Galangin suppresses HepG2 cell proliferation by activating the TGF-β receptor/Smad pathway

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ABSTRACT

Galangin can suppress hepatocellular carcinoma (HCC) cell proliferation. In this study, we demonstrated that galangin induced autophagy by activating the transforming growth factor (TGF)-β receptor/Smad pathway and increased TGF-β receptor I (RI), TGF-βRII, Smad1, Smad2, Smad3 and Smad4 levels but decreased Smad6 and Smad7 levels. Autophagy induced by galangin appears to depend on the TGF-β receptor/Smad signalling pathway because the down-regulation of Smad4 by siRNA or inhibition of TGF-β receptor activation by LY2109761 blocked galangin-induced autophagy. The down-regulation of Beclin1, autophagy-related gene (ATG) 16L, ATG12 and ATG3 restored HepG2 cell proliferation and prevented galangin-induced apoptosis. Our findings indicate a novel mechanism for galangin-induced autophagy via activation of the TGF-β receptor/Smad pathway. The induction of autophagy thus reflects the anti-proliferation effect of galangin on HCC cells.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, especially in Asia. Chemotherapy is a common therapeutic strategy after surgery, but its use has been limited due to its toxicity to normal tissues. Natural products have long been used for anti-cancer treatment and are potentially safer alternatives (Surh, 2003).

Galangin is a polyphenolic compound with a molecular weight of 270.24 Da that is primarily derived from the rhizome of Alpinia officinarum Hance, which is used in traditional Chinese medicine and for dietary purposes (Heo et al., 2001). We have previously shown that galangin significantly decreases cell viability and induces apoptosis in HCC lines, and we proposed galangin as a potential anti-HCC agent (Zhang et al., 2012). Further, reports by Wen et al. (2012) and Zhang et al. (2013) have described galangin-induced autophagy in HepG2 cells. These observations suggest that galangin inhibits the proliferation of HepG2 cells by a novel mechanism. However, the precise mechanism by which galangin induces autophagy remains unclear.

Autophagy is an evolutionally conserved lysosomal degradation pathway. During autophagy, the isolation membrane envelops some cytoplasmic contents and then transforms into the autophagosomal membrane via fusion with the lysosome. The contents are then degraded by lysosomal enzymes (Mathew et al., 2007). Autophagy-related gene (ATG) proteins play essential roles in autophagy. Autophagosome formation is mediated by ATG protein systems, which alter the microtubule-associated protein 1 light chain 3 (LC3-I) to its phosphatidyethanolamine-conjugated membrane-bound form (LC3-II) via conjugation of the ATG5-ATG12 complex (Shibutani and Yoshimori, 2014).

Beclin1, also called ATG6/Vps30, plays an important role in initiating autophagosome formation. Beclin1 is a tumour suppressor gene because it is monoallelically deleted in 40% to 75% of human ovarian, prostate and breast cancers (Aita et al., 1999; Liang et al., 1999). Loss of Beclin1 may cause genomic instability, which ultimately initiates tumourigenesis (Mathew et al., 2007).

Transforming growth factor-β (TGF-β) superfAMILY signalling plays a critical role in the regulation of cell growth, differentiation, and migration and in the development of various cell types. Normally, signalling is initiated by the ligand-mediated oligomerisation of serine/threonine receptor kinases and phosphorylation of the cytoplasmic signalling molecules Smad2 and Smad3 for the
TGF-β pathway or Smad1/5/8 for the bone morphogenetic protein (BMP) pathway. TGF-β/Smad signalling acts as both a tumour suppressor and tumour promoter, similar to the duality of autophagy in oncogenesis (Bierie and Moses, 2006). Suzuki et al. (2010) reported that TGF-β/Smad signalling induces autophagy in HuH7 cells, and we have previously reported galangan-induced autophagy in HepG2 cells (Wen et al., 2012). In this study, we investigated whether galangan treatment activates TGF-β/Smad signalling to induce autophagy in HepG2 cells and determined a potential mechanism of activation.

2. Materials and methods

2.1. Cell culture

The human liver cancer cell line HepG2 was maintained at the Institute of Biochemistry and Molecular Biology at Guangdong Medical College. This cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% foetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere at 5% CO₂.

2.2. Agents and chemicals

Galangan (PubChem CID: 5281616) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) before being added to the cell cultures. The final concentration of DMSO in the culture medium was kept below 0.1% (v/v) after the addition of galangan. MIT and Hoechst 33258 were purchased from Sigma–Aldrich. LY2109761 was purchased from Selleck Chemicals (Houston, TX, USA). Rabbit, goat or mouse polyclonal antibodies against ATG16L, ATG12, ATG3, Beclin1, light chain 3 (LC3), TGF-βRI, TGF-βRII, Smad1, Smad2, Smad3, Smad4, Smad6, Smad7, Actin and Beclin1 as well as siRNAs against ATG16L, ATG12, ATG3 and Smad4 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). pSmad1, pSmad2 and pSmad3 were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.3. Cell viability assay

The cells were treated with galangan, and cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to a previous report (Wang et al., 2012).

2.4. Detection of autophagy by pEGFP-LC3

HepG2 cells were transfected with pEGFP-LC3, a highly specific fluorescence marker of autophagy, to measure autophagy levels. FuGENE HD (Roche Diagnostics, Basel, Switzerland) was used to transfect the HepG2 cells. After the induction of autophagy by galangan, the cellular localisation pattern of green fluorescence protein (GFP)-LC3 was photographed using a Nikon fluorescence microscope. When autophagy occurs, the percentage of GFP-LC3-positive cells with GFP-LC3 punctate foci increases, and the foci redistribute from a diffuse pattern to a punctate cytoplasmic pattern (GFP-LC3 foci) that specifically labels preautophagosomal and autophagosomal membranes, respectively.

2.5. Cell apoptosis and caspase-3 activity analysis

Apoptosis was evaluated by in situ uptake of Hoechst 33258 as reported by Zhang et al. (2012). The apoptotic index was determined by dividing the number of apoptotic nuclei by the total number of nuclei in each visual field × 100%. In addition, the cells were collected after treatment by centrifugation, washed twice with PBS, and fixed with ice-cold 70% ethanol overnight. Prior to flow cytometry analysis for cell apoptosis, the fixed cells were washed once with PBS and incubated with 100 μg/ml propidium iodide plus 200 μg/ml RNase. The apoptotic level was determined by analysing the peak of the subG1 ratio.

Caspase-3 activity was measured using the Colorimetric Assay Kit according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). After treatment, the cells were collected and lysed in chilled lysis buffer, which contained 50 mMHEPES (pH 7.5), 150 mM NaCl, 20 mM EDTA, 0.2% Triton X-100, 10 μg/ml apotinin, 1 mM PMSF and 5 mM dithiothreitol, for 15 min on ice. The supernatant, containing 100 μg of protein, was incubated with 0.2 mM AcDEVD-pNA, a specific substrate for caspase-3. Caspase-3 activity was measured at 405 nm, with background subtraction at 570 nm using the Varioscan Flash Reader spectrophotometer (THERMO, MA, USA), and expressed as Ac-pNA cleavage or released absorbance.

2.6. Real-time polymerase chain reaction (RT-PCR)

The HepG2 cells were treated with 148 μM galangan for 24 h. Total RNA was isolated using Trizol reagent and digested with DNase I. A quantitative comparison of mRNA levels was conducted using real-time PCR with beta-actin as the endogenous control. The amplification reactions were carried out according to the one-step SYBR<sup>®</sup> prime Script<sup>®</sup> RT-PCR II kit instructions (perfect real-time

<table>
<thead>
<tr>
<th>Primer sequences for the genes.</th>
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<tr>
<td>Gene names</td>
<td>Primers</td>
<td>Primers</td>
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<tr>
<td>Beclin1</td>
<td>5’-GGTGGTCCTGCGGATC1TCAC-3’</td>
<td>5’-TCTGTTCTGCGGTTGAGGTTCT-3’</td>
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<tr>
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<td>5’-ACTCTGCAACGCGGTCAGA-3’</td>
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<tr>
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<td>5’-TCTGAGCAGTAGACTGCACTGCA-3’</td>
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<tr>
<td>TGF-βRII</td>
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<tr>
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<td>β-actin</td>
<td>5’-CCACACCTCCTCAATGAGG-3’</td>
<td>5’-TGAAGACAGGCTGAC-3’</td>
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PCR, TAKARA Biotechnology Co., Ltd., Dalian, China). The final specific primer concentration was 400 nM, and the amount of total RNA template was 100 ng. Amplification was performed with an ABI7300 real-time PCR thermocycler (Applied Biosystems, USA) at 42 °C for 5 min. Denaturation was performed at 95 °C for 10 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The differential expression of mRNAs was analysed using ABI PRISM Sequence Detection software. The primer sequences that were used are shown in Table 1.

2.7. Transmission electron microscopy

The HepG2 cells were treated with different concentrations of galangin for 24 h. Cells were harvested by trypsinisation and washed twice with phosphate-buffered saline (PBS). Then, the cells were pelleted by centrifugation, carefully fixed with a buffer containing 3% glutaraldehyde and 0.1 M cacodylate to prevent the loss of cell aggregation, and re-fixed in osmium tetroxide. The cells were embedded in Epon and cut into sections with a thickness of 1.0 μm. Before being viewed with a CM-120 Philips electron microscope, the sections were stained with methylene buffer Arumell as described previously (Wen et al., 2012).

2.8. Down-regulation of Smad4 by short interfering (si)RNAs

Twenty-four hours prior to transfection, the HepG2 cells were seeded onto 6-well plates containing DMEM without antibiotics. The cells were transfected with Smad4 siRNAs and control siRNA using FuGENE HD (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s protocol. The transfected cells were treated with 148 μM galangin for another 24 h. The target protein levels were measured by western blot analysis.

2.9. TGF-β receptor inhibitor treatment

HepG2 cells were pre-incubated with 10 μM LY2109761, a TGF-β receptor inhibitor, for 2 h and then treated with 148 μM galangin for 24 h. Cell autophagy was determined using the pEGFP-LC3 assay and the target protein levels were measured by western blot analysis.

2.10. Western blot analysis

The cells were resuspended in cell lysis buffer (pH 8.0) that contained 50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.05% PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. Approximately 30 μg of protein/well was loaded onto the gels. The targeted protein levels were determined using western blotting, as described previously (Wang et al., 2012).

2.11. Statistical analysis

The values given are presented as the mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a least significant difference (LSD) test. P < 0.05 was considered significant.

3. Results

3.1. Induction of HepG2 cell autophagy by galangin

To explore the effect of galangin, HepG2 cells were treated with different concentrations of galangin for 24 h. Autophagy was detected by pEGFP-LC3 focus formation and confirmed by transmission electron microscopy. As shown in Fig. 1A and B, after incubation with galangin for 24 h, the morphological evaluation with fluorescence microscopy revealed a significant increase in the number of cells with LC3 foci. Autophagic cells can be identified by the localisation of LC3 proteins in autophagosomal membranes. LC3-II was detected by western blot to determine galangin-induced autophagy levels because LC3-II can be distinguished from LC3-I by the increased mobility of LC3-II. After galangin treatment, LC3-II levels increased in a dose-dependent manner (Fig. 1C), indicating that galangin effectively induced autophagy in HepG2 cells. Autophagy was also confirmed by transmission electron microscopy (Fig. 1D). Our results indicated

![Image](image-url)

**Fig. 1.** Autophagy is induced by galangin. (A) HepG2 cells transfected with GFP-LC3 were treated with different concentrations of galangin for 24 h. Fluorescence microscopy images at 400×; GFP-LC3-positive cells with GFP-LC3 foci. (B) The data are presented as the mean ± standard deviation (SD); n = 6. (C) The changes in LC3 expression were detected by western blot analysis. (D) Transmission electron microscopy images.
that galangin treatment induces autophagy in HepG2 cells in a dose-dependent manner.

3.2. ATG inhibition suppresses the galangin-induced initiation of autophagy

We next explored whether galangin could alter ATG expression levels in HepG2 cells. Quantitative reverse transcription PCR indicated that treatment with galangin for 24 h significantly increased Beclin1, ATG16L, ATG12 and ATG3 expression levels (Fig. 2A). The western blot analysis revealed that the levels of Beclin1, ATG16L, ATG12 and LC3-II were increased by galangin (Fig. 2B). The downregulation of Beclin1, ATG16L, ATG12 and ATG3 by siRNA inhibited the galangin-induced increase in LC3-II (Fig. 2C) and GFP-LC3 focus formation in HepG2 cells (Fig. 2D). These results suggested that galangin regulates ATG expression levels to prompt HepG2 cell autophagy.

3.3. Autophagy induction by galangin is mediated by the TGF-β/Smad pathway

We then investigated the signalling pathways mediating autophagy induction during galangin treatment. Quantitative reverse transcription PCR showed that treatment with galangin for 24 h significantly increased TGF-βRI and TGF-βRII expression levels (Fig. 3A). Western blot analysis also revealed that the protein levels of TGF-β RI and TGF-β RII were increased (Fig. 3B). Furthermore, following galangin treatment, the Smad1, Smad2, Smad3 and Smad4 expression levels increased, whereas the Smad6 and Smad7 levels decreased. The phosphorylation of...
Fig. 3. Galangin treatment induces autophagy in HepG2 cells via the TGF-β/Smad pathway. (A) The expression of TGF-β/Smad pathway-associated genes was measured by real-time PCR after HepG2 cells were treated with 148 μM galangin for 24 h. (B) Protein levels were detected by western blot after HepG2 cells were treated with 148 μM galangin for different lengths of time. (C) HepG2 cells were pre-treated with 10 μM LY2109761 for 2 h followed by 148 μM galangin treatment for another 24 h. After treatment, the number of GFP-LC3-positive cells with GFP-LC3 foci was measured. The data are presented as the mean ± SD; n = 6. (D) After the 10 μM LY2109761 and 148 μM galangin treatments, total protein was isolated for western blot analysis. (E) Total proteins were isolated for western blot analysis after the HepG2 cells had been treated with Smad4 siRNA and 148 μM galangin or either agent alone for 24 h. (F) After treatment with Smad4 siRNA and 148 μM galangin or either agent alone for 24 h, the number of GFP-LC3-positive cells with GFP-LC3 foci was measured. The data are presented as the mean ± SD; n = 6.
Smad1, Smad2 and Smad3 also increased. These results suggested that the TGF-β receptor/Smad pathway is involved in galangin-induced autophagy in HepG2 cells.

Further, LY2109761, an inhibitor of TGF-β receptor kinase, blocked the galangin-induced increase in LC3-II and attenuated GFP-LC3 focus formation in HepG2 cells (Fig. 3C). The phosphorylation of Smad1, Smad2 and Smad3 was decreased compared with LY2109761-free groups (Fig. 3D). LY2109761 also suppressed the galangin-induced increase of Beclin1, ATG16L, ATG12 and ATG3 (Fig. 3D). These results confirmed that TGF-βRI and TGF-βRII are required for galangin-induced autophagy.

We also examined the involvement of the Smad pathway. Smad4 was selected for knockdown because it is the common mediator of Smads. Smad4 knockdown abolished the galangin-induced increase of Beclin1, ATG16L, ATG12 and ATG3 (Fig. 3E), indicating the importance of gene transcription for galangin-induced autophagy. Accordingly, GFP-LC3 focus formation due to galangin treatment was suppressed by Smad4 knockdown (Fig. 3F). Thus, the Smad pathway may at least partially mediate autophagy via the transcriptional regulation of ATGs, which may support sustained autophagy activation.

### 3.4. Blockade of ATGs attenuates galangin-mediated growth inhibition and decreases galangin-induced apoptosis

We examined the possible contribution of autophagy to galangin-mediated growth inhibition. As shown in Fig. 4A, knockdown of Beclin1, ATG16L, ATG12 or ATG3 by siRNA attenuated galangin-induced growth inhibition in a dose-dependent manner in HepG2 cells. Furthermore, knockdown of Beclin1, ATG16L, ATG12 or ATG3 suppressed the apoptosis induced by galangin in HepG2 cells. These results were then verified by flow cytometry analysis. The subG1 ratio was significantly decreased in HepG2 cells after siRNA-mediated knockdown of ATG expression compared with control siRNA cells (Fig. 5A and B). Furthermore, the caspase-3 activity of the cells paralleled the results of the flow cytometry analysis. The caspase-3 activity in the cells was not significantly affected by incubation with 37 μM galangin (Fig. 5C).

These results suggest that the effects of autophagy inhibition on galangin-mediated proliferation inhibition can be attributed to the induction of apoptosis in HepG2 cells. We concluded that autophagy activation partially contributes to galangin-mediated growth inhibition or apoptosis in HepG2 cells.

### 4. Discussion

Previous studies have shown that galangin can exhibit anticancer activity (Zhang et al., 2012; Jaiswal et al., 2012; Kim et al., 2012; Gwak et al., 2011); in HCC, galangin has been shown to induce both apoptosis and autophagy (Zhang et al., 2013). Su et al. (2013) reported that galangin-induced endoplasmic reticulum (ER) stress in liver cancer cells leads to proliferation inhibition. In the present study, galangin at least partially induced autophagy in hepatocellular carcinoma cells through the TGF-β/Smad pathway and the transcriptional activation of ATGs. Accordingly, autophagy induction in ER stress is mediated by an increase in ATG transcription (Lamb et al., 2013; Song et al., 2014; Morita and Yoshimori, 2012). Galangin may induce autophagy in HepG2 cells via the TGF-β/Smad pathway and increase ATG expression under ER stress.

Galangin treatment significantly increased ATG expression levels for many proteins at both the transcriptional and the proteins levels, including Beclin1, ATG16L, ATG12 and ATG3. siRNA treatment confirmed that galangin-induced autophagy in HepG2 cells is dependent on ATG expression and indicated that galangin-mediated autophagy in HepG2 cells is associated with increased ATG expression. Previous studies have confirmed that autophagy is a membrane trafficking mechanism that delivers cytoplasmic cargo to the vacuole/lysosome for degradation and recycling (Reggiori and Klionsky, 2002). ATGs play important roles in autophagosome formation.

A critical protein in the autophagy process is Beclin1 (also known as ATG6), which can stimulate autophagy when overexpressed in mammalian cells (Yue et al., 2003). Mammalian Beclin1 has been shown to interact with Bcl-2 and Bcl-XL, which can effectively antagonise Beclin1-mediated autophagy by binding and blocking its phosphorylation (Nikolopoulou et al., 2013; Fu et al., 2013). This finding also demonstrates that Bcl-2 and Bcl-XL play anti-apoptotic roles and are able to suppress autophagy, which suggests cross-talk between the apoptotic and autophagic pathways.

The phosphorylation of Beclin1 and Bcl-2 can dissociate the Beclin1-Bcl-2 complex, which leads to autophagy inhibition. Death-associated protein kinase (DAPK), a serine/threonine kinase that plays an important role in apoptosis and autophagy regulation, phosphorylates Thr119 in the BH3 domain of Beclin1, dissociating Beclin1 from Bcl-XL and increasing autophagosome formation (Zalciver et al., 2009). DAPK activation with an increase in DAPK mRNA expression but a decrease in protein levels (data not shown) was also observed during galangin treatment, which suggested that DAPK may be involved in regulating

![Fig. 4. Inhibition of autophagy attenuates galangin-mediated growth inhibition. (A) Growth inhibition assay. HepG2 cells were transfected with siRNAs and treated with different doses of galangin for 24 h after transfection. Cell viability was determined by an MTT assay. (B) Apoptosis assay. After siRNA transfection and treatment with different doses of galangin for 24 h, apoptosis was evaluated by the in situ uptake of Hoechst 33258. The apoptotic index was calculated as the ratio of the number of apoptotic nuclei to the total number of nuclei in each visual field.](image-url)
Beclin1-induced autophagy during galangin treatment. Similarly, the phosphorylation of Bcl-2 (Thr69, Ser70, Ser87) by Jun N-terminal kinase 1 (JNK1) (Park et al., 2009) abrogates the binding of Bcl-2 to Beclin1, thereby inducing autophagy. It was also demonstrated that galangin inhibits the proliferation of hepatoma carcinoma cells by activating JNK1 (Su et al., 2013), which may dissociate the Beclin1-Bcl-2 complex during this process. The significant increase in Beclin1 expression levels with galangin treatment indicates that Beclin1 plays an important role in galangin-induced autophagy of HepG2 cells. The activation of JNK1 (Su et al., 2013) and DAPK by galangin may indicate that the function of Beclin1 in autophagy is activated by JNK1 and DAPK. Currently, little is known about the transcriptional regulation of Beclin1. Some reports have shown that several transcription factors, such as FoxO3, nuclear factor (NF)-κB, hypoxia-inducible factor (HIF) 1α, c-Jun and E2F1 drive Beclin1 expression (Wirawan et al., 2012). Further investigation is needed to determine how Beclin1 mRNA levels are increased by galangin treatment.
ATG12, ATG16L and ATG3 play important roles in autophagosome formation. ATG12 is essential for autophagy and localises to the cytoplasm where it is covalently bound to ATG5 and targeted to autophagosomes vesicles. The ATG12–ATG5 complex functions as an important regulator of the autophagic process and is required for the changes in membrane morphology and the development of autophagosomes (Mizushima et al., 1998a,b,b; Suzuki et al., 2001).

ATG16L binds the ATG12–ATG5 complex, forming a multimeric complex that is involved in the formation of mature autophagosomes (Mizushima et al., 2003; Fujita et al., 2008). ATG3, an E2-like enzyme, catalyses the formation of ATG8-phosphatidylethanolamine (ATG8–PE) conjugate (Tanida et al., 2002; Sou et al., 2008). Our data show that the galangin-mediated increases in ATG12, ATG16L and ATG3 expression may prompt autophagosome formation during autophagy in HepG2 cells.

Smad family members are critical intracellular transduction molecules that transmit signals from the cell surface to the nucleus. Three classes of Smads have been characterised: the receptor-regulated Smads (R-Smads), including Smad1, Smad2, Smad3, Smad5, and Smad8; the common mediator Smad (co-Smad), Smad4; and the inhibitory Smads (I-Smads), Smad6 and Smad7. Briefly, activated TGF-β RI and TGF-β RII oligomerise and then phosphorylate specific R-Smads, which can be inhibited by I-Smads. The phosphorylated R-Smad dissociates from the oligomerised receptors and forms a heteromeric complex with Smad4. The heteromeric complex moves to the nucleus and then targets a variety of DNA binding proteins to regulate transcriptional responses (Attisano and Wrana, 2002). Lien et al. (2013) found that shear-induced autophagy is mediated by Smad1 and Smad5 in Hep3B cells, and Suzuki et al. (2010) reported that hepatocellular carcinoma cells induce autophagy through the TGF-β/Smad pathways. Therefore, it has been suggested that the Smad pathways are involved in autophagy activation.

TGF-βRI and TGF-βRII expression levels increased significantly with galangin treatment. The expression levels of the R-Smads (Smad1, Smad2, and Smad3) and Smad4 also increased, but the Smad6 and Smad7 levels decreased. This result implies that galangin activated TGF-βRs and R-Smads and suppressed the I-Smads, leading to enhanced TGF-β/Smad pathway signalling. Activated Smads can regulate diverse biological effects by partnering with transcription factors, resulting in the modulation of target gene transcription levels. Based on the results obtained from siRNA-mediated downregulation Smad4, Smads participated in the galangin-induced increase in Beclin1, ATG16L, ATG12 and ATG3 to mediate autophagy via the transcriptional regulation of ATGs and support sustained autophagy activation. These results were consistent with those of a previous report (Suzuki et al., 2010) and suggest that the Smad pathways are involved in galangin-induced autophagy in HepG2 cells.

The present results show that autophagy plays an important role in galangin–mediated growth inhibition and indicate that ATGs may be involved in autophagy and apoptosis. Interestingly, Sue et al. (2013) observed that galangin induced the stress-responsive transcription factor CHOP in a previous study. Thus, galangin-mediated autophagy may be additionally regulated in a CHOP-dependent manner. Some previous reports demonstrated the upregulation of p53 (Wen et al., 2012) and the activation of AMPK (Zhang et al., 2013) during galangin-mediated HepG2 cell autophagy. Although we have no direct evidence for cross-talk between these pathways, previous reports have shown that activation of p53 (Yuan et al., 2014) and AMPK (Kumar et al., 2013) also induce apoptosis and autophagy. Therefore, we speculate that galangin induces changes in AMPK/TAN and the activation of p53, which may increase AMPK activity and regulate Smad expression to induce ATG expression.

Autophagy is a cytoprotective mechanism under stressful conditions, but some evidence has shown a role for autophagy in suppressing cell proliferation (Kumar et al., 2013; Law et al., 2014); furthermore, excessive autophagy can cause autophagic cell death. Many studies have shown that the induction of autophagy can also contribute to apoptosis (Kumar et al., 2013; Law et al., 2014; Laussmann et al., 2011; He et al., 2014). The inhibition of autophagy significantly attenuates cell death in many cellular circumstances (Shimizu et al., 2004; He et al., 2014). Suzuki et al. (2010) also showed that knockdown of Beclin1, ATG5 or ATG7 attenuates the TGF-β-mediated induction of apoptosis in hepatoma cells, similar to the results of our studies in which autophagy was induced in HepG2 cells by galangin.

Although galangin inhibited the growth of HepG2 cells mainly by autophagy rather than by apoptosis in the concentration range of galangin that was used in this study (Zhang et al., 2013), the present data also showed that downregulation of ATGs attenuates the apoptosis induction function of galangin. It is possible that a decrease in Beclin1 cannot effectively antagonise the functions of Bcl-2 and Bcl-xL (Nikoletopoulou et al., 2013; Fu et al., 2013). An understanding of the precise molecular mechanisms is needed to determine why the downregulation of ATGs attenuates the apoptosis induction function of galangin. This discrepancy may be partially explained by the hypothesis that extensive autophagy activation contributes to cell apoptosis and that the induction of autophagy in a restricted range supports cell survival (Rubinstein and Kimchi, 2012). We observed that galangin potently induces continuous autophagy in HepG2 cells, inhibiting cell proliferation and inducing cell apoptosis. Thus, higher levels of and prolonged autophagy induction contribute to galangin-mediated growth inhibition in HepG2 cells.

In brief, we showed that galangin at least partially stimulates autophagy to suppress HCC growth in certain HepG2 cells through the increased expression of some ATGs and that the Smad pathways are involved in this process. In accordance with our previous reports, our current data support the notion that galangin is a potential anti-cancer agent.

Conflict of interest

The authors state no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Lien, Morita, Laussmann, Nikoletopoulou, Law, protein, molecular


