Hispidulin potently inhibits human glioblastoma multiforme cells through activation of AMPK

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Title

Hispidulin potently inhibits human glioblastoma multiforme cells through activation of AMPK

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common and lethal type of primary brain tumor. Despite recent therapeutic advances in other cancers, the treatment of GBM remains ineffective and essentially palliative. The current focus lies in the finding of components that activate the AMP-activated protein kinase (AMPK), one key enzyme thought to be activated during the caloric restriction (CR). In the present study, we found that treatment of hispidulin, a flavone isolated from *Saussurea involucrate* Kar. et Kir., resulted in dose-dependent inhibition of GBM cellular proliferation. Interestingly, we show that hispidulin activated AMPK in GBM cells. The activation of AMPK suppressed downstream substrates, such as the mammalian target of rapamycin (mTOR) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and a general decrease in mRNA translation. Moreover, hispidulin activated AMPK decreases the activity and/or expression of lipogenic enzymes, such as the fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC). Furthermore, hispidulin blocked the progression of the cell cycle at G1 phase and induced apoptosis by inducing p53 expression and further up-regulating p21 expression in GBM cells. Based on these results, we demonstrated that hispidulin has the potential to be a chemoprevention and therapeutic agent against human GBM.

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KEYWORDS: Hispidulin; AMPK, *Saussurea involucrate* Kar. et Kir.; Traditional Chinese Medicine; Glioblastoma multiforme

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and aggressive class of malignant brain tumors. Standard therapy for GBM consists of surgical resection, radiotherapy, and chemotherapy (*1*). Compared to the advances in the treatment of other types of tumors, the overall prognosis for GBM patients with this disease remains dismal, the average time for recurrence of the tumor is only 6.9 months, and the 5-year survival rate for GBM patients is still less than 5% (*2*). Therefore, new chemotherapeutic agents on the treatment of GBM are still an energetic topic.

Caloric restriction (CR) is a 20-40% lowering of caloric intake, known to retard aging processes and to lengthen life in many organisms (*3*). It has been suggested that both dietary restriction and decreased nutrient-sensing pathway activity can lower the incidence of age-related loss of function and disease by reducing the levels of DNA damage and mutations that accumulate with age (*4*). Cancer is an age-related disease in organisms with renewable tissues, as the incidence of most cancers increase with age following an accumulation of mutations. Moderate CR lowered the incidence of cancer.

The AMP-activated protein kinase (AMPK) is a critical monitor of cellular energy status, thought to be activated during CR. AMPK is a heterotrimeric serine/threonine protein kinase that is composed of a catalytic α-subunit, and

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regulatory β- and γ-subunits. AMPK activity is regulated allosterically by AMP and through phosphorylation in the activation loop of the α-subunit (*5*). AMPK controls processes relative to tumor development, including cell growth, survival, cell cycle progression, and protein synthesis. The AMPK pathway is linked to tumor growth and proliferation through regulation of the mammalian target of rapamycin (mTOR) pathway. AMPK activation inhibits the growth of a broad spectrum of cancers via mTOR, reduces the proliferation of certain tumor cells and can cooperate with other agents to induce apoptosis. The best-understood roles of mTOR in mammalian cells are related to the control of mRNA translation by the 4E-BP1 (*6*). In the hypophosphorylation form, 4E-BP1 by mTOR ultimately results in the initiation of translation of certain mRNAs, including those that are needed for cell cycle progression and are involved in cell cycle regulation (*7*).

Defects in fatty acid synthesis or processing contribute to the development of many diseases, including insulin resistance, type 2 diabetes, obesity, non-alcoholic fatty liver disease, and cancer (*8*). Fatty acid synthase (FASN), a key enzyme for lipogenesis, provides the best opportunity for therapeutic applications because of its tissue distribution and unusual enzymatic activity. FASN is downregulated in most normal human tissues because of the fat in our diet, with the exception of lactating breasts and cycling endometrium. In contrast, FASN is often highly expressed in

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human cancers, including breast, colorectum, prostate, bladder, ovary, oesophagus, stomach, lung, oral tongue, oral cavity, head and neck, thyroid and endometrium, and also in mesothelioma, nephroblastoma, retinoblastoma, soft tissue sarcomas, Paget's disease of the vulva, cutaneous melanocytic neoplasms including melanoma, and hepatocellular carcinoma (*9*). This differential tissue distribution makes FASN an attractive target for cancer cells. Moreover, acetyl-CoA carboxylases (ACC) are rate-limiting enzymes in *de novo* fatty acid synthesis, catalyzing ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. Recently, ACC up-regulation has been recognized in multiple human cancers, not only in advanced breast carcinomas but also in preneoplastic lesions associated with increased risk for the development of infiltrating breast cancer (*10*). Therefore, FASN and ACC might be effective as potent targets for cancer intervention, and the inhibitors developed for the treatment of metabolic diseases would be potential therapeutic agents for cancer therapy.

Hispidulin (4',5,7-trihydroxy-6-methoxyflavone) is a naturally occurring flavone commonly found in *Saussurea involucrata* Kar. et Kir., a rare traditional Chinese medicinal herb (*11*). Several *in vitro* studies have demonstrated its potent antioxidative, antifungal, anti-inflammatory, antimutagenic, and antineoplastic properties (*12-14*). Recently, hispidulin is identified as a potent ligand of the central human BZD receptor *in vitro* (*15*). It also acts as a partial positive allosteric modulator

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at $GABA_A$ receptors, penetrates the blood–brain barrier (BBB) and possesses anticonvulsant activity in the central nervous system (CNS) (*16*). Based on more observations it has been found that hispidulin acts as a potential modulator of CNS activity, prompted us to investigate its antineoplastic activity against GBM. In this work, we examined the effects of hispidulin on GBM cells. We present here, for the first time that AMPK is activated by hispidulin in GBM cells. The activation of AMPK suppresses protein synthesis, lipogenesis, and cell cycle progression in GBM cells. Our study suggests that hispidulin may be useful as a GBM chemopreventive or therapeutic agent.

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MATERIALS AND METHODS

Chemicals. MTT, compound c, PI and antibodies for β-Actin were purchased from Sigma (St. Louis, MO). Hispidulin was purchased from Tocris Bioscience (Bristol, UK). Antibodies for FASN, phospho-ACC (Ser 79), phospho-mTOR (Ser2448), pohspho-4E-BP1 (Thr 37/46), phosphor-AMPK (Thr 172), PARP, p21 and p53 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for mouse and rabbit conjugated with horseradish persdish peroxidase were purchased from Chemicon (Temecula, CA). Immobilon Western Chemiluminescent HRP Substrate was from Milliore Corporation (Billerica, MA).

Cell culture. GBM8401 and GBM8901 human GBM cells were obtained from Bioresources Collection and Reasearch Center (Hsin Chu, Taiwan). These cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and were grown at 37°C in a humidified atmosphere of 5% CO_2.

Cell Proliferation Assays. As described previously (*17*), the effects of hispidulin on cell proliferation were examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method.

Western blot. Cells (2×10^6) were seeded onto a 100-mm tissue culture dish containing 10% FBS DMEM/F12 and cultured for 24 h. Then cells were incubated in

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10% FBS DMEM/F12 treating with various agents as indicated in figure legends. After treatment, cells were placed on ice, washed with cold PBS, and lysed in lysis buffer. Western Blot was done as described previously (*18*). The intensity of the bands was scanned and quantified with NIH image software.

Cell cycle analysis. Cells (5×10^5) were cultured in 60-mm cell culture dish and incubated for 24 h. Then cells were harvested in 15 mL tube, washed with PBS, resuspended in PBS, and fixed in 2 mL of iced 100 % ethanol at -20 ℃ overnight. Cell pellets were collected by centrifugation, resuspended in 0.5 mL of hypotonic buffer (0.5 % Triton X-100 in PBS and 0.5 μg/ml RNase), and incubated at RT for 30min. Then 1 mL of propidium iodide solution (50 μg/ mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide-DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry (BD Biosciences, San Jose, CA).

Short hairpin RNA. RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, supported by the National Research Program for Genomic Medicine Grants of NSC (NSC 97-3112-B-001-016). Short hairpin RNAs (shRNAs) were designed to target specific sequences of human AMPK (Clone ID: TRCN000000861; Target sequence: 5'-GTT GCC TAC CAT CTC ATA ATA-3'). One

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day before transfection, cells were seeded at the density of 30-40% without antibiotics. 20 nM AMPK shRNAs were transfected into cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were incubated for an additional 24h before addition of hispidulin as previously described. The effects of hispidulin on cell proliferation were examined by MTT method.

Statistical analysis. All valves were expressed as mean ±SD. Each value is the mean of at least three separate experiments in each groups, Student's *t*-test was used for statistical comparison. Asterisks indicate that the values are significantly different from the control (*, $P < 0.05$; **, $P < 0.01$).

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RESULTS

Hispidulin exhibits potent antiproliferative activity against human GBM cells. To investigate the bioactivity of hispidulin (**Figure 1A**) in human GBM cells, we treated GBM8401 and GBM8901 cells with different concentrations of hispidulin at 37℃ for 48 h, and assessed cell proliferation by MTT assay. The results showed that GBM8401 **(Figure 1B)** and GBM8901 **(Figure 1C)** cells were inhibited by hispidulin in a dose-dependent manner. The IC_{50} values of hispidulin against GBM8401 and GBM8901 cells were 60 μM and 40 μM, respectively.

Hispidulin suppresses protein synthesis by activating AMPK to inbibit the mTOR pathway. The current focus lies in the finding of components that activate AMPK. We next identify whether the antiproliferative effects of hispidulin is by activating AMPK in human GBM cells. GBM8401 and GBM8901 cells were treated with 60 μM and 40 μM hispidulin at 37℃ for different durations, respectively. Western blot analysis indicated that hispidulin stimulates AMPK phosphorylation in a time-dependent manner (**Figure 2A**). Those results showed that hispidulin up-regulated AMPK activity and suppressed cell proliferation in human GBM cells. mTOR/4E-BP1 pathway controls the protein translation/synthesis in various types of cells. 4E-BP1 is phosphorylated by mTOR upon growth factor stimulation, and then the cells undergo cell cycle progression and proliferation (*7*). To determine whether

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hispidulin suppresses the protein synthesis by activating AMPK to inhibit the mTOR pathway, phospho-mTOR and 4E-BP1 were detected by western blotting. The phosphorylation of mTOR (**Figure 2B**) and 4E-BP1 (**Figure 2C**) were decreased at 12h in GBM8401 and GBM8901 cells. Next, we added the compound c, an AMPK inhibitor, in the absence or presence of hispidulin. The AMPK activity was suppressed by compound c in the presence of hispidulin and the mTOR activity was recovered in GBM8401 (**Figure 3A**) and GBM8901 (**Figure 3B**) cells. We hypothesized that hispidulin, by up-regulating AMPK activity, would inhibit mTOR activation and downstream events in human GBM cells.

Hispidulin decreases lipid synthesis by decreasing FASN expression and inhibiting ACC activity. The activity of FASN and ACC were known to be negatively regulated by AMPK (*19*). In the present study, the FASN protein level was decreased (**Figure 4A**) and ACC was phorsphorylated (**Figure 4B**) in a time dependent fashion when GBM8401 and GBM8901 cells were treated with 60 μM and 40 μM hispidulin, respectively. To further study the effect of AMPK in regulating the activity of fatty acid synthesis enzymes. We added the compound c, in the absence or presence of hispidulin. After the treatment of hispidulin, the protein levels of FASN were decreased and phospho-ACC was increased. However, the activities of enzymes of fatty acid synthesis were restored in the presence of compound c in GBM8401

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(**Figure 5A**) and GBM8901 (**Figure 5B**) cells. These results demonstrate that hispidulin inhibits the activity of fatty acid synthesis enzymes through the activation of the AMPK pathway.

Hispidulin induces growth arrest and apoptosis. We examined effects of hispidulin on the cell cycle to clarify the mechanism of hispidulin-induced inhibition of proliferation. Hispidulin caused the accumulation of the G0/G1 phase followed by an increase in hypodiploid cells as indicated by apoptotic cells in GBM8401 (**Figure 6A**) and GBM8901 (**Figure 6B**) cells. Moreover, we examined the expression of G1 related cell cycle control proteins and apoptosis related proteins on western blot analysis. GBM8401 and GBM8901 cells were treated with 60 μM and 40 μM hispidulin for indicated durations and used 50 μg of whole-cell extracts on Western blot analyses. After 12 hours of hispidulin treatment, we found increased levels of p53 and p21 in GBM8401 and GBM8901 cells (**Figure 6C**). Moreover, hispidulin showed a clear apoptosis within 12 hours, showing cleavages for PARP in Western blot analyses (**Figure 6D**). To further determine whether hispidulin induced inhibition of proliferation by activating AMPK, we added the compound c, an AMPK inhibitor, in the absence or presence of hispidulin. The inhibition of proliferation by hispidulin was resumed in the presence of compound c in GBM8401 (**Figure 7A**) and GBM8901 (**Figure 7B**) cells. In addition, we also treated cells with AMPK shRNA to

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285 silence the expression of AMPK. AMPK shRNA recovered the inhibition of
286 proliferation by hispidulin in GBM8401 (**Figure 7A**) and GBM8901 (**Figure 7B**)
287 cells. We hypothesized that hispidulin, by up-regulating AMPK activity, would inhibit
288 GBM cells proliferation.
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DISCUSSION

The previous study illustrates that flavone hispidulin probably acts as a partial positive allosteric modulator at GABAA receptors, penetrates the BBB and possesses anticonvulsant activity in the CNS (*15*, *16*). These observations encouraged us to investigate its antineoplastic activity against GBM. In the field of food and nutrition, the current focus lies in the finding of components that activate AMPK. Here, we show that hispidulin activated AMPK in GBM cells. The activation of AMPK suppressed protein synthesis, lipogenesis, and cell cycle progression. Targeting AMPK signaling by hispidulin may have potential therapeutic implications for GBM and age-related diseases.

mTOR, a serine-threonine kinase, plays a key role in the regulation of cellular growth. The mTOR pathway is aberrantly activated in many human cancers. The role of mTOR in tumor acts as a sensor for energy, growth factors and nutrients, all of which are required for protein translation. Thus, approaches to block the pathway are being actively pursued in many laboratories and pharmaceutical companies. Activation of AMPK results in a decrease of mTOR signaling. The AMPK signal system contains some tumor suppressor genes including LKB1, TSC1, TSC2 and p53, and suppresses tumor growth by inhibiting the activity of various proto-oncogene such as PI3K, Akt and ERK (*20*). Both TSC1 (also named hamartin) and TSC2 (also

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named tuberin) tumor suppressor protein control the protein synthesis of cell. Activation of AMPK induces activation of the TSC2-TSC1 complex to inhibit mTOR (*21*). The Eukaryotic translation initiation factor 4E-BP1 is the downstream effector of mTOR. Through this effector mTOR controls the protein translation (*22*). Data presented here show that the inhibition of protein translation via the AMPK-mTOR pathway by hispidulin in GBM cells is effective.

AMPK acts as a fuel gauge by monitoring cellular energy levels (*23*). FASN and ACC are key enzymes for lipogenesis. AMPK specifically regulated both the phosphorylation and dephosphorylation cycles of ACC and the expression levels of FASN. Acutely activated AMPK phosphorylates and inhibits ACC. Chronically activated AMPK decreases the expression of SREBP1c, thus suppressing the synthesis of ACC, FASN and other lipogenic enzymes (*24*). A recent study identified that pharmacologically inducing a 'low-energy status' in tumour cells can result in AMPK-induced ACC phosphorylation, FASN downregulation and marked decrease of endogenous lipogenesis. Cancer cells, thus, stopped proliferating and lost their invasive and tumorigenic properties *in vitro* and *in vivo* (*25*). In this study, we show that AMPK is activated by hispidulin, and is required for hispidulin suppression of lipogenesis. From a clinical perspective, these findings justify further work exploring the ability of 'low-energy mimickers' to therapeutically manage lipogenic carcinomas.

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AMPK also plays the role of energy sensor in cell cycle (*26*). It seems rational to view AMPK as a survival factor for cancer cells. AMPK raises energy production via the activation of glucose uptake, glycolysis, and fatty acid oxidation in response to ATP-depleting stresses (*27*). Recent study shows that AMPK is critical for cancer cell adaptation in response to hypoxia or glucose deprivation (*28*). Solid tumors that outgrow the existing vasculature are continuously exposed to a microenvironment in which the supply of both oxygen and nutrition are quite limited. In accordance with the aforementioned reports and the data documented herein, it seems reasonable to conclude that the inhibition of AMPK in cancer cells may prove useful as an approach for the increased induction of apoptosis in tumor cells after hispidulin treatment. However, some have concluded that AMPK activation may be employed as a component of an anticancer therapy (*29*). The logic of this approach is predicated on recent observations that AMPK also strongly suppresses cell proliferation. This effect is mediated, in part, by several tumor suppressor proteins associated with the AMPK signaling network, including LKB1 and the tuberous sclerosis complex (TSC2). Jones *et al.* recently reported that the activation of AMPK induces p53-Ser15 phosphorylation in response to glucose deprivation, resulting in replicative senescence (*30*). The ability of AMPK to promote senescence or to inhibit cell proliferation in response to energy starvation has been interpreted as a check point that couples

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glucose availability to the progression of the cell cycle; it was implied that the activation of AMPK might promote the conservation of the remaining energy to support the survival and physiological functions of the cell during cell cycle arrest. Our results indicated that hispidulin inhibited the proliferation of GBM cells via the activation of AMPK. Hispidulin treatment inhibited the progression of cell cycle in the G1 phase. Hispidulin increased the expression level of p53 and subsequently enhanced the expression level of p21 resulting in cell cycle arrest in GBM cells. It is likely that induction of p21 promotes growth arrest and exerts a protective effect after AMPK activation.

In conclusion, AMPK is activated by hispidulin in GBM cells. When this occurs, a key enzyme involved in protein synthesis, mTOR, is inhibited. In addition, the activity and/or expression of lipogenic enzymes, such as FASN and ACC are decreased. Interestingly, hispidulin blocked the progression of cell cycle at G1 phase and induced apoptosis in GBM cells (**Figure 8**). Taken together, our study suggests that hispidulin may be useful as a GBM chemopreventive agent. Nevertheless, additional studies are required to evaluate the efficacy of hispidulin in suitable experimental animal systems.

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ABBREVIATIONS

ACC, Acetyl-CoA carboxylase; AMPK, AMP-activated preotin kinase; CR, Caloric restriction; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dimethyl sulfoxide; 4E-BP1, Eeukaryotic initiation factor 4E-binding protein-1; FASN, Fatty acid synthase; FBS, Fetal bovine serum; mTOR, Mmammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide.

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513 **Figure Legends**

514 **Figure 1. Hispidulin inhibits the proliferation of human GBM cells.** (**A**) Chemical
515 structures of hispidulin. (**B**) GBM8401 and (**C**) GBM8901 cells were seeding into
516 24-well plates in the presence of 10% FBS and after 24 hr treated with various
517 concentrations of hispidulin at 37 ^{o} C for 48 hr. The effect on cell growth was
518 examined by MTT assay, and the percentage of cell proliferation was calculated by
519 defining the absorption of cells without of hispidulin treatment as 100%. This
520 experiment was repeated three times. Bar represents the SD.

521 **Figure 2. Hispidulin up-regulates AMPK activity.** GBM8401 and GBM8901 cells
522 were treated with 60 μM and 40 μM hispidulin for indicated duration, respectively.
523 After harvesting, cells were lysed and prepared for western blotting analysis using
524 antibodies against (**A**) phospho-AMPK (Thr172), (**B**) phospho-mTOR (Ser2448), (**C**)
525 phospho-4E-BP1 (Thr37/46) and β-actin. Western blot data presented are
526 representative of those obtained in at least three separate experiments. The values
527 below the figures represent the change in protein expression normalized to β-actin.

528 **Figure 3. Hispidulin decreases the protein synthesis by activating AMPK to**
529 **inhibit mTOR pathway.** (**A**) GBM8401 and (**B**) GBM8901 cells were incubated with
530 15 μM compound c in the absence or presence of hispidulin for 24 hr.
531 Phospho-AMPK (Thr172), phospho-mTOR (Ser2448), and β-actin were detected by

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western blot. Western blot data presented are representative of those obtained in at least three separate experiments. The values below the figures represent the change in protein expression normalized to β-actin.

Figure 4. Hispidulin decreases the activity of fatty acid synthesis by inhibiting the expression of FASN and the activity of ACC. GBM8401 and GBM8901 cells were treated with 60 μM and 40 μM hispidulin for indicated duration, respectively. After harvesting, cells were lysed and prepared for western blotting analysis using antibodies against (**A**) FASN, (**B**) pospho-ACC (Ser79) and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The values below the figures represent the change in protein expression normalized to β-actin.

Figure 5. Hispidulin decreases the activity of fatty acid synthesis via activating of AMPK. (**A**) GBM8401 and (**B**) GBM8901 cells were incubated with 15 μM compound c in the absence or presence of hispidulin for 24 hr. After harvesting, cells were lysed and prepared for western blotting analysis using antibodies against FASN, phospho-ACC (Ser79), and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The values below the figures represent the change in protein expression normalized to β-actin.

Figure 6. Hispidulin induces cell cycle arrest and apoptosis in GBM cells. (**A**)

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551 GBM8401 and (**B**) GBM8901 cells were treated with 60 μM and 40 μM hispidulin
552 for the indicated duration, respectively. After harvesting, cells were analyzed for
553 propidium iodide-stained DNA content by flow cytometry. The indicated percentages
554 are the mean of three independent experiments, each in duplicate. Bar represent the
555 S.D. GBM8401 and GBM8901 cells were treated with 60 μM and 40 μM hispidulin
556 for indicated duration, respectively. After harvesting, cells were lysed and prepared
557 for western blotting analysis using antibodies against (**C**) p53, and p21, (**D**) PARP and
558 β-actin. Western blot data presented are representative of those obtained in at least
559 three separate experiments. The values below the figures represent the change in
560 protein expression normalized to β-actin.

561 **Figure 7. Hispidulin decreases the activity of fatty acid synthesis via activating of**
562 **AMPK.** (**A**) GBM8401 and (**B**) GBM8901 cells were incubated with 15 μM
563 compound c in the absence or presence of hispidulin for 48 hr. GBM8401 and
564 GBM8901 cells were transfected with 50 nmol/L AMPKα1 shRNA using
565 lipofectamine. After twenty-four hour transfection, cells were treated with hispidulin
566 for 48 hr. The effect on cell growth was examined by MTT assay, and the percentage
567 of cell proliferation was calculated by defining the absorption of cells without of
568 hispidulin treatment as 100%. This experiment was repeated three times. Bar
569 represents the SD. Asterisks indicate that the values are significantly different from

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570 that of the control (*, $P < 0.05$; **, $P < 0.01$).

571 **Figure 8. A schematic summary for the anti-GBM cells mechanisms of hispidulin**

572 **shown in the present study.**

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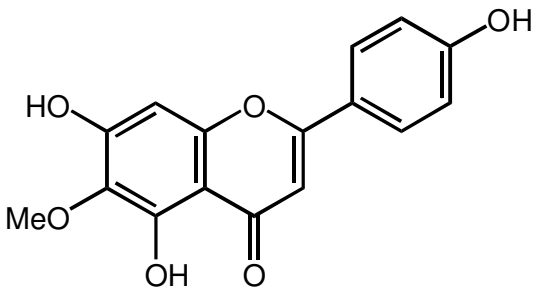
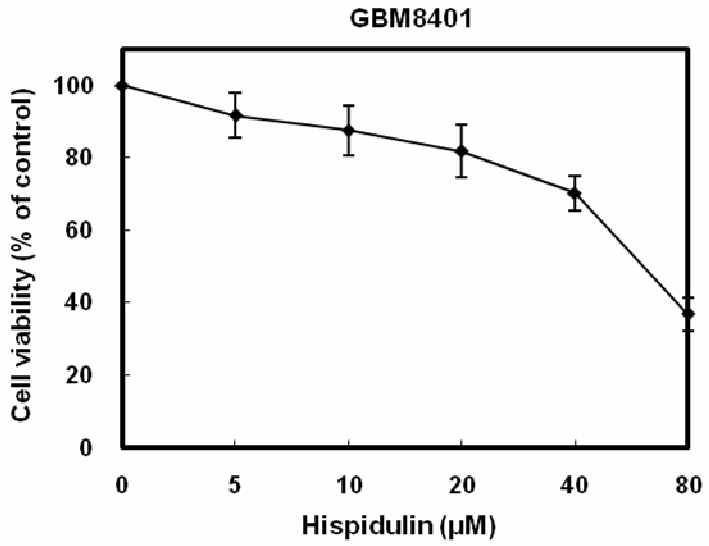
Figure 1

(A)


OH
HO
O
MeO
OH
O

(B)


GBM8401
Cell viability (% of control)
100
80
60
40
20
0
0
5
10
20
40
80
Hispidulin (μM)

(C)

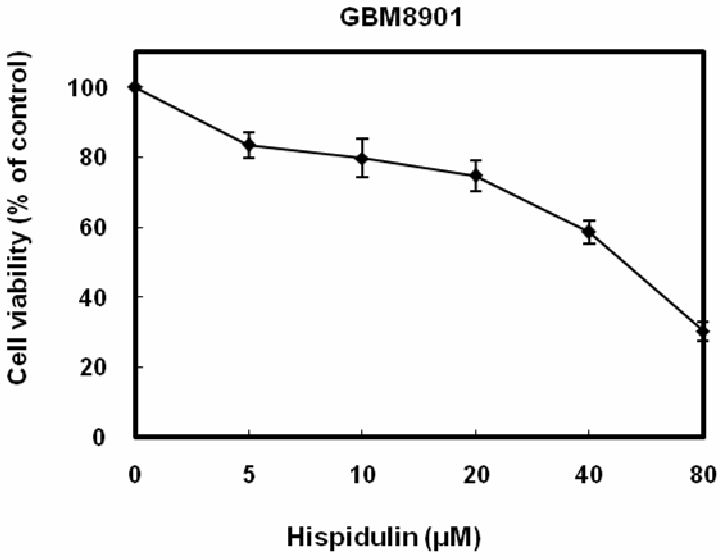

GBM8901
Cell viability (% of control)
100
80
60
40
20
0
0
5
10
20
40
80
Hispidulin (μM)

Figure 2

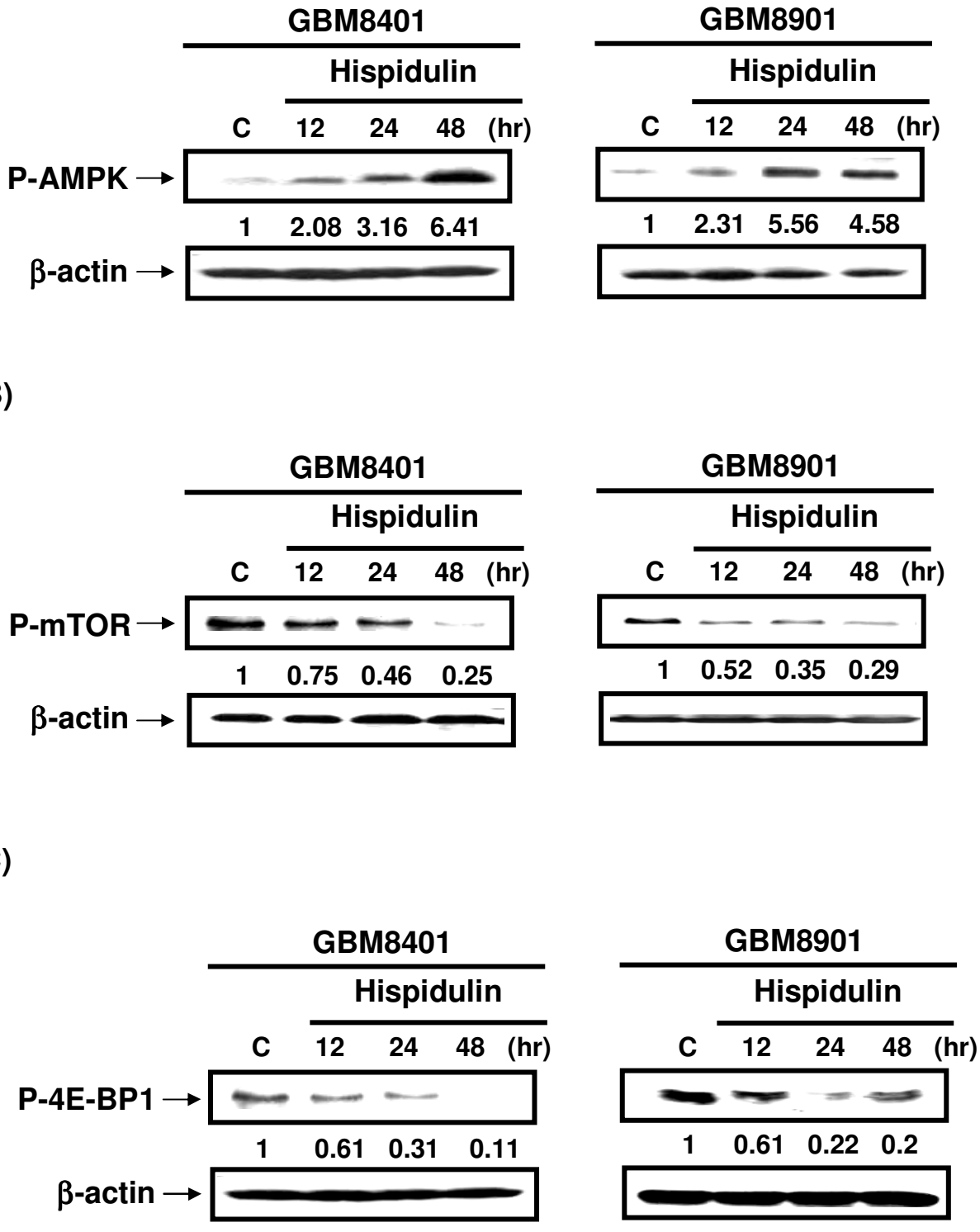

(A)
GBM8401
Hispidulin
C 12 24 48 (hr)
P-AMPK
1 2.08 3.16 6.41
β-actin
GBM8901
Hispidulin
C 12 24 48 (hr)
1 2.31 5.56 4.58
(B)
GBM8401
Hispidulin
C 12 24 48 (hr)
P-mTOR
1 0.75 0.46 0.25
β-actin
GBM8901
Hispidulin
C 12 24 48 (hr)
1 0.52 0.35 0.29
(C)
GBM8401
Hispidulin
C 12 24 48 (hr)
P-4E-BP1
1 0.61 0.31 0.11
β-actin
GBM8901
Hispidulin
C 12 24 48 (hr)
1 0.61 0.22 0.2

Figure 3

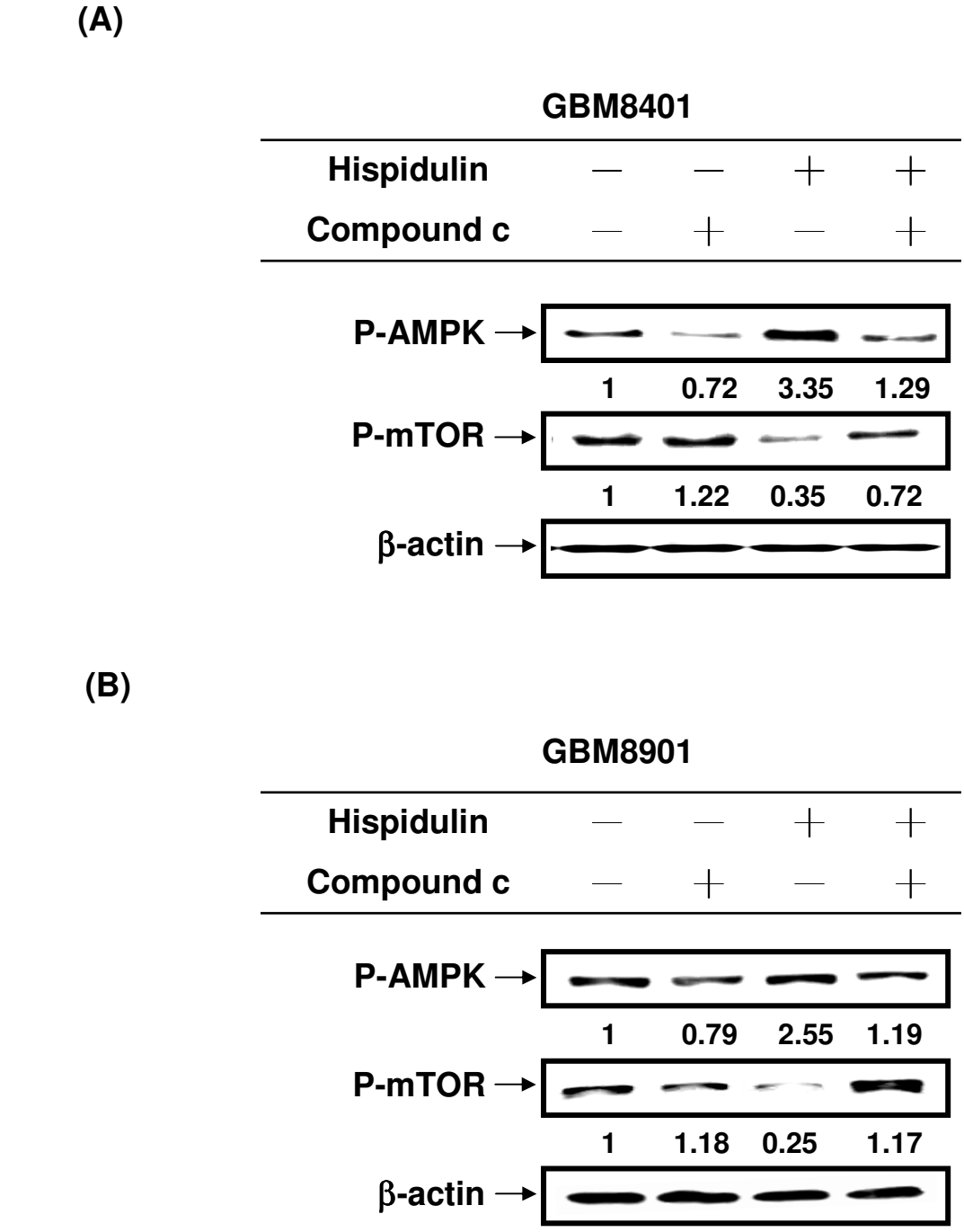

(A)
GBM8401
Hispidulin — — + +
Compound c — + — +
P-AMPK
1 0.72 3.35 1.29
P-mTOR
1 1.22 0.35 0.72
β-actin
(B)
GBM8901
Hispidulin — — + +
Compound c — + — +
P-AMPK
1 0.79 2.55 1.19
P-mTOR
1 1.18 0.25 1.17
β-actin

Figure 4

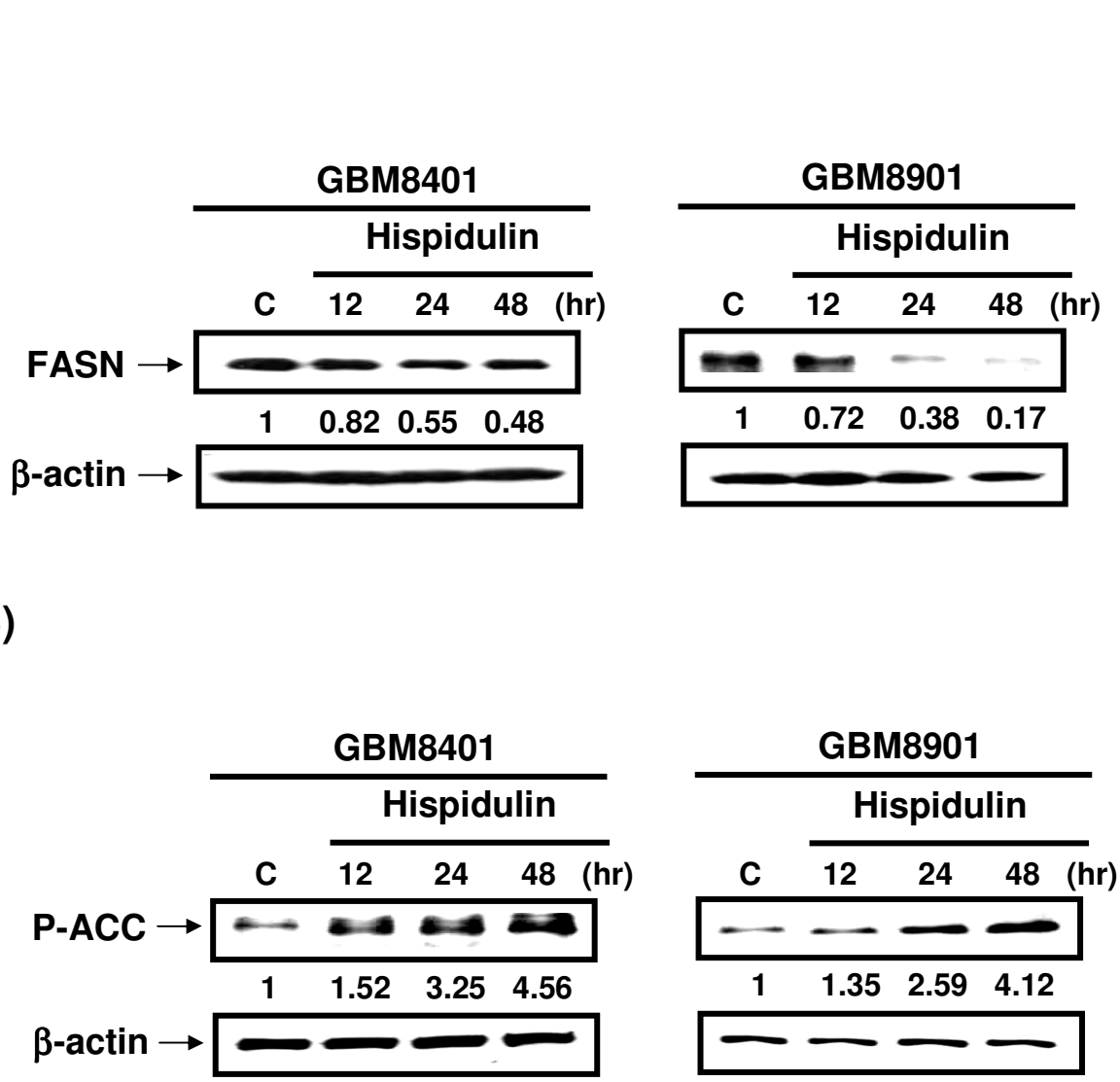

(A)
GBM8401
Hispidulin
C 12 24 48 (hr)
FASN
1 0.82 0.55 0.48
β-actin
GBM8901
Hispidulin
C 12 24 48 (hr)
1 0.72 0.38 0.17
(B)
GBM8401
Hispidulin
C 12 24 48 (hr)
P-ACC
1 1.52 3.25 4.56
β-actin
GBM8901
Hispidulin
C 12 24 48 (hr)
1 1.35 2.59 4.12

Figure 5

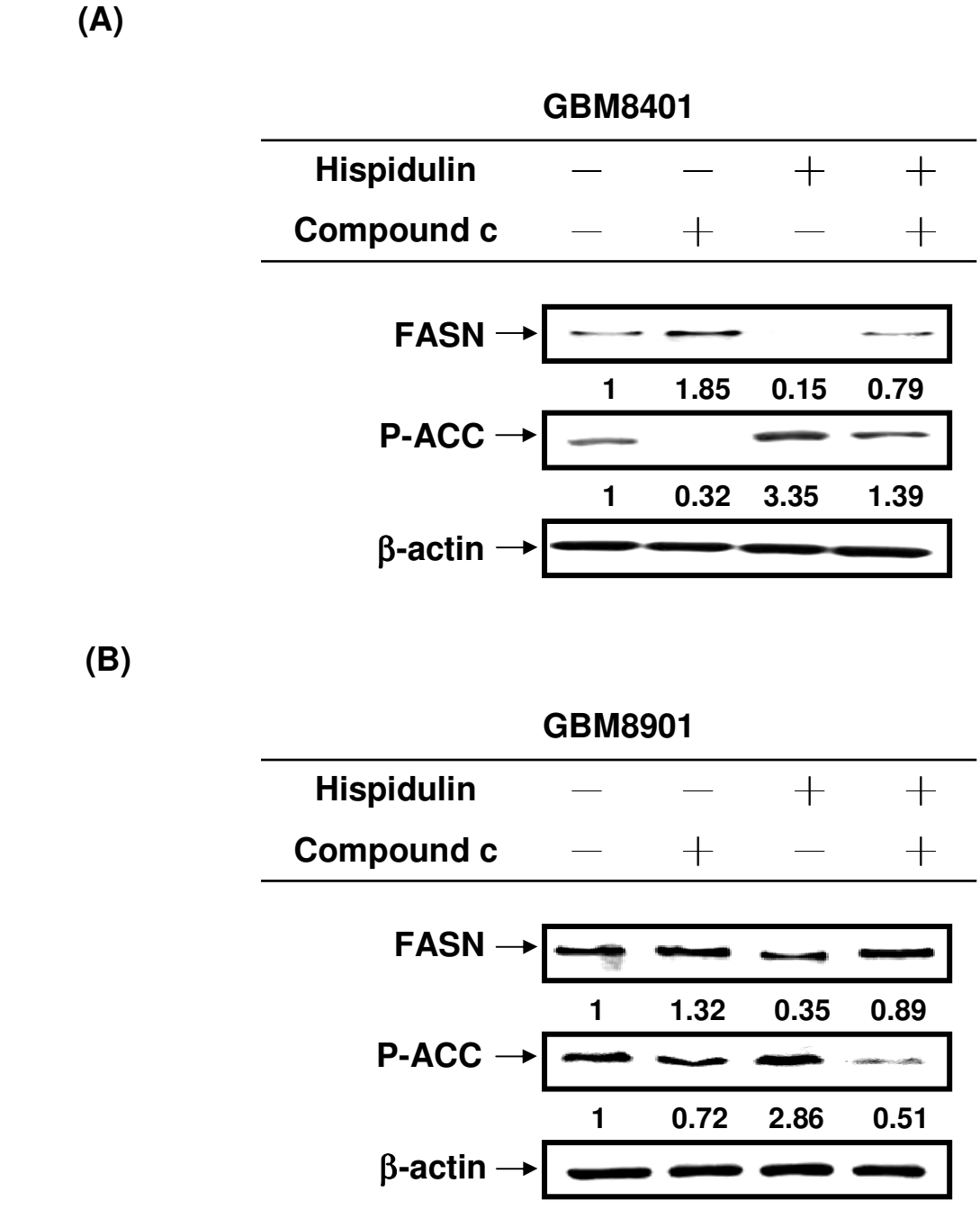
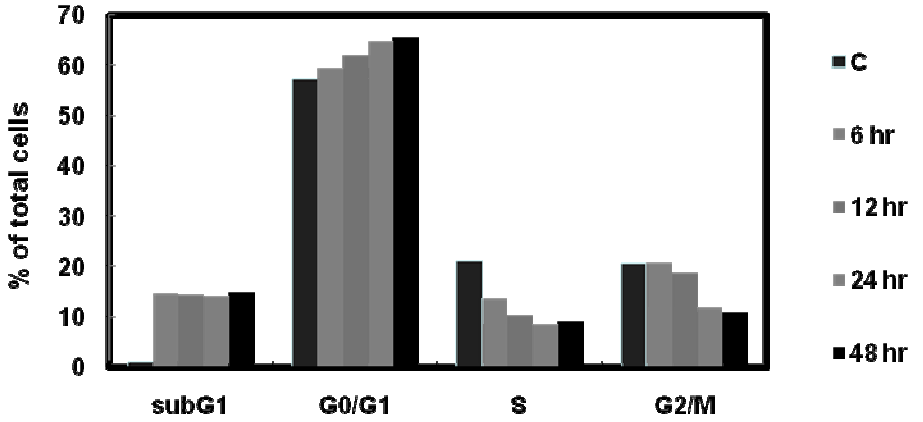
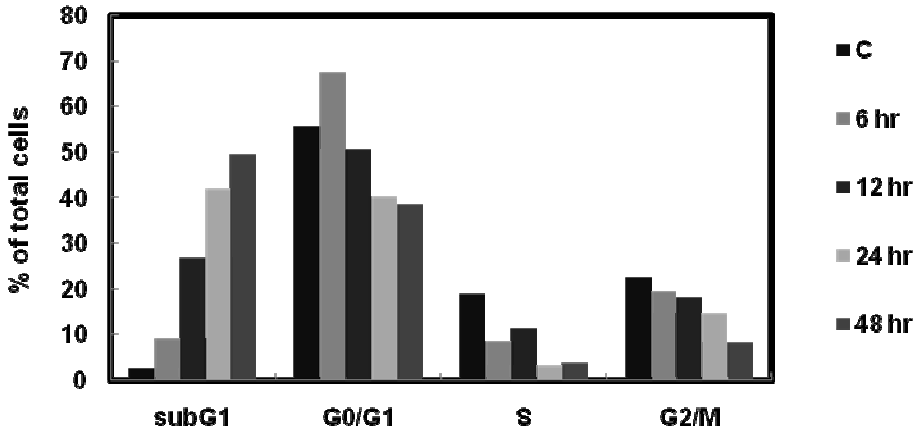

(A)
GBM8401
Hispidulin — — + +
Compound c — + — +
FASN
1 1.85 0.15 0.79
P-ACC
1 0.32 3.35 1.39
β-actin
(B)
GBM8901
Hispidulin — — + +
Compound c — + — +
FASN
1 1.32 0.35 0.89
P-ACC
1 0.72 2.86 0.51
β-actin

Figure 6

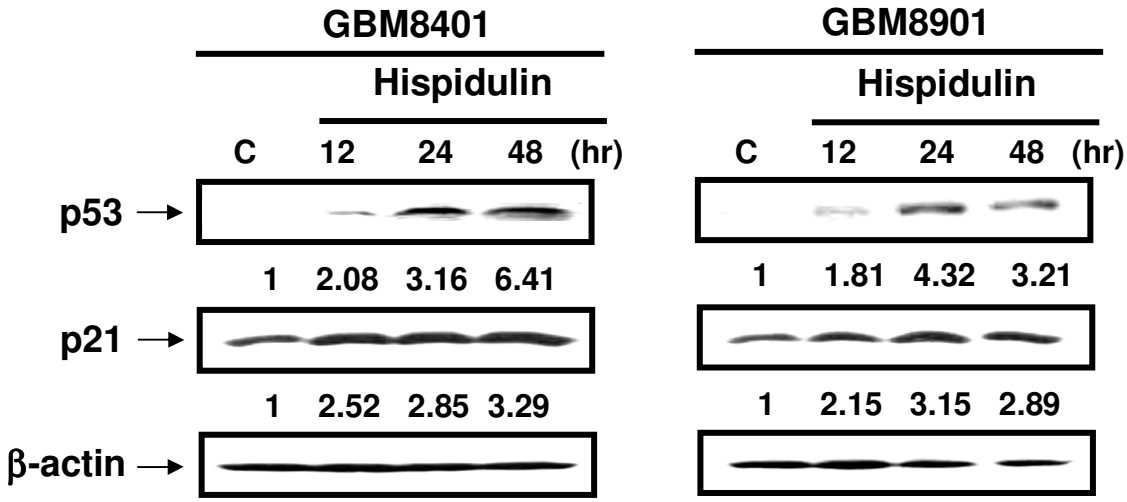
(A)


70
60
50
40
30
20
10
0
% of total cells
subG1
G0/G1
S
G2/M
C
6 hr
12 hr
24 hr
48 hr

(B)


80
70
60
50
40
30
20
10
0
% of total cells
subG1
G0/G1
S
G2/M
C
6 hr
12 hr
24 hr
48 hr

(C)


GBM8401
Hispidulin
C 12 24 48 (hr)
p53
1 2.08 3.16 6.41
p21
1 2.52 2.85 3.29
β-actin
GBM8901
Hispidulin
C 12 24 48 (hr)
1 1.81 4.32 3.21
1 2.15 3.15 2.89

(D)

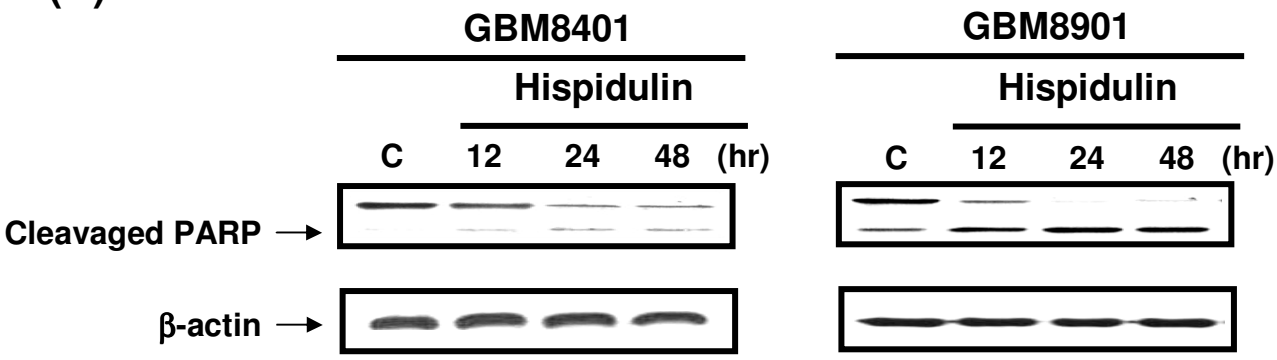
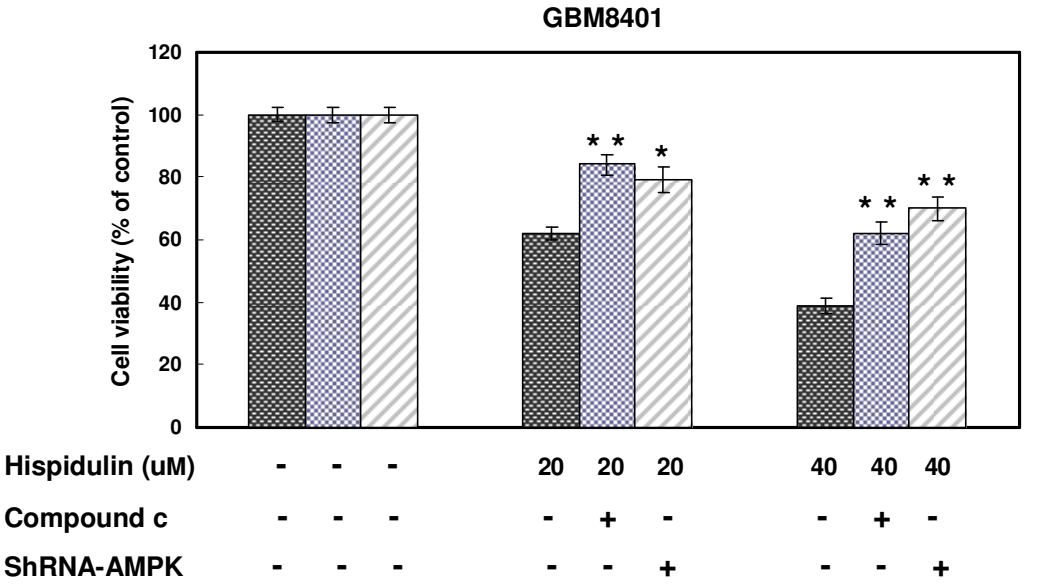

GBM8401
Hispidulin
C 12 24 48 (hr)
GBM8901
Hispidulin
C 12 24 48 (hr)
Cleavaged PARP
β-actin

Figure 7

(A)


GBM8401
Cell viability (% of control)
120
100
80
60
40
20
0
* *
*
* *
* *
Hispidulin (uM) - - - 20 20 20 40 40 40
Compound c - - - - + - - + -
ShRNA-AMPK - - - - - + - - +

(B)

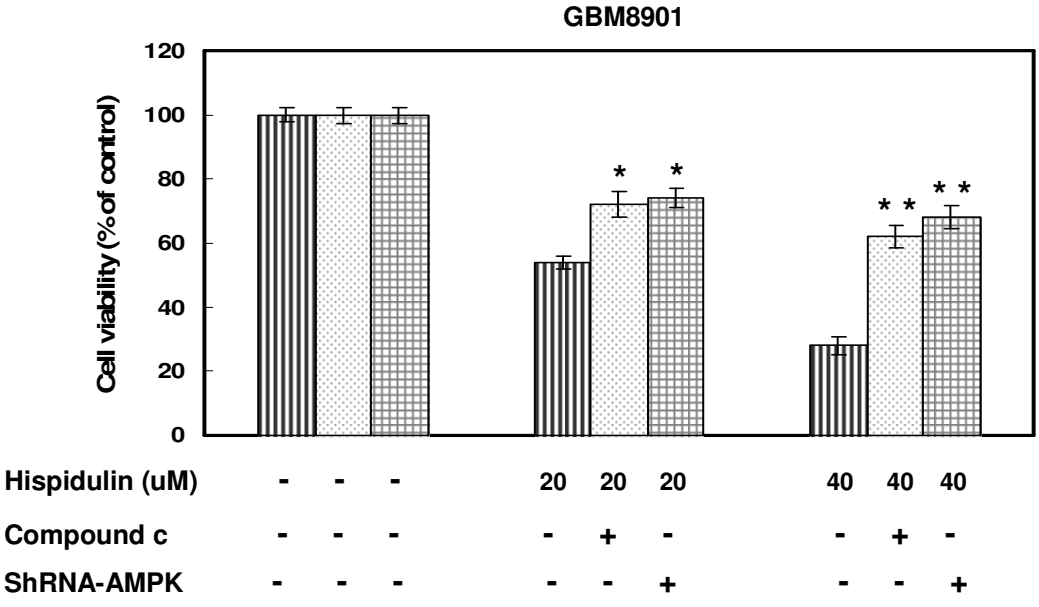
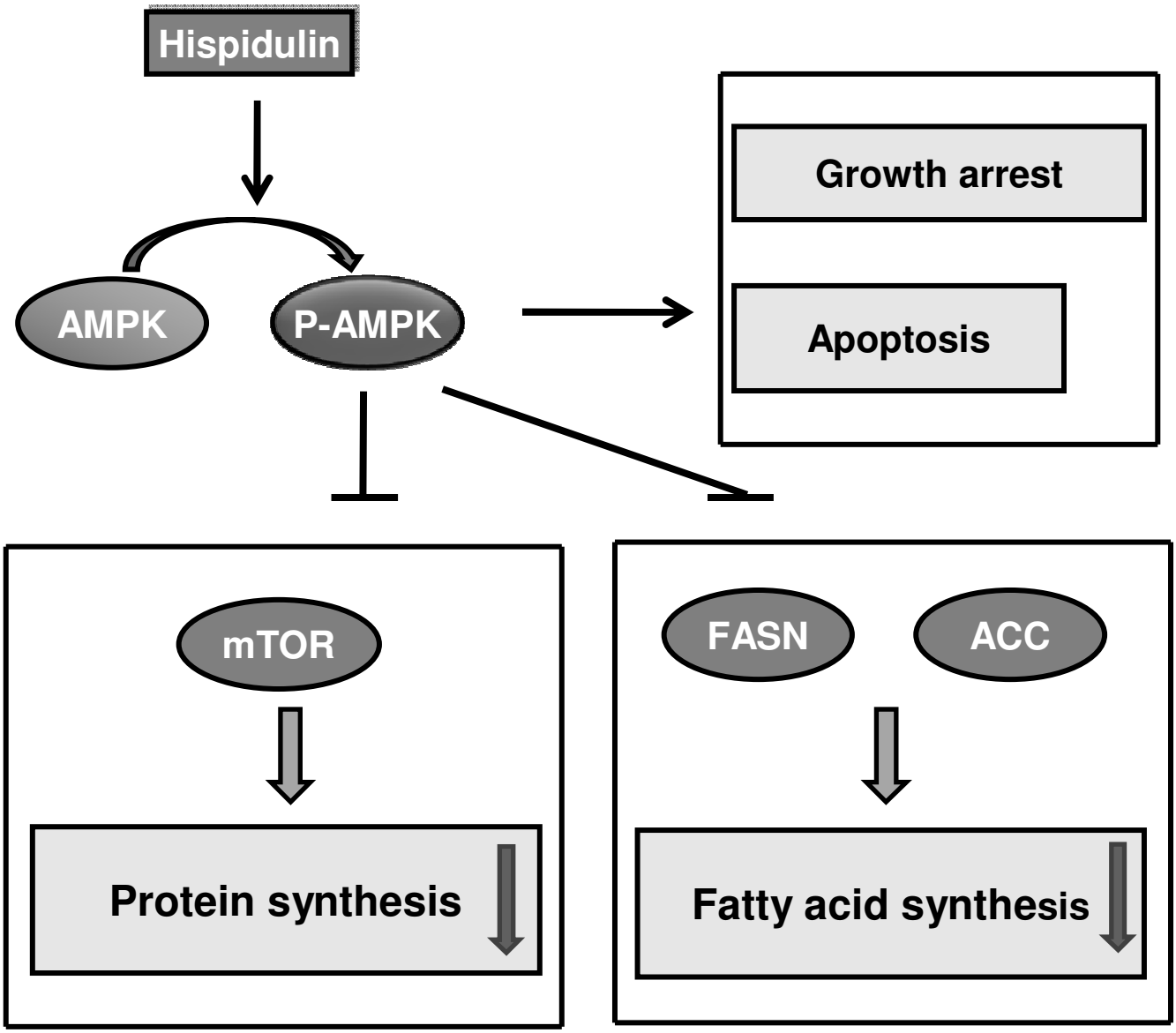

GBM8901
Cell viability (%of control)
120
100
80
60
40
20
0
*
*
* *
* *
Hispidulin (uM) - - - 20 20 20 40 40 40
Compound c - - - - + - - + -
ShRNA-AMPK - - - - - + - - +

Figure 8


Hispidulin
AMPK
P-AMPK
Growth arrest
Apoptosis
mTOR
Protein synthesis
FASN
ACC
Fatty acid synthesis