Cell proliferation effects of *S*-allyl-L-cysteine are associated with phosphorylation of Janus kinase 2, insulin-like growth factor type-I receptor tyrosine kinase, and extracellular signal-regulated kinase 2 in primary cultures of adult rat hepatocytes

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Abstract

The cell proliferation effect of *S*-allyl-L-cysteine (SAC) and its mechanisms were examined in primary cultures of adult rat hepatocytes. In serum-free cultivation, SAC (10^{-6} M)-stimulated hepatocytes showed significant proliferation compared to control at 5-h culture; the effect was dependent on the culture time and the dose of SAC (EC_{50} value 8.58×10^{-8} M). In addition, SAC-stimulated hepatocytes significantly increased mRNA expression levels of *c-Myc* and *c-Fos* at 1 h and *cyclin B1* at 3.5 and 4 h, respectively. In contrast, alliin and allicin, structural analogs of SAC, did not show these effects observed with SAC. The SAC-induced hepatocyte proliferation effects were completely suppressed by monoclonal antibodies against growth hormone receptor and insulin-like growth factor type-I (IGF-I) receptor, respectively. Furthermore, the Janus kinase 2 (JAK2) inhibitor TG101209, phospholipase C (PLC) inhibitor U-73122, IGF-I receptor tyrosine kinase (RTK) inhibitor AG538, PI3 kinase inhibitor LY294002, MEK inhibitor PD98059, and mTOR inhibitor rapamycin completely suppressed the SAC-induced hepatocyte proliferation. JAK2 (p125 kDa) phosphorylation in cultured hepatocytes peaked 5 min after SAC stimulation. SAC-induced IGF-I RTK (p95 kDa) and ERK2 (p42 kDa) phosphorylation had slower rises than JAK2, peaking at 20 and 30 min, respectively. These results indicate that SAC promoted cell proliferation by growth hormone receptor/JAK2/PLC pathway activation followed by activation of the IGF-I RTK/PI3K/ERK2/ mTOR pathway in primary cultures of adult rat hepatocytes.

Keywords

S-Allyl-L-cysteine (SAC), proliferation, Janus kinase 2 (JAK2), Insulin-like growth factor type I (IGF-I), Extracellular signal-regulated kinase 2 (ERK2)

1. Introduction

The liver has a self-regenerative function called liver regeneration, which causes rapid regeneration following liver damage or surgical resection (Fausto, 2000; Michalopoulos and DeFrances, 1997). The mitogen-activated protein (MAP) kinase pathway is a series of phosphorylation cascade reactions induced by the activation of receptor tyrosine kinase (RTK) during liver regeneration. This pathway ultimately activates a serine/threonine kinase called extracellular signal-regulated kinase (ERK) that promotes cell proliferation and differentiation (Fausto et al., 2006; Morrison, 2012).

S-Allyl-L-cysteine (SAC) is a water-soluble compound in which an allyl group is bonded to the sulfur atom of cysteine. SAC is contained in a minuscule quantity in fresh garlic, but its content increases up to about 36 times during aging of garlic for 3 months (Colín-González et al., 2012). In the garlic aging process, SAC is biosynthesized from γ-glutamyl-S-allyl cysteine by a γ-glutamyl transferase. The synthesized SAC is oxidized to alliin (*S*-allyl-L-cysteine sulfoxide), and it is hydrolyzed to allyl sulfenic acid (Borlinghaus et al., 2014; Colín-González et al., 2012). Allicin (diallylthiosulfinate) is produced by the condensation of this allyl sulfenic acid (Fig. 1). There is also a pathway in which alliin and allicin are synthesized without going through SAC (Amagase et al., 2001).

Extracted substances of aged garlic and SAC have an antioxidant effect based on the removal of active oxygen or activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) (Colín-González et al., 2015, 2012; Kalayarasan et al., 2008). Therefore, SAC is used as a supplement in Japan because it is effective for hypertension and hypercholesterolemia (Colín-González et al., 2012; Kim et al., 2006; Sohn et al., 2012). In addition, pharmacological effects of SAC other than these are becoming clear, and one

of them is a cell proliferation effect. For example, SAC has been demonstrated to promote neuroblast division and differentiation by increasing the expression of serotonin 5-HT_{1A} receptors in the dentate gyrus of mice (Nam et al., 2011). Furthermore, we have previously reported that SAC, but not *S*-methyl-L-cysteine or cysteine, accelerated liver regeneration by promoting DNA synthesis in remnant liver and restoring liver function in two-thirds partially hepatectomized rats (Kurihara et al., 2020). However, it is not clear by what mechanism of action SAC exerts a cell proliferation-promoting effect on hepatic parenchymal cells.

The aim of this study was to examine the detailed signal transduction pathways of cell proliferation induced by SAC in primary cultures of adult rat hepatocytes. Thus, the cell proliferation effect of SAC was compared to that of alliin or allicin by measuring the number of hepatocyte nuclei, the progression of S-phase (DNA synthesis phase) in the cell cycle, and the expression levels of genes, such as *c-Myc*, *c-Fos*, and *cyclin B1* associated with liver regeneration. Moreover, the activity of growth-related signaling elements such as RTK or MAPK involved in hepatocyte proliferation was measured using various inhibitors of SAC-induced cell proliferation, and the mechanism of SAC on cell proliferation in primary cultured hepatocytes was considered.

2. Materials and Methods

2.1 Animals

Male Wistar rats weighing 180-200 g were obtained from Sankyo Labo Service Corp. (Tokyo, Japan). The pre-experimental acclimatization was set for 3 days, and during this period, rats had *ad libitum* access to food and water. The handling of all rats used in this study complied with the Guidelines for the Care and Use of Laboratory Animals of Josai

University (Nos. JU 20035 and JU 21035).

2.2 Isolation and culture of hepatocytes

Rat hepatocytes were isolated and purified by two-step *in situ* collagenase perfusion according to the method of Seglen (Seglen, 1975). In brief, under sodium pentobarbital (45 mg/kg, i.p.) anesthesia, cannulation to the portal vein was performed. Then, the liver was refluxed in Ca^{2+}-free Hanks as perfusion buffer for 10 min at 37 °C. Next, the solution was changed to perfusion buffer containing 0.025% collagenase Type II (Worthington Biochemical Corp., Freehold, NJ, USA.) and 5.0 mM $CaCl_2$ refluxed for 10-11 min. Isolated hepatocytes were used at more than 96% cell viability by trypan blue exclusion. Hepatocytes (3.3×10^4 cells/cm^2) were plated and cultured for 3 h with Williams' medium E (Sigma-Aldrich Co., St. Louis, MO, USA) containing newborn bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA.) and 10^{-10} M dexamethasone. After a 3-h attachment period, the medium was exchanged to serum-free medium with various reagents. The reagents added to hepatocytes were SAC, alliin, allicin (Tokyo Chemical Industry Co., Tokyo, Japan), and growth-related signal-transducing factor inhibitors or monoclonal antibodies. The inhibitors used were AG538 (Blum et al., 2003), LY294002 (Vlahos et al., 1994), PD98059 (Li et al., 2008), rapamycin (Dixon et al., 1999), TG101209 (Pardanani et al., 2007), SH-4-54 (Haftchenary et al., 2013), and U-73122 (Thompson et al., 1991), which were obtained from Sigma-Aldrich Co. The monoclonal antibodies used were against growth hormone receptor, insulin-like growth factor (IGF)-I receptor, epidermal growth factor (EGF) receptor, and hepatocyte growth factor (HGF) receptor, which were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.3 Counting the number of nuclei and hepatocytes

The cell proliferation effect of SAC was evaluated by measuring the number of hepatocyte nuclei using a slight modification of the method of Nakamura (Nakamura et al., 1983). Briefly, hepatocyte nuclei isolated in 0.1% Triton X-100 containing 0.1 M citric acid were stained with 0.3% trypan blue, and the number of nuclei was measured with a hemocytometer. In this study, the cell proliferation effect was evaluated by measuring the number of nuclei rather than the number of cells, because the attachment between plates or cell-cell contact is tight in hepatocytes, and a single cell cannot be isolated quantitatively.

The numbers of total hepatocytes per 0.01 cm^2 (namely, $\times 10^2$ cells/cm^2) were counted in a phase-contrast microscopic image. The values were measured from cells in microscopic images taken at any three locations, and the average was taken as one example.

2.4 RNA extraction and quantification of *c-Myc*, *c-Fos*, and *cyclin B1* mRNA

The mRNA expression levels of *c-Myc*, *c-Fos*, and *cyclin B1* in cultured hepatocytes were quantified using the TaqMan assay. The total RNA of the cultured hepatocytes was extracted with Direct-zol RNA Miniprep (Zymo Research Co., Tustin, CA, USA), and then a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used for reverse transcription from the obtained RNA to cDNA. Next, 5 μg/μL of cDNA mixed with Fast Advanced Master Mix (Applied Biosystems) and TaqMan probe (*c-Myc*, *c-Fos, cyclin B1*, or glyceraldehyde 3-phosphate dehydrogenase [*GAPDH*]) were put in a StepOne Real-Time PCR System (Thermo Fisher Scientific, Inc.), and each mRNA expression level was determined. *GAPDH* mRNA expression

levels were used as an endogenous control, and relative mRNA expression levels were calculated by the ΔΔCt method (Livak and Schmittgen, 2001).

2.5 Detection of the S-phase and the G_0/G_1-phase of the cell cycle

DNA synthesis was determined by detecting the S-phase of the cell cycle. The S-phase and the G_0/G_1-phase in cultured hepatocytes were measured with a Muse™ cell analyzer, using a slight modification to the manufacturer's instructions (Merck Millipore, Darmstadt, Germany) (Kimura et al., 2014). Briefly, nuclei were isolated by lysing hepatocytes as described in section 2.3 above. The isolated nuclei were mixed with 5% propidium iodide (Fujifilm Wako Pure Chemical Corp., Tokyo, Japan) and incubated for 15 min. The Muse™ cell analyzer is a cell analysis apparatus-based flow cytometer, and the cell cycle, including the S-phase or G_0/G_1-phase, was measured by this device.

2.6 Measurement of phosphorylated p125 kDa Janus kinase (JAK) 2, p95 kDa receptor tyrosine kinase (RTK), and p44/42 kDa MAP kinase (ERK1/2)

Phosphorylated p125 kDa JAK2, p95 kDa RTK, and p44/42 kDa MAPK (ERK1/2) were measured by Western blotting analysis (Kurihara et al., 2021b; Towbin et al., 1979). Briefly, the cultured hepatocytes were lysed by Lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) and centrifuged, and then the supernatant was mixed with a Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein samples (30 μg/lane) were divided by polyacrylamide gel electrophoresis and transferred to a PVDF transfer membrane (PerkinElmer, Inc., Waltham, MA, USA). Next, they were immunoblotted with monoclonal antibodies against phosphorylated p125 kDa JAK2, p95 kDa RTK, or ERK1/2 (Cell Signaling Technology). The phosphorylated ERK1/2

antibody was diluted 2000-fold, and the phosphorylated p125 kDa JAK2 and p95 kDa RTK antibodies were diluted 1000-fold. After incubation with HRP-labeled secondary antibody (Cell Signaling Technology), the blot was detected by Western Lightning Plus-ECL (PerkinElmer, Inc.). Emission intensity was quantified by ChemiDoc Touch MP (Bio-Rad Laboratories, Inc.). Phosphorylated p125 kDa JAK2, p95 RTK, or ERK1/2 activity ratios were calculated by dividing the respective expression levels (p125 kDa JAK2, p95 kDa RTK, or ERK1/2 antibodies [Cell Signaling Technology]) by the total activity. Each of these antibodies was diluted 1000-fold.

2.7 Statistical analysis

Values are shown as means ± standard error of the mean (SEM) of three separate experiments. For comparison with the control, Dunnett's test was performed, and the significance level was defined as 5%.

3. Results

3.1 Time- and dose-dependent cell proliferation effects of SAC and its analogs on cultured hepatocytes

The cell proliferation effects of SAC and its analogs (alliin or allicin) were investigated in cultured hepatocytes. Hepatocytes attached to the plate and exchanged for serum-free medium were cultured with SAC, alliin, allicin, or a combination of SAC with a selective JAK2 inhibitor TG101209 or a selective signal transducer and activator of transcription 3/5 (STAT3/5) inhibitor SH-4-54. Fig. 2 shows a phase-contrast microscopic image of cultured hepatocytes for 5 h. It can be confirmed that the number of SAC-stimulated hepatocyte nuclei was greater than control (medium alone) (Fig. 2A, B). As shown in Fig.

2G, the number of total hepatocytes per field of view (per 0.01 cm^2[$\times 10^2$ $cells/cm^2$]) under the microscope in the control treatment (medium only) was 309 ± 15. In contrast, the number of total hepatocytes stimulated with 10^{-6} M SAC was 407 ± 19, which was a significant increase of approximately 1.32-fold compared to the control (Fig. 2G). The correlation coefficient between the number of hepatocytes and nuclei stimulated by SAC was 0.95 (Fig.2.H). On the other hand, no increase in the number of hepatocytes was observed with alliin or allicin stimulation (Fig. 2C, D and G). In addition, the number of total hepatocytes treated with SAC and TG101209 was significantly reduced to control levels, but no reduction in the number of hepatocytes was observed in the combination of SAC with SH-4-54 (Fig. 2E-G).

Next, the number of hepatocyte nuclei was measured to assess the time- and dose-dependent proliferative effects of SAC or its analogs (Fig. 3A, B). As shown in Fig. 3A, SAC-stimulated hepatocyte nuclei were observed to proliferate depending on the culture time. In 10^{-6} M SAC-treatment, a significantly higher number of nuclei on cultured hepatocytes was observed at about 4 h, peaked at approximately 5 h, and then continued for up to 21 h. In contrast, allicin and alliin did not show a significant increase in hepatocyte nuclei (Fig. 3A). Next, fixing the culture time at 5 h in serum-free culture, effects of SAC or its derivatives at concentrations of 10^{-10} to 10^{-5} M on the number of hepatocyte nuclei were examined. SAC-stimulated hepatocyte nuclei were increased dose-dependently and plateaued at 10^{-6} M, with the 50% effective concentration (EC_{50}) value of 8.58×10^{-8} M. In contrast, no hepatocyte proliferation effect of alliin or allicin was observed in the range of 10^{-10} to 10^{-5} M (Fig. 3B).

3.2 Effects of SAC and its analogs on *c-Myc*, *c-Fos*, and *cyclin B1* mRNA expression

levels in cultured hepatocytes

It is known that oncogenes such as *c-Myc* and *c-Fos* are involved in cultured hepatocyte proliferation and liver regeneration (Fausto, 2000). The StepOne Real-Time PCR System was used to investigate whether *c-Myc* and *c-Fos* mRNAs were expressed in hepatocytes stimulated with SAC and its analogs. Fig. 4A shows the time course associated with *c-Myc* mRNA expression levels in hepatocytes stimulated with SAC and its analogs. The expression level of *c-Myc* mRNA was significantly increased in SAC-stimulated hepatocytes. The peak was observed 1 h after addition of SAC (10^{-6} M), and the expression level increased approximately 1.62-fold compared with time 0. On the other hand, stimulation with alliin (10^{-6} M) or allicin (10^{-6} M) did not significantly increase *c-Myc* mRNA expression levels. Fig. 4B shows *c-Fos* mRNA expression levels in SAC- or its analog-stimulated hepatocytes. The *c-Fos* mRNA expression levels showed a time course similar to those of *c-Myc*, and the increase was approximately 1.85-fold in SAC-stimulated hepatocytes 1 h after stimulation (Fig. 4B).

In addition, mRNA expression levels of *cyclin B1* were measured as a G_2/M phase marker in SAC or analog-stimulated hepatocytes. As shown in Fig. 4C, *cyclin B1* mRNA expression levels in SAC (10^{-6} M)-stimulated hepatocytes began to increase at 3 h and peaked at 3.5 to 4 h. At the peak times, the proportion of SAC-induced *cyclin B1* was significantly increased by approximately 1.72-fold compared to controls (Fig.4C). Alliin (10^{-6} M) or allicin (10^{-6} M) did not show a significant increase in *cyclin B1* mRNA expression in hepatocytes (Fig.4C).

3.3 Time course effects of SAC and its analogs on S- and G_0/G_1-phase progression in cultured hepatocytes

To investigate whether SAC promotes DNA synthesis in cultured hepatocytes, the S-phase in the cell cycle of hepatocytes stimulated with SAC and its analogs was measured. As shown in Fig. 5A, the proportion of S-phase of SAC (10^{-6} M)-stimulated hepatocytes was significantly higher over 3 to 5 h compared to controls. In contrast, alliin (10^{-6} M)- or allicin (10^{-6} M)-stimulated hepatocytes did not show an effect on the proportion of S-phase (Fig. 5A). Furthermore, the proportion of hepatocyte nuclei for the G_0/G_1-phase further decreased significantly in SAC (10^{-6} M) than in control at 3 and 4 h (Fig. 5B).

3.4 Effects of monoclonal antibodies against growth factor receptors on SAC-induced hepatocyte proliferation

To investigate whether SAC showed a cell proliferation effect via growth factor receptors expressed in cultured hepatocytes, the number of nuclei and the progression of the S-phase of SAC-stimulated hepatocytes in the presence of monoclonal antibodies to growth factor receptors associated with cell proliferation were measured. As shown in Fig. 6A, B, SAC-stimulated progression of the S-phase and increase in hepatocellular nuclei were completely suppressed by monoclonal antibodies to GH receptor and IGF-I receptor, respectively. In contrast, monoclonal antibodies against HGF and EGF receptors did not suppress the SAC-induced hepatocyte proliferation (Fig. 6A, B).

3.5 Effects of SAC on hepatocyte proliferation in the presence of specific signal transduction factor inhibitors

Next, to investigate intracellular signaling pathways associated with SAC-induced cell proliferation and DNA synthesis, the number of nuclei and progression of S-phase of SAC-stimulated hepatocytes in the presence of specific signaling factor inhibitors

associated with cell proliferation were measured. Fig. 7A, B show that the hepatocyte proliferation effects of SAC were completely inhibited to control level by TG101209, a selective PLC inhibitor (U-73122), a selective IGF-I RTK inhibitor (AG538), a selective phosphoinositide 3-kinase inhibitor (LY294002), a selective MAPK/ERK kinase (MEK) inhibitor (PD98059), and a selective mammalian target of rapamycin (mTOR) inhibitor (rapamycin). On the other hand, SH-4-54 did not inhibit the SAC-induced hepatocyte proliferation (Fig. 7A, B).

3.6 Time courses of the effects of SAC on p125 kDa JAK2, p95 kDa IGF-I RTK, and ERK1/2 phosphorylation in cultured hepatocytes

JAK2, IGF-I RTK, and ERK1/2 phosphorylation patterns of the SAC-stimulated hepatocytes were measured by Western blot analysis. Fig. 8A shows that phosphorylated p125 kDa JAK2 increased sharply after the addition of SAC and peaked at 5 min; p125 kDa JAK2 phosphorylation activity by SAC at the peak was approximately 2.3-fold the activity ratio compared to the control. Then, the phosphorylation activities began to decrease to control levels after 30 and 60 min of culture (Fig. 8A). Phosphorylation by SAC of IGF-I RTK and ERK2 was observed later than that of JAK2, with peaks 20 and 30 min after SAC addition, respectively. However, no increase in ERK1 phosphorylation was observed with SAC (Fig. 8B, C).

3.7 Effects of specific signal transduction factor inhibitors on phosphorylation of JAK2, p95 kDa IGF-I RTK, and ERK2 induced by SAC

Next, the effects of specific inhibitors of growth-related signal transducers on JAK2, IGF-I RTK, and ERK2 phosphorylated by SAC were investigated. Fig. 9 shows that SAC-

induced JAK2 phosphorylation was suppressed only by TG101209 (Fig. 9A). SAC-induced IGF-I RTK phosphorylation was suppressed not only by TG101209, but also by U-73122 and AG538 (Fig. 9B). SAC-induced ERK2 phosphorylation was suppressed by LY294002 and PD98059, in addition to the above inhibitors (Fig. 9C). SH-4-54 or rapamycin did not affect SAC-induced phosphorylation of both IGF-I RTK and ERK2 (Fig. 9A-C).

4. Discussion

In our previous studies, we demonstrated that SAC accelerated liver regeneration and promoted cell proliferation *in vivo* in two-thirds partially hepatectomized rats (Kurihara et al., 2020). The aim of this study was to examine the mechanism of the proliferative effect of SAC compared to its analogues in primary cultures of adult rat hepatocytes *in vitro*.

Fig. 2 showed that SAC, but not alliin or allicin, significantly increased the number of hepatocytes at 5 h of culture. These results showed that there was a close correlation between the increase in the number of cells and the number of nuclei shown in Figs. 2H, 3, and 7. Furthermore, the effects of SAC on hepatocyte numbers were completely suppressed by TG101209, but not SH-4-54. The results suggest that the effects of SAC are mediated by JAK2, but not STATs.

c-Myc and *c-Fos* are proto-oncogenes expressed during early liver regeneration and are involved in the transition from the G_0 phase to the G_1-phase in the cell cycle of regenerated hepatocytes (Corral et al., 1988; Morello et al., 1990). Accordingly, since SAC significantly increased mRNA expression levels of *c-Myc* and *c-Fos*, SAC can advance the G_0 phase cells to G_1-phase (Fig. 4A and B). In addition, since SAC also

313 increased mRNA expression levels of *cyclin B1*, SAC promoted progression to S- and
314 G_2/M-phases in cultured hepatocytes (Fig. 4C, 5).

315 Rapid cell proliferation effects on hepatocytes have been demonstrated not only with
316 SAC, but also with growth factors such as HGF and IGF-I (Kimura and Ogihara, 1998,
317 1997). It has been shown that the proliferative effects of growth factors in cultured
318 hepatocytes are affected by cell density and dexamethasone. Nakamura *et al.* reported
319 that incorporation of [^{3}H]-thymidine into the nucleus (as a DNA synthesis index) in
320 hepatocytes treated with epidermal growth factor and insulin was faster at low cell density
321 (2.5×10^4 cells/cm^2) than at high cell density (1.0×10^5 cells/cm^2) (Nakamura et al., 1983).
322 Furthermore, we demonstrated that the hepatocellular proliferation effect of HGF was
323 suppressed depending on the dose of dexamethasone (10^{-10}-10^{-7} M) (Kimura et al., 2011).
324 The rapid cell proliferation effect of SAC may be affected by the cell density (3.3×10^4
325 cells/cm^2) and dexamethasone (10^{-10} M) concentration in the culture conditions of this
326 study.

327 Alliin has a structure in which oxygen is added to the sulfur part of SAC, and its
328 structure is very similar to SAC (Fig. 1). Not only SAC, but also alliin and its metabolite,
329 allicin, have antioxidant effects based on the removal of reactive oxygen or activation of
330 the Nrf2-Keap pathway (Chung, 2006; Li et al., 2012b, 2012a). In other words, the results
331 in Figs. 2 to 5 imply that the proliferative effect of SAC on hepatocytes does not go
332 through the Nrf2-Keap pathway in cultured hepatocytes. In addition, we have
333 demonstrated that antioxidants such as isoascorbic acid and vitamin E do not cause
334 hepatocyte proliferation (Moteki et al., 2012). These results suggest that the hepatocyte
335 proliferation effect of SAC proceeds by a mechanism different from the antioxidant effect.

336 To analyze the target of SAC and the signal transduction pathways, effects of some

monoclonal antibodies against growth factors on SAC-induced hepatocyte proliferation were examined. Fig. 6 shows that SAC-induced progression of the S-phase and the number of nuclei in hepatocytes were completely suppressed by monoclonal antibodies to the GH receptor and IGF-I receptor. In the past, we demonstrated that GH secretes IGF-I by stimulation of the GH receptor/JAK2/PLC pathway, and that the secreted IGF-I promotes hepatocyte DNA synthesis and cell proliferation via the IGF-I RTK/PI3K/ERK2/mTOR pathway in primary cultures of adult rat hepatocytes (Kurihara et al., 2021b, 2021a). Based on these results, we hypothesized that SAC promotes cell proliferation via the GH signaling pathway. In support of this notion, the hepatocyte proliferation effect of SAC was completely suppressed by inhibitors related to the JAK2/PLC and IGF-I signaling pathways (Fig. 7). In addition, as shown in Fig. 8, Western blot analysis confirmed that SAC phosphorylated p125 kDa JAK2, p95 kDa RTK (i.e., IGF-I RTK), and ERK2 in cultured hepatocytes. The ERK2, but not ERK1, phosphorylation-enhancing effect of SAC was also observed in the remaining liver in SAC-treated partially hepatectomized rats (Kurihara et al., 2020). Fig. 8 shows that the peak of SAC-induced JAK2 phosphorylation was faster than that of SAC-induced RTK and ERK2 phosphorylation. Furthermore, Fig. 9 shows that SAC-induced RTK phosphorylation was suppressed by TG101209 and U-73122. These results indicate the presence of IGF-I RTK downstream of JAK2/PLC in the SAC-induced hepatocyte proliferation signaling pathway. It has been reported that JAK1, which is one of the JAK family, interacts with PLC (Chang et al., 2004; Clark et al., 2014). However, TG101209, which inhibited SAC-induced PLC, has high specificity for JAK2 and low affinity for JAK1 and JAK3 (Pardanani et al., 2007). Therefore, the interaction of JAK with PLC may be mainly mediated by JAK2. On the other hand, LY294002 and PD98059

suppressed SAC-induced ERK2 phosphorylation, but not SAC-induced p95 IGF-I RTK phosphorylation. In contrast, rapamycin did not suppress both SAC-induced RTK and ERK2 (Fig. 9). These results indicate that PI3K and MEK are upstream elements of ERK2, and mTOR is a downstream element of ERK2. Taken together, these results support the notion that SAC activates the IGF-I RTK/PI3K/MEK/ERK2/mTOR pathway expressed in cultured hepatocytes. In addition, we have previously demonstrated that SAC stimulates autocrine secretion of IGF-I (Moteki et al., 2022).

In conclusion, the results of the present study showed that SAC rapidly stimulated proliferation in primary cultures of adult rat hepatocytes, and that the hepatocyte proliferation effect of SAC was based on activation of the GH receptor/JAK2/PLC pathway followed by activation of the IGF-I RTK/PI3K/ERK2/mTOR pathway.

CRediT authorship contribution statement

Hajime Moteki participated in conducting all the experiments in this study and writing a draft of this manuscript. Mitsutoshi Kimura participated in supervising the planning and execution of the study. Masahiko Ogihara edited the manuscript and managed the project for this research. The final manuscript was approved by all authors.

Declarations of interest: none

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Figures

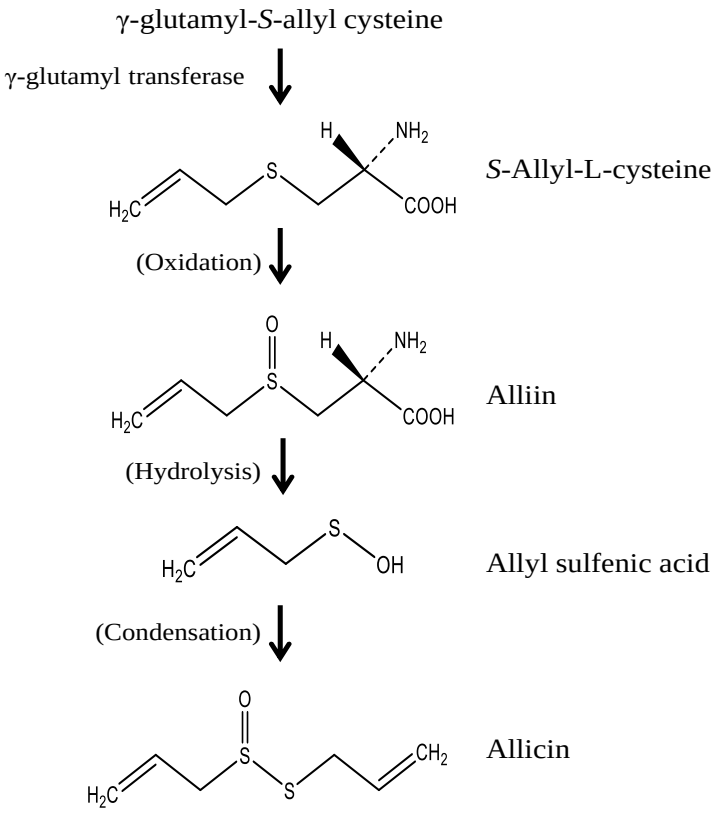
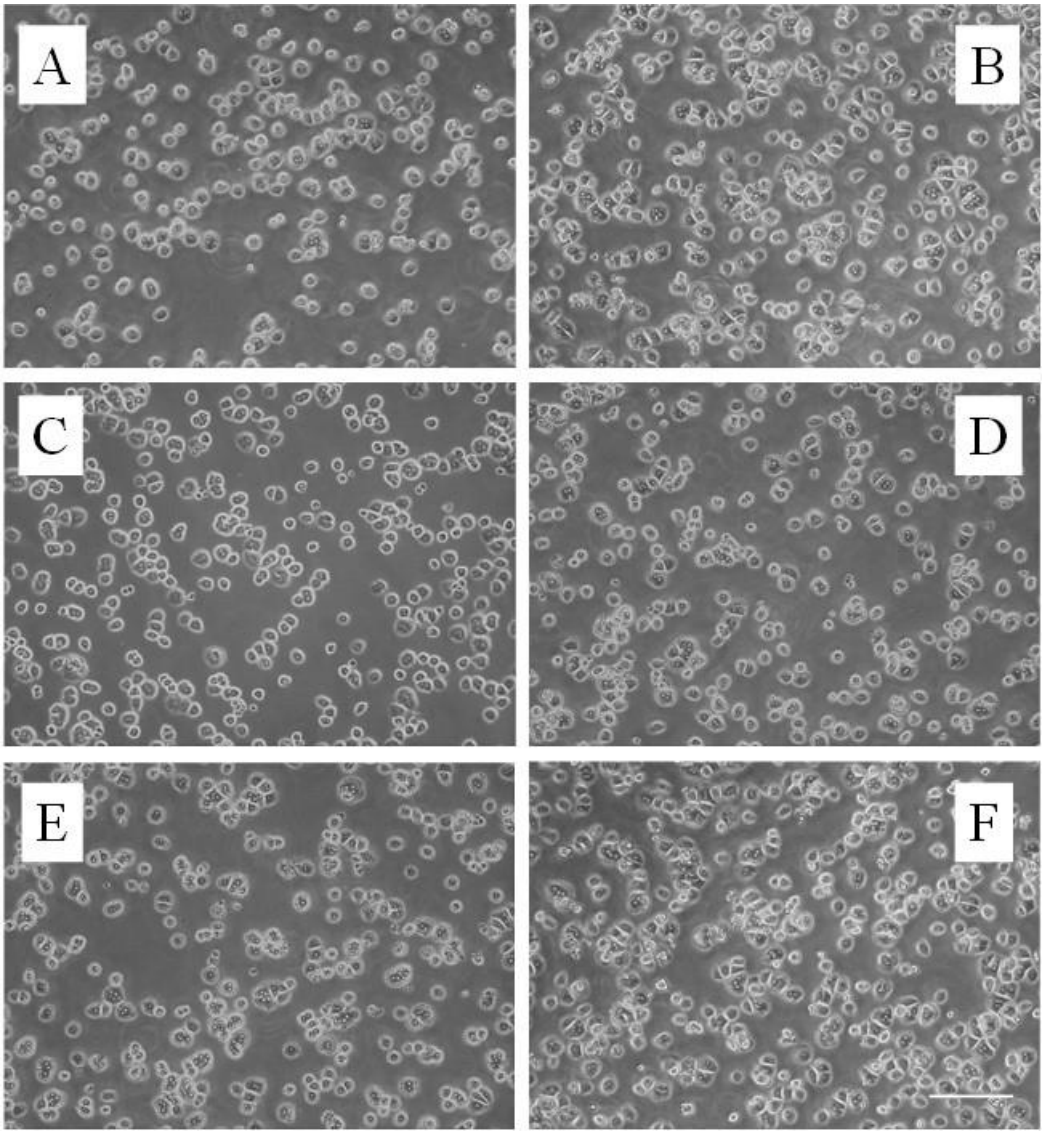
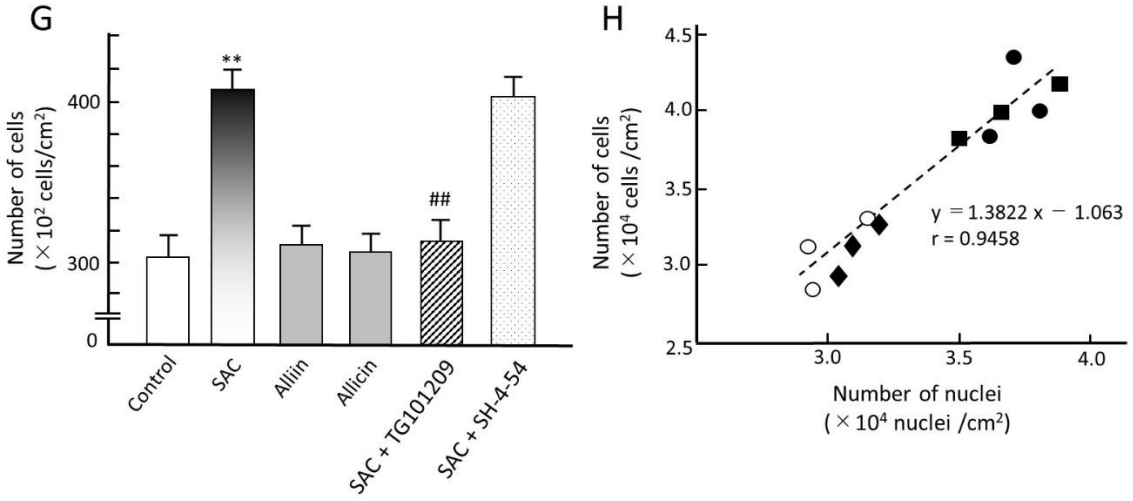


Fig. 1 Biosynthetic pathway of SAC, alliin, and allicin in garlic


A
B
C
D
E
F

14


G
Number of cells
(×10² cells/cm²)
400
300
0
**
##
Control
SAC
Alliin
Allicin
SAC + TG101209
SAC + SH-4-54
H
Number of cells
(×10⁴ cells /cm²)
4.5
4.0
3.5
3.0
2.5
y = 1.3822 x − 1.063
r = 0.9458
3.0
3.5
4.0
Number of nuclei
(×10⁴ nuclei /cm²)

15

Fig. 2 Phase-contrast micrographs of hepatocytes cultured with SAC and its analogs for 5 h. As described in the Methods section, after a 3-h attachment period, hepatocytes (3.3 × 10^4 cells/cm^2) were cultured in serum-free medium containing SAC, alliin, or allicin with growth-related signal-transducing factor inhibitors for 5 h. Photos are (A), control (medium alone); (B), SAC (10^{-6} M); (C), alliin (10^{-6} M); (D), allicin (10^{-6} M); (E), SAC (10^{-6} M) + TG101209 (10^{-6} M); (F), SAC (10^{-6} M) + SH4-54 (10^{-6} M); Scale bar, 200 μm. (G), Number of hepatocytes per field of view (per 0.01 cm^2[×10^2 cells/ cm^2]) under the microscope. Values are shown as means ± S.E.M. (n=3). ** ($P<0.01$) shows comparisons with respective control. ## ($P<0.01$) shows comparisons with respective SAC treatment. (H), Correlation between the number of hepatocytes and the number of nuclei. Open circles (○), control (medium only, n=3); closed circles (●), SAC (10^{-6} M) (n=3); closed diamonds (♦), SAC (10^{-6} M) + TG101209 (10^{-6} M) (n=3); closed squares (■), SAC (10^{-6} M) + SH4-54 (10^{-6} M) (n=3).

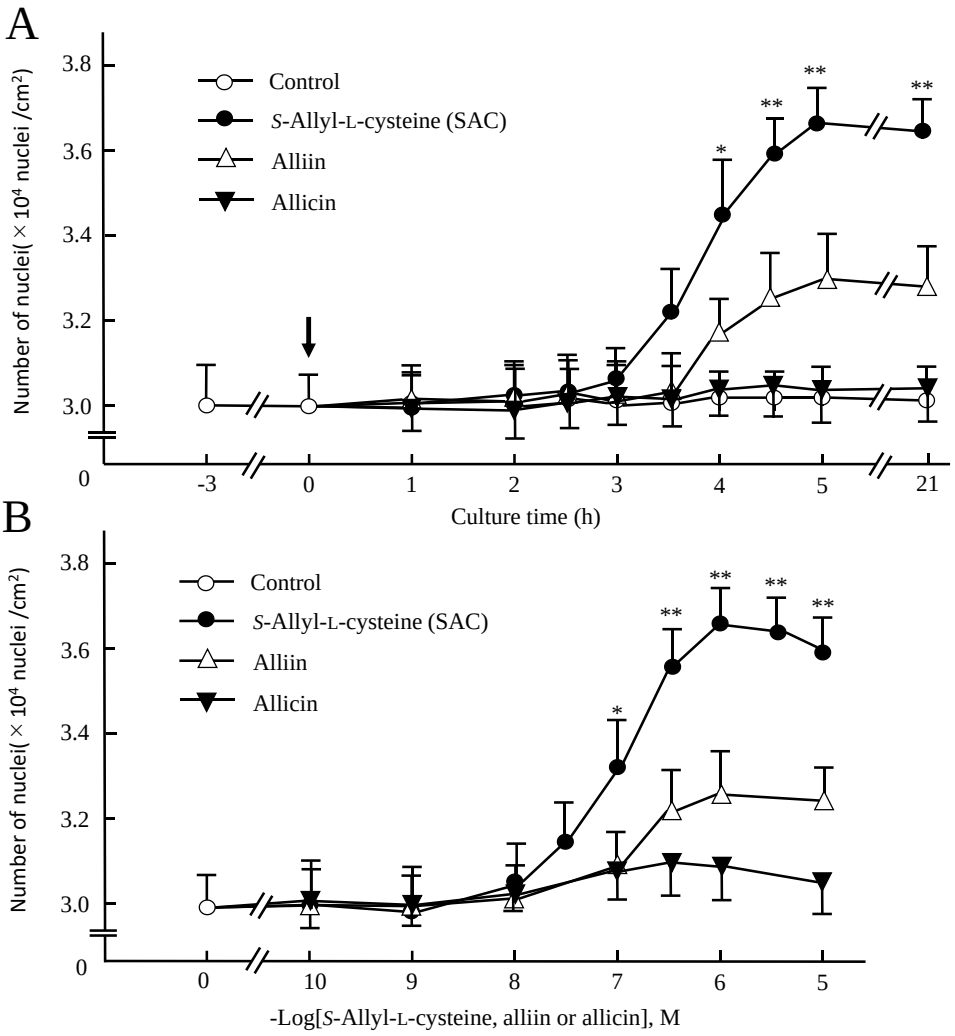
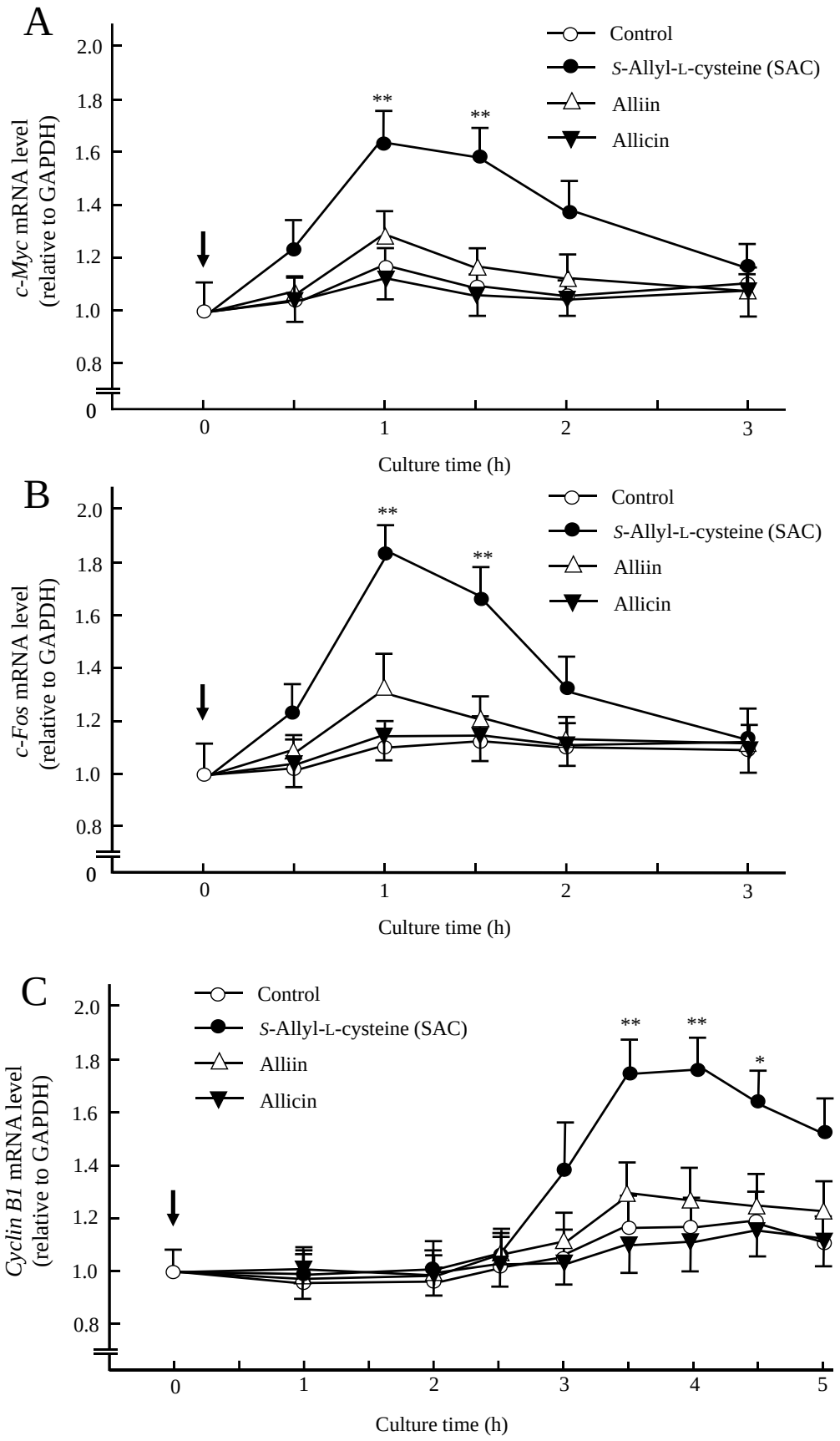


Fig. 3 Time- and dose-dependent cell proliferation effects of SAC and its analogs on cultured hepatocytes. As described in the Methods section, hepatocytes (3.3×10^4 cells/cm^2) attached to the plate were exchanged for serum-free medium and then SAC, alliin, or allicin was added. (A) Time-dependent cell proliferation effects of SAC (● 10^{-6} M), alliin (△ 10^{-6} M), or allicin (▼ 10^{-6} M) in cultured hepatocytes. The arrow shows the addition of the drugs. (B) Dose-dependent cell proliferative effects of SAC (● 10^{-10}-10^{-6} M), alliin (△ 10^{-10}-10^{-6} M), or allicin (▼ 10^{-10}-10^{-6} M) on cell proliferation in cultured hepatocytes for 5 h of drug stimulation. Values are shown as means ± S.E.M. (n=3). *($P<0.05$) and ** ($P<0.01$) show comparisons with respective control.


A
Control
S-Allyl-L-cysteine (SAC)
Alliin
Allicin
c-Myc mRNA level
(relative to GAPDH)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
**
**
0
1
2
3
Culture time (h)
B
Control
S-Allyl-L-cysteine (SAC)
Alliin
Allicin
c-Fos mRNA level
(relative to GAPDH)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
**
**
0
1
2
3
Culture time (h)
C
Control
S-Allyl-L-cysteine (SAC)
Alliin
Allicin
Cyclin B1 mRNA level
(relative to GAPDH)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
**
**
*
0
1
2
3
4
5
Culture time (h)

47

Fig. 4 Effects of SAC and its analogs on *c-Myc*, *c-Fos*, and *cyclin B1* mRNA expression levels in cultured hepatocytes. The mRNA expression levels of *c-Myc*, *c-Fos*, and *cyclin B1* in hepatocytes were quantified using the TaqMan assay, as described in the Methods section. (A) *c-Myc*; (B) *c-Fos*; (C) *cyclin B1.* Control (○ medium alone), SAC (● 10^{-6} M), alliin (△ 10^{-6} M), or allicin (▼ 10^{-6} M) were added at the arrow point. The value for each sample was normalized to the GAPDH mRNA expression levels in each sample. Values are shown as means ± S.E.M. (n=3). *($P<0.05$) and ** ($P<0.01$) show comparison with respective control.

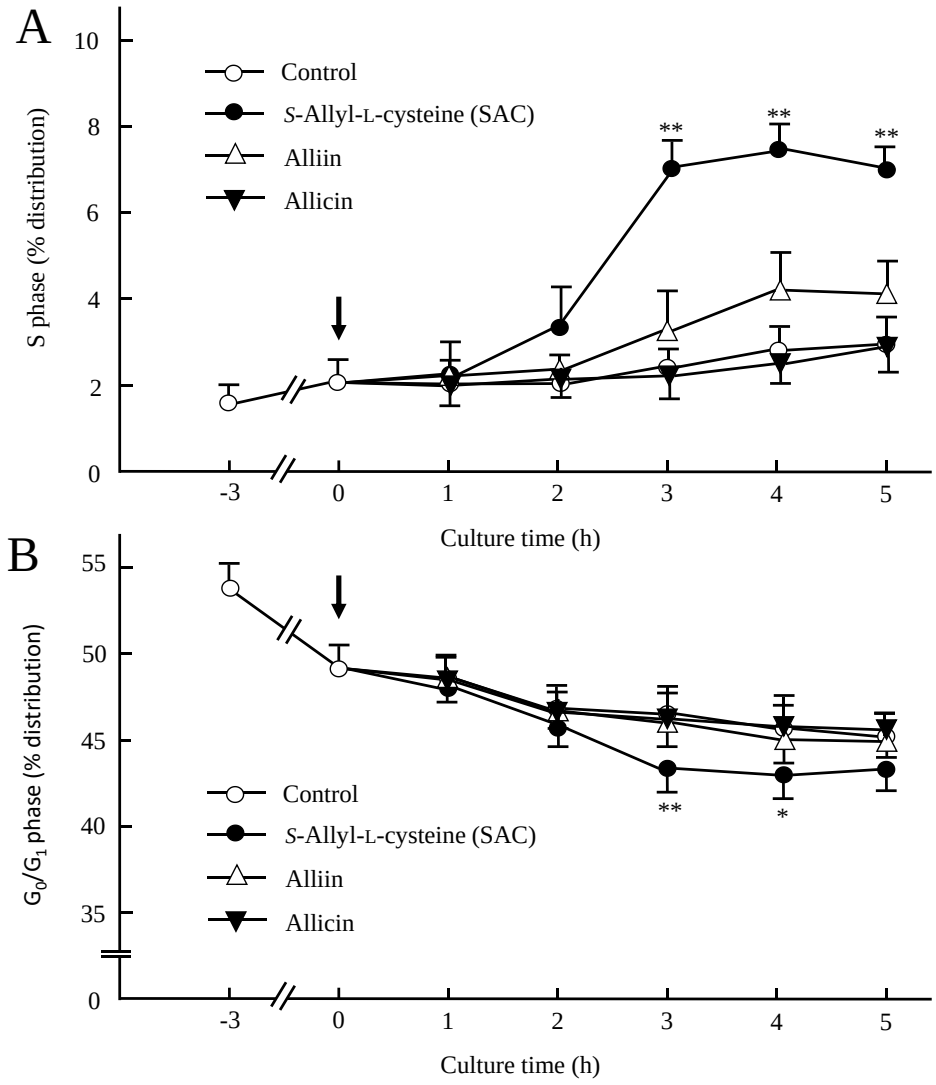


Fig. 5 Time course of the effects of SAC and its analogs on S- and G_0/G_1-phase progression in cultured hepatocytes. S- and G_0/G_1-phases in SAC- (● 10^{-6} M), alliin- (△ 10^{-6} M), or allicin- (▼ 10^{-6} M) stimulated, as described in the Methods section. (A) Time course of the percentage in the S-phase. (B) Time course of the percentage in the G_0/G_1-phase. The arrow shows the addition of the drugs. Values are shown as means ± S.E.M. (n=3). *($P<0.05$) and ** ($P<0.01$) show comparisons with respective control.

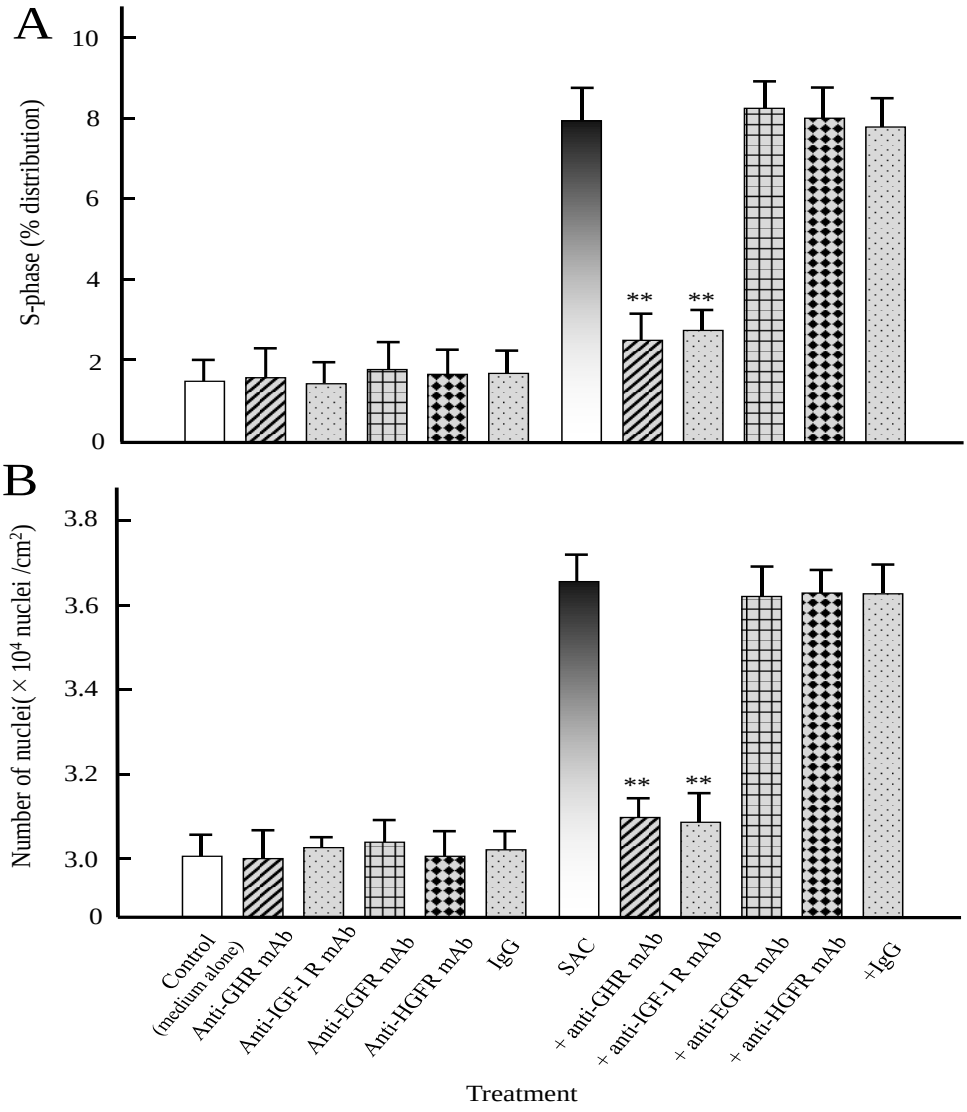


Fig. 6 Effects of monoclonal antibodies to growth factor receptors on SAC-induced hepatocyte proliferation. As described in the Methods section, hepatocytes (3.3×10^4 cells/cm^2) attached to the plate were exchanged for serum-free medium. Then, hepatocytes were cultured in the presence of SAC with or without growth-related signal-transducing inhibitors. S-phase progression (DNA synthesis [A]) and number of nuclei (cell proliferation [B]) were measured 5 h after SAC (10^{-6} M) was added. Monoclonal antibodies were to growth hormone receptor (anti-GHR mAb), insulin-like growth factor type-I receptor (anti-IGF-I R mAb), epidermal growth factor receptor (anti-EGFR mAb),

and hepatocyte growth factor receptor (anti-HGFR mAb). IgG (as a negative control). The antibody concentrations are 100 ng/mL each. Values are shown as means ± S.E.M. (n=3). ** ($P<0.01$) shows comparisons with respective SAC treatment.

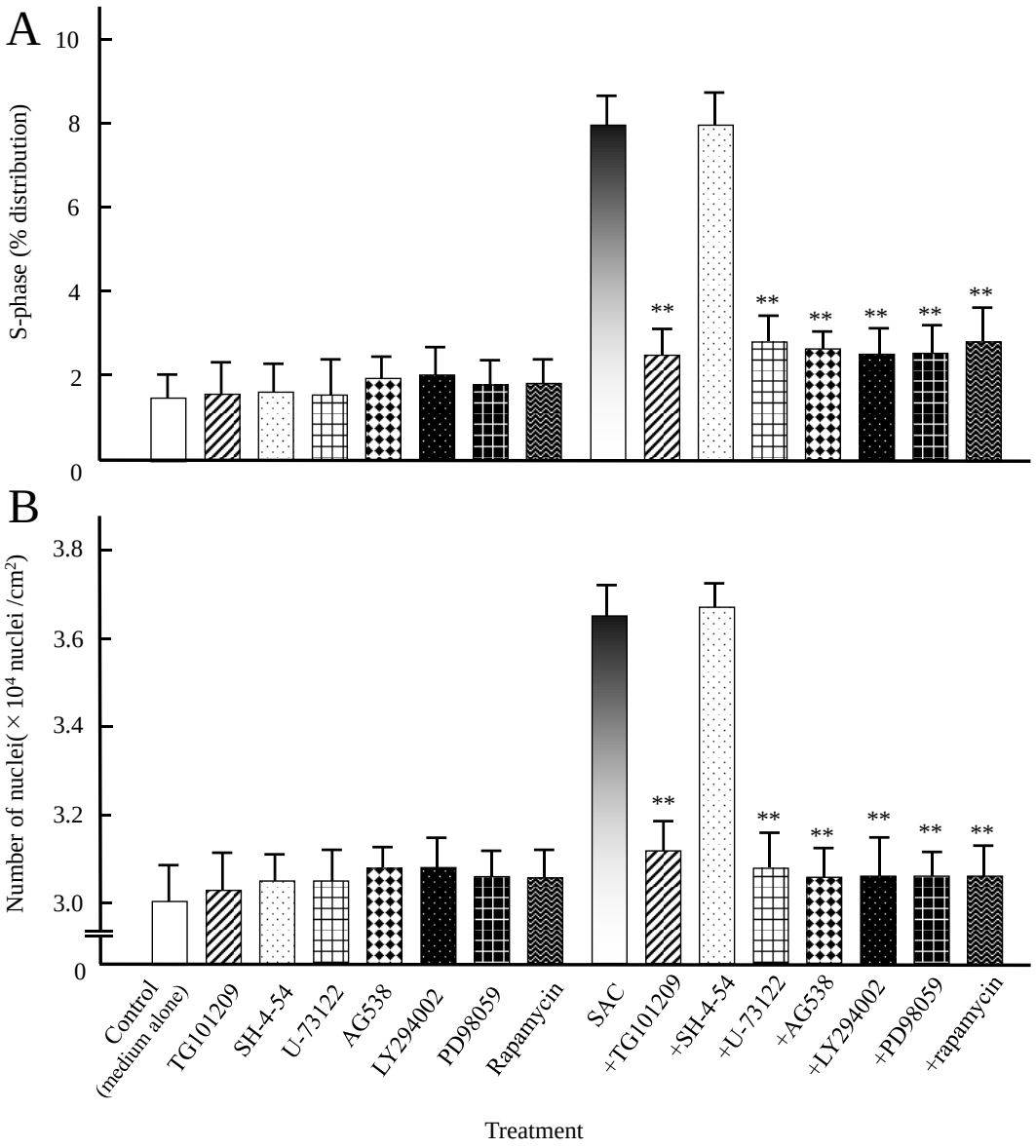
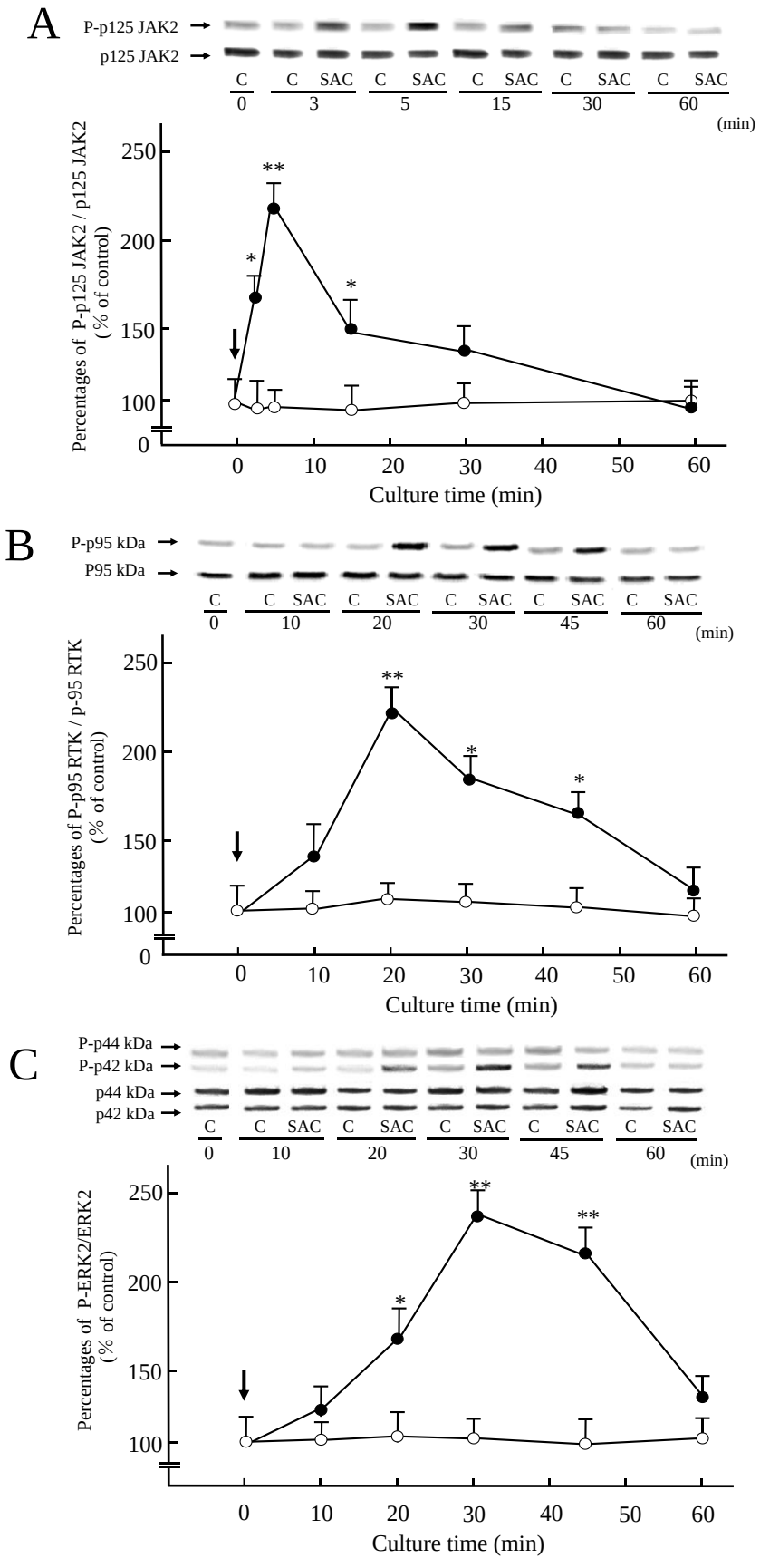


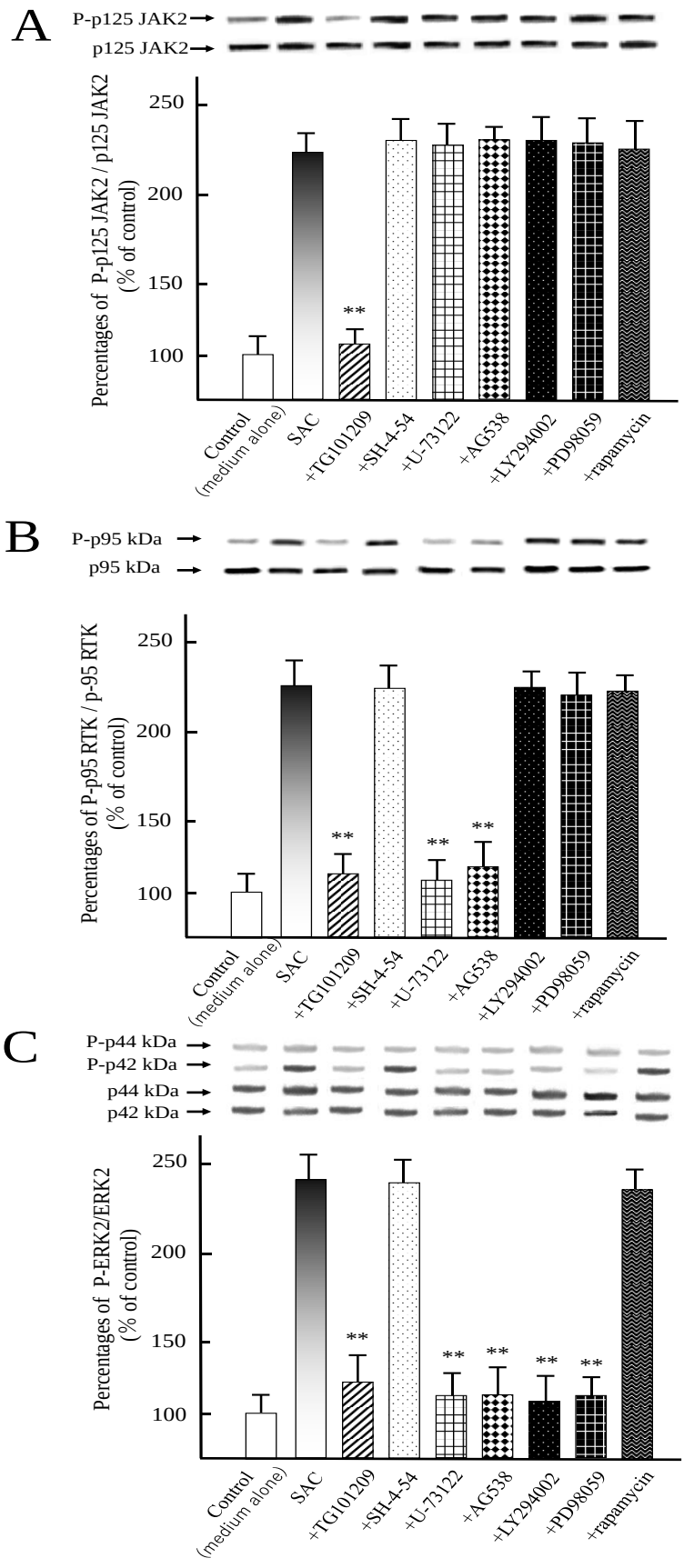
Fig. 7 Effects of SAC on hepatocyte proliferation in the presence of specific signal transduction factor inhibitors. After cell attachment and exchanging for serum-free medium as described in the Methods section, hepatocytes were cultured in the presence of SAC with or without growth-related signal-transducing factor inhibitors. S-phase progression (DNA synthesis [A]) and number of nuclei (cell proliferation [B]) were measured 5 h after SAC (10^{-6} M) was added. Inhibitors used were TG101209 (10^{-6} M), SH-4-54 (10^{-6} M), U-73122 (10^{-6} M), AG538 (10^{-7} M), LY294002 (10^{-7} M), PD98059

(10^{-6} M), and rapamycin (10 ng/mL). Values are shown as means ± S.E.M. (n=3).** ($P<0.01$) shows comparisons with respective SAC treatment.


A
P-p125 JAK2
p125 JAK2
C C SAC C SAC C SAC C SAC C SAC
0 3 5 15 30 60
(min)
Percentages of P-p125 JAK2 / p125 JAK2 (% of control)
Culture time (min)
B
P-p95 kDa
P95 kDa
C C SAC C SAC C SAC C SAC C SAC
0 10 20 30 45 60
(min)
Percentages of P-p95 RTK / p-95 RTK (% of control)
Culture time (min)
C
P-p44 kDa
P-p42 kDa
p44 kDa
p42 kDa
C C SAC C SAC C SAC C SAC C SAC
0 10 20 30 45 60
(min)
Percentages of P-ERK2/ERK2 (% of control)
Culture time (min)

130

Fig. 8 Time course of the effects of SAC on p125 JAK2, p95 kDa IGF-I RTK, and ERK1/2 phosphorylation in cultured hepatocytes. Hepatocytes (3.3×10^4 cells/cm^2) attached to the plate were replaced with serum-free medium as described in the Methods section. Phosphorylation of p125 JAK2, p95 kDa RTK, and ERK1/2 was determined by Western blot analysis, as described in the Methods. (A) Percentages of phosphorylated p125 JAK2 (P-p125 kDa JAK2) / total p125 JAK (p125 kDa JAK2); (B) Percentages of phosphorylated p95 RTK (P-p95 kDa RTK) / total p95 RTK (p95 kDa RTK); (C) Percentages of phosphorylated ERK (P-ERK) 2 / total ERK (ERK) 2. Western blot images are shown at the top of each graph. The arrow shows the addition of SAC (10^{-6} M). Values are shown as means ± S.E.M. (n=3). *($P<0.05$) and ** ($P<0.01$) show comparisons with respective control.


A
P-p125 JAK2
p125 JAK2
Percentages of P-p125 JAK2 / p125 JAK2 (% of control)
250
200
150
100
**
Control (medium alone)
SAC
+TG101209
+SH-4-54
+U-73122
+AG538
+LY294002
+PD98059
+rapamycin
B
P-p95 kDa
p95 kDa
Percentages of P-p95 RTK / p-95 RTK (% of control)
250
200
150
100
**
**
**
Control (medium alone)
SAC
+TG101209
+SH-4-54
+U-73122
+AG538
+LY294002
+PD98059
+rapamycin
C
P-p44 kDa
P-p42 kDa
p44 kDa
p42 kDa
Percentages of P-ERK2/ERK2 (% of control)
250
200
150
100
**
**
**
**
**
Control (medium alone)
SAC
+TG101209
+SH-4-54
+U-73122
+AG538
+LY294002
+PD98059
+rapamycin

155

Fig. 9 Effects of specific signal transduction factor inhibitors on p125 JAK2, p95 kDa IGF-I RTK, and ERK2 phosphorylation induced by SAC. After cell attachment and exchanging for serum-free medium as described in the Methods, hepatocytes were cultured in the presence of SAC with or without growth-related signal-transducing inhibitors. Phosphorylation of p125 JAK2, p95 kDa RTK, and ERK1/2 was determined by Western blot analysis, as described in the Methods. (A) Phosphorylation of p125 kDa JAK2 5 min after SAC (10^{-6} M) stimulation. (B) Phosphorylation of p95 kDa RTK 20 min after SAC (10^{-6} M) stimulation. (C) Phosphorylation of ERK2 30 min after SAC (10^{-6} M) stimulation. Western blot images are shown at the top of each graph. Inhibitors used were TG101209 (10^{-6} M), SH-4-54 (10^{-6} M), U-73122 (10^{-6} M), AG538 (10^{-7} M), LY294002 (10^{-7} M), PD98059 (10^{-6} M), and rapamycin. Values are shown as means ± S.E.M. (n=3).** ($P<0.01$) shows comparisons with respective SAC treatment.

CRediT Author Statement

Title of Manuscript:

Cell proliferation effects of *S*-allyl-L-cysteine are associated with phosphorylation of Janus kinase 2, insulin-like growth factor type-I receptor tyrosine kinase, and extracellular signal-regulated kinase 2 in primary cultures of adult rat hepatocytes.

Hajime Moteki: participated in conducting all the experiments in this study and writing a draft of this manuscript. **Mitsutoshi Kimura**: participated to supervise the planning and execution of the study. **Masahiko Ogihara:** edited the manuscript and managed the project for this research. The final manuscript was approved by all authors.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: