti-HCV activity was clearly inhibited in mTOR-siRNA transfected cells (**Fig.4**, lane 2, 4) in comparison to the control cells (**Fig.4**, lane 1, 3).

***GGA induced mTOR activity and mTOR phosphorylation in OR6 cells***

The phosphorylation of the serine-2448 residues of mTOR by 50  $\mu\text{mol/L}$  of GGA was detected 30 min after GGA treatment. The band intensity of serine 2448 phosphorylated mTOR

decreased by pretreatment with rapamycin but was almost same as with GGA alone following pretreatment with LY294002 (**Fig. 5**). Furthermore, an mTOR activity assay was conducted to confirm the activity mechanism of GGA (**Fig. 6**). The mTOR activity was increased by treatment with GGA alone (Fig. 6, lane 2) and was inhibited by pretreatment with rapamycin (**Fig. 6**, lane 4), whereas pretreatment with LY94002 did not suppress the mTOR activity (**Fig. 6**, lane 3)

## DISCUSSION

GGA demonstrated the anti-HCV activity in this study. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity. An additive effect was observed with the combination of IFN and GGA.

GGA is a non-toxic heat shock protein (HSP) 70 inducer (13). Various GGA activities outside of the stomach are also related to HSP induction (14,15,16). GGA induced HSP-70 exerts an anti-ischemic stress activity in the heart and liver (16,17), an anti-inflammatory activity in various cell types (18) and promotes liver regeneration (19). GGA induces thioredoxin as well as HSP-70 in hepatocytes and other cells (20). Thioredoxin anti-virus activity, is induced by AP-1 and NF- $\kappa$ B but not HSP-70 (21). GGA has potent antiviral activity via the enhancement of antiviral factors and can clinically provide protection from influenza virus infection (22). GGA significantly inhibits the synthesis of influenza virus-associated proteins and prominently enhances the expression of human myxovirus resistance 1 (MxA) followed by increased HSP-70 transcription (22). Moreover, GGA augments the expression of an interferon-inducible double-strand RNA-activated protein kinase (PKR) gene and promotes PKR autophosphorylation and concomitantly alpha subunit of eukaryotic initiation factor 2 phosphorylation during influenza virus infection (22). These anti-virus activities are related to GGA induced HSP-70. HSP-70 protein and PKR were not induced by GGA in OR6 cells in the current study. There is apparently no relationship between the GGA induced anti-HCV activity and GGA induced HSP induction in OR6 cells.

GGA induction of anti-viral protein is dependent upon STAT-1 tyrosine phosphorylation in HuH-7 and HepG2 (10). However, GGA did not induce STAT-1 tyrosine phosphorylation and anti-virus protein, PKR, in OR6 cells in this study. Moreover, the GGA induced anti-HCV activity depended on mTOR activity, not STAT-1. OR6 cells are full length HCV replicon transfected HuH-7 cells (11). HCV virus products inhibit the Jak-STAT pathway (24). The mechanism of inhibition of the Jak-STAT pathway is multi-factorial including the suppressor of cytokine signaling 3 (SOCS-3) expression (26), protein phosphatase 2A (PP2A) induction (27), STAT-3 expression (28) and IL-8 expression (29). GGA induced STAT-1 tyrosine phosphorylation and inducible PKR protein levels are also minor. Generally, the replicon transfection induces the intrinsic IFN (30), but STAT-1 tyrosine phosphorylation was not detected in combined OR6 cells. HCV replicon produced viral product might be inhibiting GGA-induced STAT-1 tyrosine phosphorylation.

mTOR is associated with the IFN induced anti- HCV signal (31). The IFN activated mTOR pathway exhibits important regulatory effects in the generation of the IFN responses, including the anti-encephalo myocarditis virus effect (32). IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE) dependent promoter gene activity. A relationship has been observed between the replication of the hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70 S6 kinase pathway and regulates the replication of HCV (33). The IFN induced mTOR activity, independent of PI3K and Akt, is the critical factor for its anti-HCV activity and Jak independent TOR activity involves STAT-1 phosphorylation and nuclear localization, and then PKR is expressed in hepatocytes (31).

No relationship between GGA and mTOR has been reported. However, GGA induced anti-HCV activity depended on mTOR activity independent of PI3-K-Akt, as observed with IFN induced mTOR activity.

GGA, a drug that can be safely administered orally, has mTOR dependent anti-HCV activity. The combination of IFN and GGA has additive effect on anti-HCV activity. The current results suggest that combination therapy with GGA and IFN is therefore expected to improve the anti-HCV activity. It will therefore be necessary to examine the clinical effectiveness of the combination with GGA and IFN for HCV patients in the future.

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**FIGURE LEGENDS**

**Figure 1. The effect of GGA on the genome-length HCV RNA replication system. (A) Dose dependent effect of GGA. (B) Time course of of GGA suppressed HCV replication. (C) The additive effect of GGA with IFN- $\alpha$  suppressed HCV replication.**

(A) The OR6 cells were treated with 1-100  $\mu\text{mol/l}$  of GGA (lanes 2-5) and lane 1 was not treated. One day later, Renilla luciferase activity was determined by luminometer ( $n = 4$ ). The data are expressed as the mean  $\pm$  SD and are representative of four similar experiments. The differences between lane 3 versus 4, lane 3 versus 5 and lane 3 versus 5 were statistically significant. (B) The OR6 cells were treated 50  $\mu\text{mol/L}$  of GGA and at the indicated time, the HCV replicon assay was done ( $n=4$ ). The differences between lane 1 versus 3-5 and lane 2 versus 4, 5 were statistically significant. (C) The OR6 cells were treated with 10 IU/mL of IFN- $\alpha$  in the absence (lane 1) or presence of treatment with 1-100  $\mu\text{mol/l}$  of GGA (lanes 2-5). Non-treatment OR6 cells has 100% of relative *Renilla* luciferase light unit. The differences between lane 1 versus 4, 5 were statistically significant.

Statistical significance was accepted as a p value of  $< 0.05$ . The data are expressed as the mean  $\pm$  SD and are representative of four similar experiments

**Figure 2. Effect of GGA on STAT-1 (A) and HSP-70 (B).**

(A) The OR6 cells were either untreated (lane 1) or treated with 10 IU/mL of IFN- $\alpha$  (lane 2) for 30 min or treated with 50  $\mu\text{mol/L}$  GGA (lane 3) and then were phosphorylated STAT-1 at tyrosine-701

residue (upper panel) and at serine-727 residue (middle panel), the expression STAT-1 (lower panel) was analyzed by Western blotting. (B) The OR6 cells were either untreated (lane 1) or given heat shock (at 42 degrees C 15minutes, overnight recovery at 37degrees C) (lane 2, 4) or treated with 50  $\mu\text{mol/L}$  of GGA (lane3, 4) and then the expression HSP-70 (upper panel) was analyzed by Western blotting.  $\beta$ -actin (lower panel) protein is the internal control.

**Figure 3. Changes in GGA suppressed HCV replication by rapamycin, but not PI3-K inhibitor and Akt inhibitor.**

OR6 cells were treated with 1-100  $\mu\text{mol/l}$  of GGA in the absence (lane 2-5) or presence of pretreatment (lanes 6–17) for 3 h. Lane 1 was not treated GGA. One day latter, Renilla luciferase activity was determined by luminometer (n = 4). The data are expressed as the mean  $\pm$  SD and are representative of four similar experiments

**Figure 4. Changes in GGA suppressed HCV replication by mTOR-siRNA.**

The OR6 cells were transfected with mTOR-siRNA (lanes 2 and 4) and the non-targeted siRNA (lanes 1 and 3). One day later, the cells were treated with GGA (lanes 1–4). The HCV replicon assay is the same as Figure 3. Non-treatment OR6 cells has 100% of relative *Renilla* luciferase light unit. Statistical significant were lane 1 versus 2 and lane 3 versus 4. The data are expressed as the mean  $\pm$  SD and are representative example of four similar experiments. Statistical significant was accepted as a p value of  $< 0.05$ .

**Figure 5. Effect of GGA on mTOR and effect of LY294002 and Rapamycin on GGA-induced serine phosphorylated mTOR.**

After pretreatment with 10 nmol/L LY294002 (lane 3) and 1  $\mu$ mol/L Rapamycin (lane 4) for 3 hours, the OR6 cells were either untreated (lane 1) or treated with 50  $\mu$ mol/L GGA (lanes 2–4) for 30 min and then were phosphorylated mTOR at Serine-2448 residue (upper panel), the expression of mTOR (lower panel) was analyzed by Western blotting.

**Figure 6. Effect of GGA on the mTOR kinase assay.**

After pretreatment with 10 nmol/L LY294002 (lane 3) and 1  $\mu$ mol/L Rapamycin (lane 4) for 3 hours, the OR6 cells were either untreated (lane 1) or treated with 50  $\mu$ mol/L GGA (lanes 2–4) for 30 min. The mTOR kinase activity was determined by ELISA-based mTOR kinase activity assay kit (n=4). The differences between lane 1 versus 2 and lane 2 and 3 versus 4 were statistically significant. The data are expressed as the mean  $\pm$  SD and are representative of four similar experiments

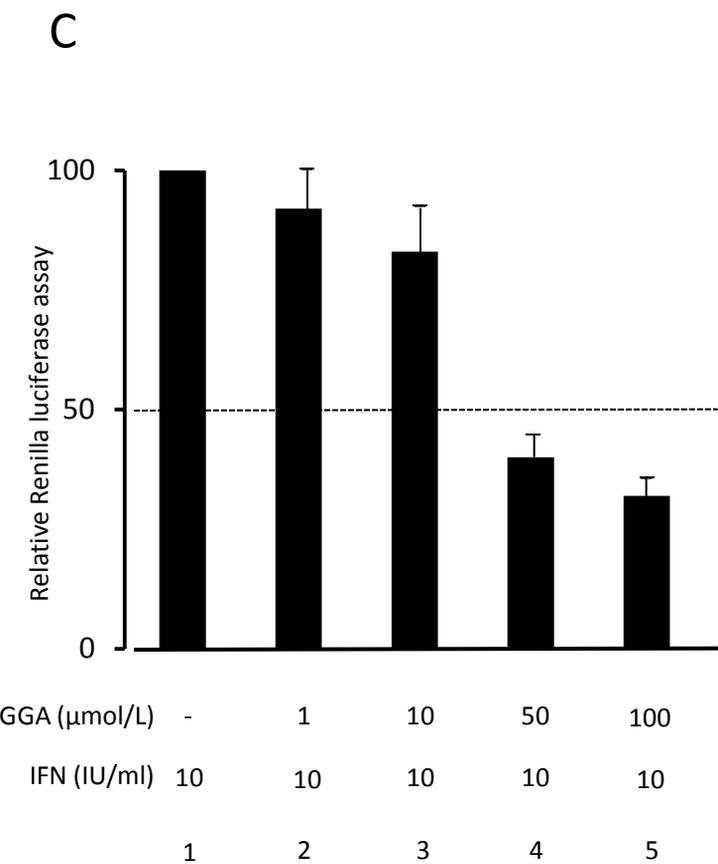
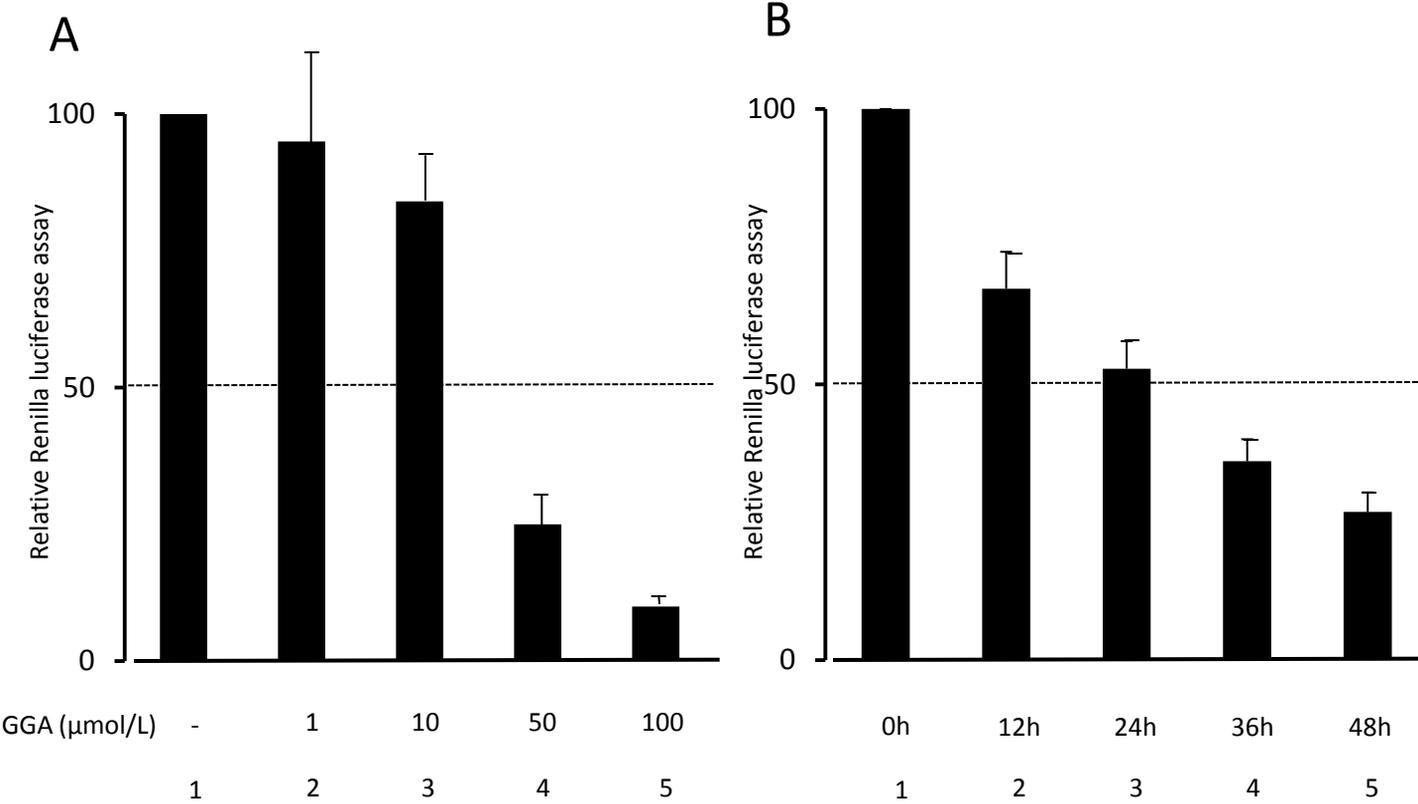


Figure.1

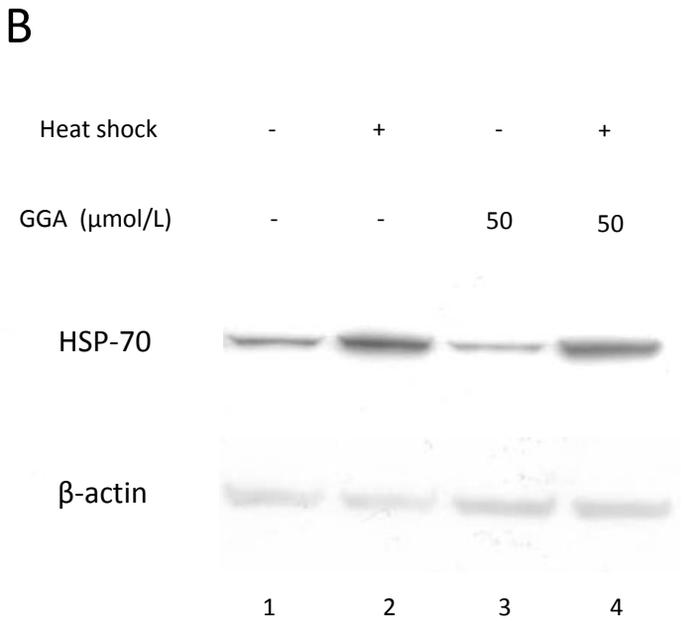
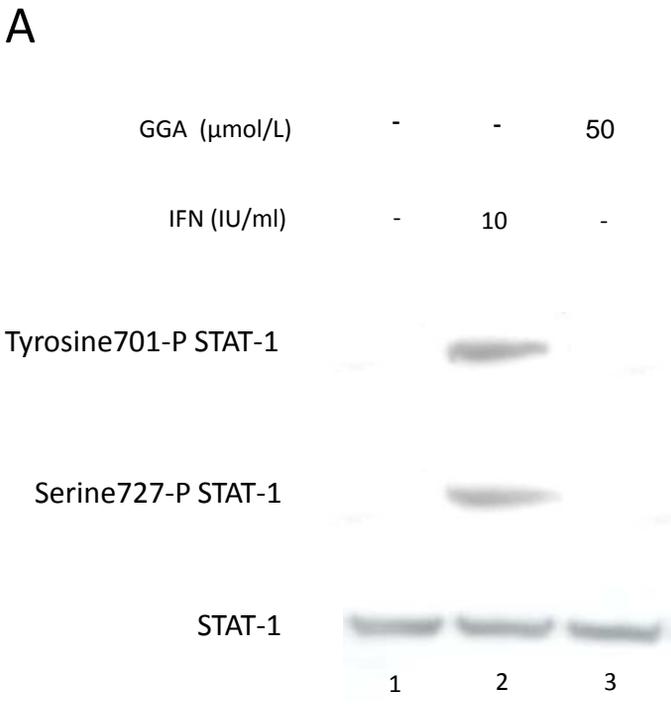


Figure.2

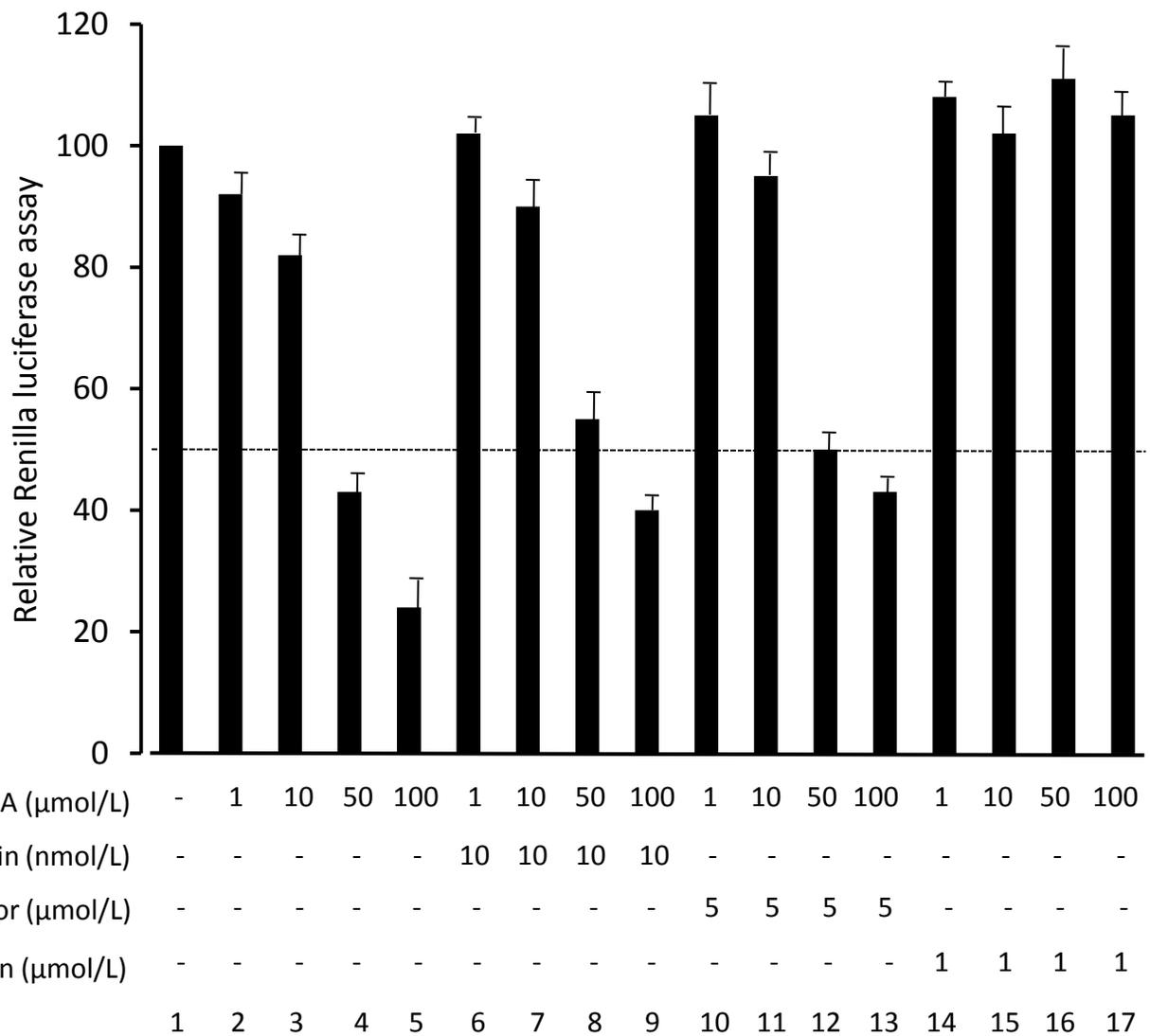


Figure.3

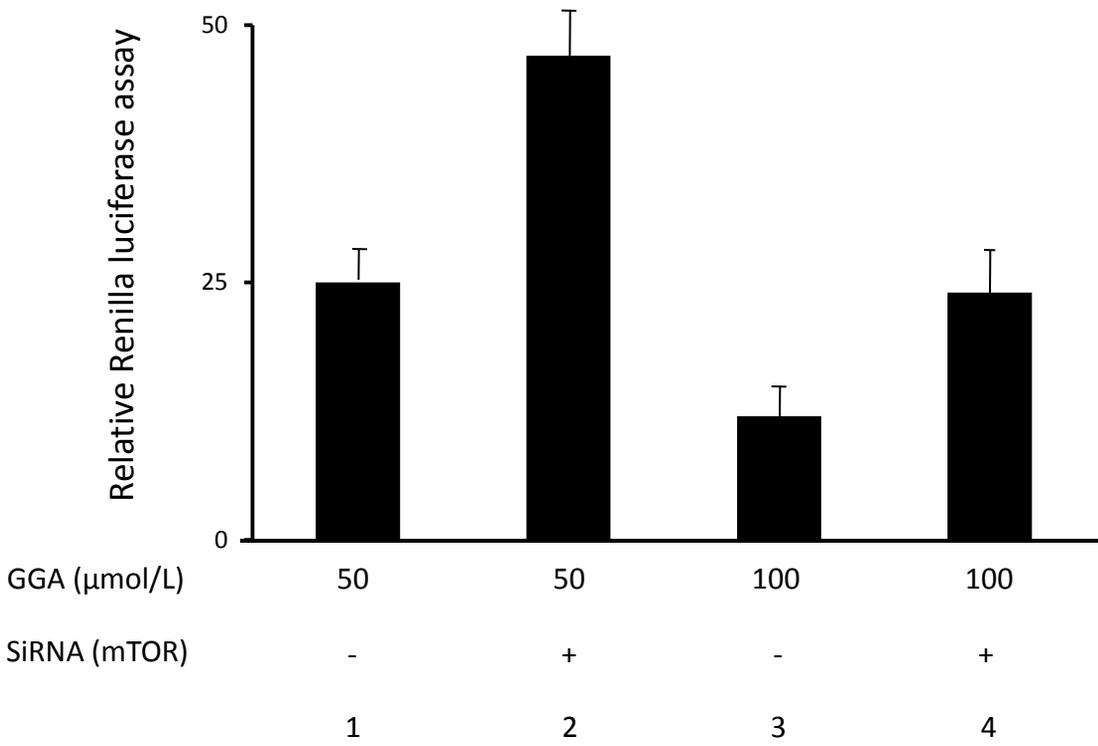


Figure.4

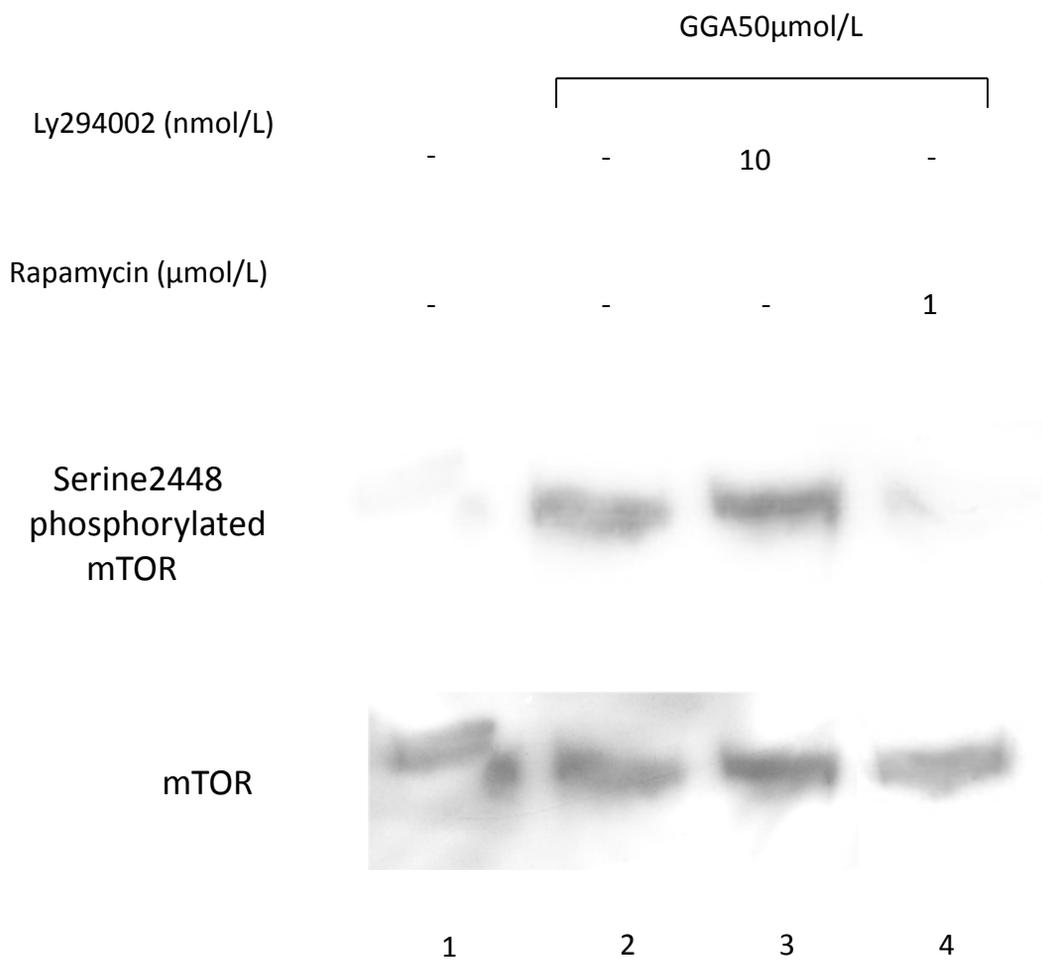


Figure.5

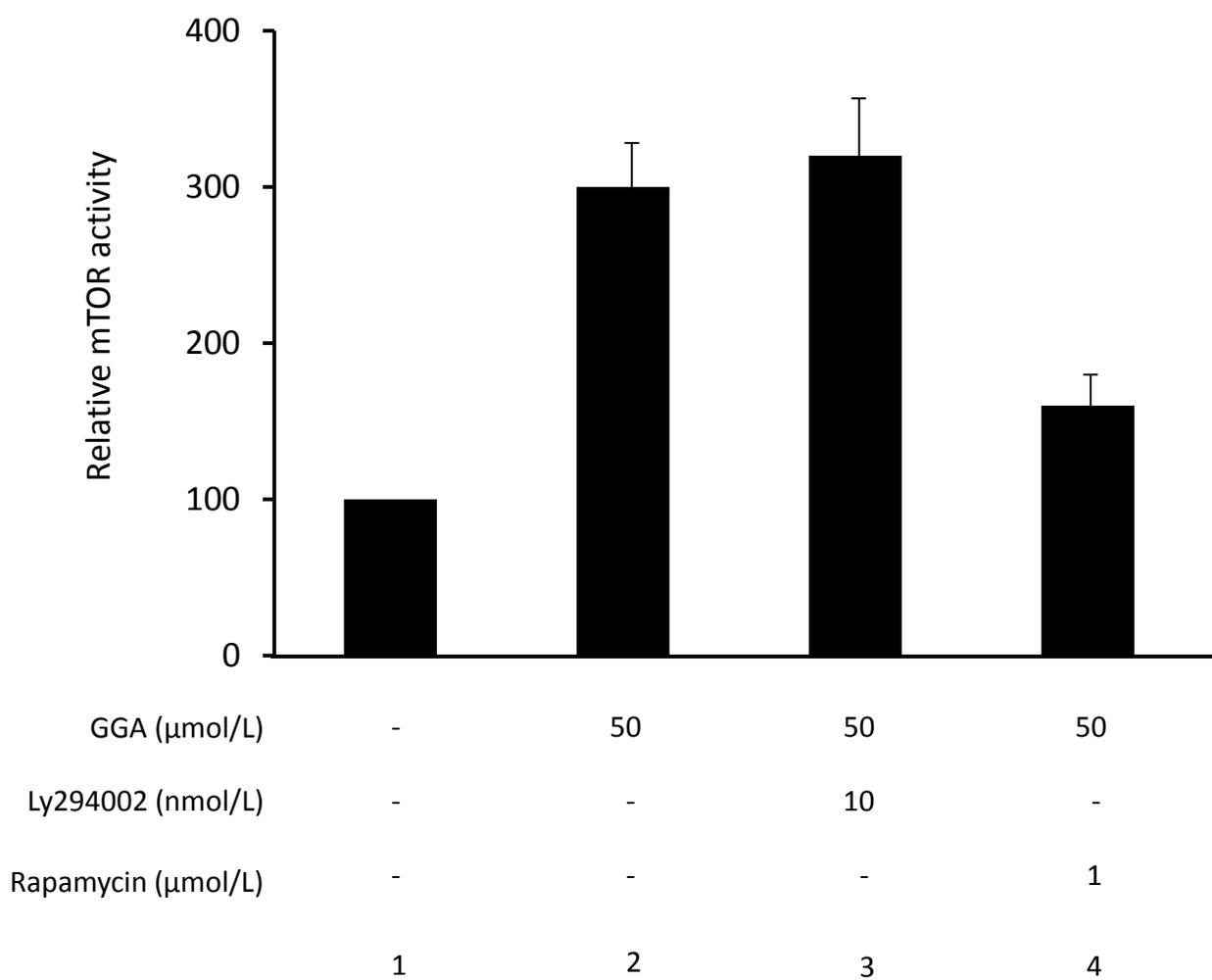


Figure.6