Hinokitiol Induces Autophagy in Murine Breast and Colorectal Cancer Cells

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ABSTRACT: Hinokitiol is found in the heartwood of cupressaceous plants and possesses several biological activities. Hinokitiol may play an important role in anti-inflammation and antioxidant processes, making it potentially useful in therapies for inflammatory-mediated disease. Previously, the suppression of tumor growth by hinokitiol has been shown to occur through apoptosis. Programmed cell death can also occur through autophagy, but the mechanism of hinokitiol-induced autophagy in tumor cells is poorly defined. We used an autophagy inhibitor (3-methyladenine) to demonstrate that hinokitiol can induce cell death via an autophagic pathway. Further, we suggest that hinokitiol induces autophagy in a dose-dependent manner. Markers of autophagy were increased after tumor cells were treated with hinokitiol. In addition, immunoblotting revealed that the levels of phosphoprotein kinase B (P-AKT), phosphomammalian target of rapamycin (P-mTOR), and phospho-p70 ribosomal s6 kinase (P-p70S6K) in tumor cells were decreased after hinokitiol treatment. In conclusion, our results indicate that hinokitiol induces the autophagic signaling pathway via downregulation of the AKT/mTOR pathway. Therefore, our findings show that hinokitiol may control tumor growth by inducing autophagic signaling. Environ Toxicol 31: 77–84, 2016.

Keywords: hinokitiol; antitumor; autophagy

INTRODUCTION

Hinokitiol is a bioactive compound with diverse biological and pharmacological activities that is found in the wood of cupressaceous plants. Its biological properties include antifungal, antibacterial, and antitumor activities (Shih et al., 2014). Hinokitiol has been shown to induce apoptosis in tumor cells through the activation of caspase-3 (Ido et al., 1999). Further, hinokitiol has been shown to inhibit

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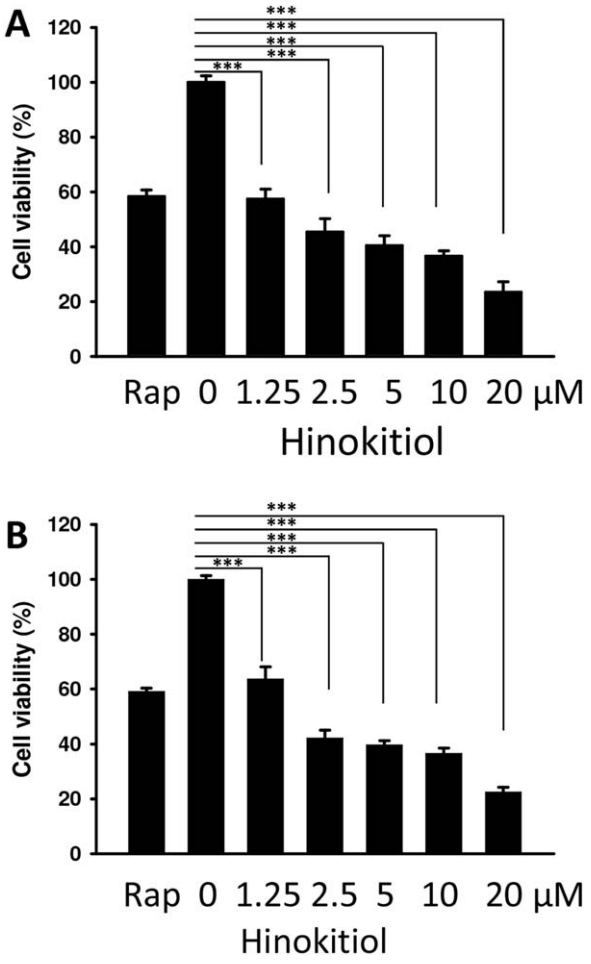

Fig. 1. Effects of hinokitiol on tumor growth *in vitro*. The (**A**) 4T1 (10^6) and (**B**) CT26 (10^6) tumor cells were treated with various concentrations of hinokitiol or rapamycin (10 nM) for 2 h. Cells were harvested and stained with trypan blue for assessing cell viability. Data are expressed as the mean ± SD of hexaplicate determinations. ***, $P < 0.001$. Each experiment was repeated three times with similar results.

colorectal cancer through cell-cycle arrest and the downregulation of cleaved caspase-9 (Lee et al., 2013b).

Breast and colorectal cancers are the most common cancers worldwide. Mortality usually results from the metastatic spread of these tumors to other organs. Metastatic breast and colorectal cancers pose significant health risks in terms of both their poor prognosis and their high incidence. Breast and colorectal cancers are characterized by a high potential to spread to almost any region of the body, with the most common sites being the lung and liver. 4T1 cells are triple-negative (lacking the expression of estrogen, progesterone, and Her2/neu receptors) murine breast carcinoma cells that closely mimic human breast cancer in both tumor growth and metastasis (Chen et al., 2014). CT26 cells share molecular features with aggressive, undifferentiated, refractory human colorectal carcinoma cells (Castle et al., 2014). These two cell lines were chosen to mimic malignant tumors in humans. A better understanding of the molecular pathways that regulate tumor cell death is essential to guiding the development of more effective therapies.

Autophagy is a cellular process that mediates the degradation of long-lived proteins and unwanted organelles in the cytosol. Autophagy is regulated by numerous factors, including nutritional status, hormones, and intracellular signaling pathways (Lee et al., 2014). Malignant cells frequently display lower levels of basal autophagic activity than their normal counterparts and fail to increase autophagic activity in response to stresses (Pattingre and Levine, 2006). The microtubule-associated protein light chain 3 (LC3) is known to exist on autophagosomes; therefore, this protein serves as a widely used marker for autophagosomes (Kabeya et al., 2000). The identification of the autophagic machinery has greatly facilitated the detection of autophagy phenotypes. Beclin 1 participates in the regulation of autophagy and plays important roles in development, tumorigenesis, and neurodegeneration (Zhang et al., 2009). Sequestosome-1 (p62/SQSTM1) is an adaptor protein that localizes to sites of autophagosome formation and can associate with the autophagosome-localizing protein LC3 and ubiquitinated proteins (Bjorkoy et al. 2005). To date, the possible interaction of hinokitiol with tumor cells has not been fully examined. Herein, using autophagic markers, we propose a role for hinokitiol in controlling tumor growth by inducing autophagy.

MATERIALS AND METHODS

Cell Lines, Reagents, and Plasmid

Murine 4T1 and CT26 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 μg/mL gentamicin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2. Hinokitiol, rapamycin, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Sigma Aldrich, St. Louis, MO). The autophagy inhibitor 3-methyladenine (3-MA) was purchased from Merck (Darmstadt, Germany). Constitutively active AKT plasmid was kindly provided by Dr. Chiau-Yuang Tsai (Department of Molecular Immunology, Osaka University, Japan) (Shiau et al., 2014).

Cell Viability Assay

Cells were pretreated with inhibitors for 4 h, and then hinokitiol (0–20 μM) or rapamycin (10 nM) was added to cells for 2 h. In a parallel experiment, the adherent cells were measured to determine cell survival. Cell survival was assessed using the trypan blue exclusion assay (Lee et al., 2014).

Immunoblot Analysis

The protein content in each sample was determined with the bicinchoninic acid (BCA) protein assay (Pierce

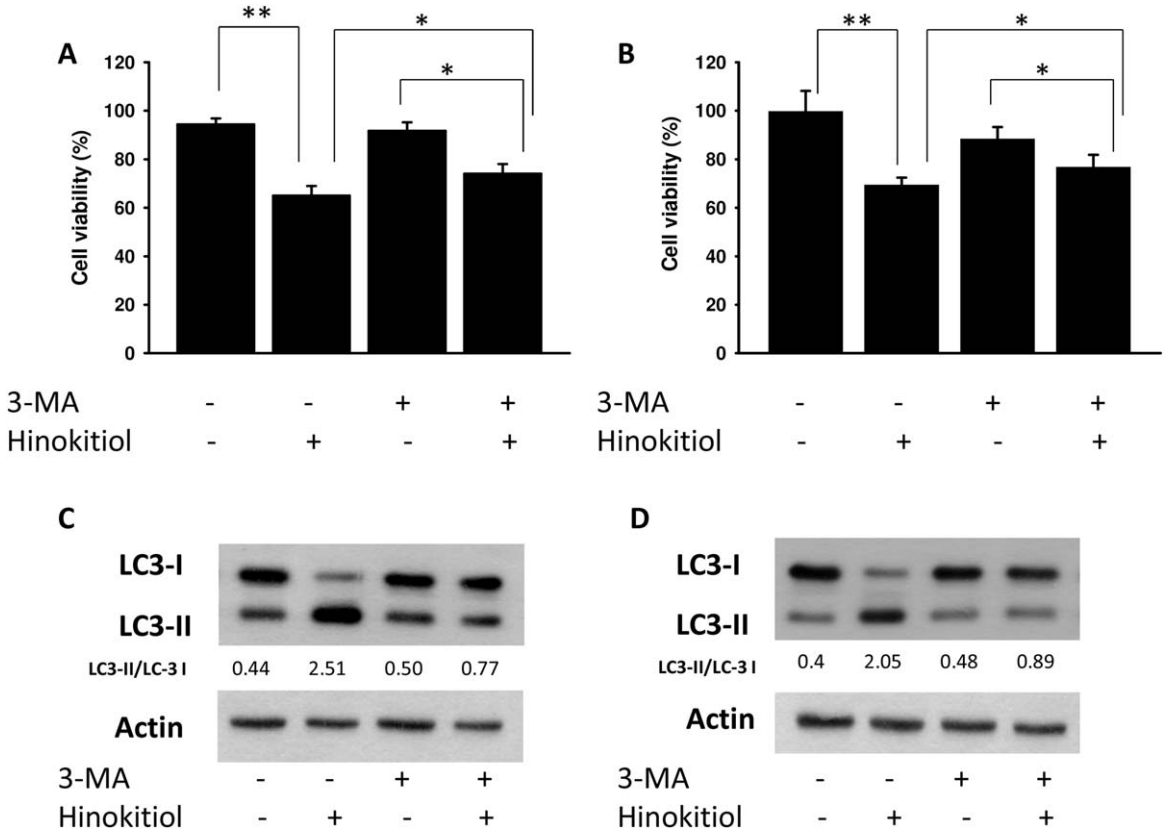

Fig. 2. Hinokitiol-induced nonapoptotic cell death. The (**A**, **C**) 4T1 and (**B**, **D**) CT26 cells were treated with 3-MA (5 mM) for 4 h and then treated with hinokitiol (1.25 μM) for 2 h. Cells were harvested and stained with trypan blue for viability assays (A, B). The expression levels of LC3 in (C) 4T1 and (D) CT26 cells were determined by immunoblot analysis. Inset values indicate protein expression levels normalized to β-actin. *, $P < 0.05$; **, $P < 0.01$. Data are expressed as the mean ± SD of hexaplicate determinations. Each experiment was repeated three times with similar results.

Biotechnology, Rockford, IL). Proteins were fractionated on SDS-PAGE, transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont, UK), and probed with antibodies against LC3 (Novus Biologicals, Littleton, CO), beclin 1 (Novus Biologicals), p62 (Novus Biologicals), the mammalian target of rapamycin (mTOR) (Cell Signaling, Danvers, MA), phospho-mTOR (Cell Signaling), protein kinase B (AKT) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), phospho-AKT (Santa Cruz Biotechnology, Inc.), p70S6 kinase (p70S6K) (Cell Signaling), phospho-p70S6K (Cell Signaling), or β-actin (AC-15, Sigma-Aldrich). Horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson, West Grove, PA) was used as the secondary antibody, and protein-antibody complexes were visualized with an enhanced chemiluminescence system (Amersham). The signals were quantified with ImageJ software (rsbweb.nih.gov/ij/) (Liu et al., 2013).

Analysis of Intracellular Autophagic Vacuoles

Green fluorescent protein (GFP)-fused-LC3 (GFP-LC3) was used to detect autophagy as described previously (Kabeya et al., 2000). pTCY-GFP was the negative control (Yo et al., 2009). The tumor cells were transfected with 5 μg of the GFP-LC3 or GFP expression plasmid using Lipofectamine 2000. The transfected cells were treated with hinokitiol for 2 h, and the fluorescence of GFP-LC3 or GFP was visualized by fluorescence microscopy. Cell numbers were counted to normalize the measurement, and the percentage of fluorescent cells was calculated (Lee et al., 2014).

Statistical Analysis

One-way analysis of variance (one-way ANOVA) was used to identify differences between experimental groups and the control group. A *P*-value less than 0.05 was considered to be statistically significant.

RESULTS

Hinokitiol Decreased Tumor Cell Growth *In Vitro*

First, we evaluated the potential cytotoxic effects of hinokitiol in the range of 0–20 μM with a proliferation assay. To

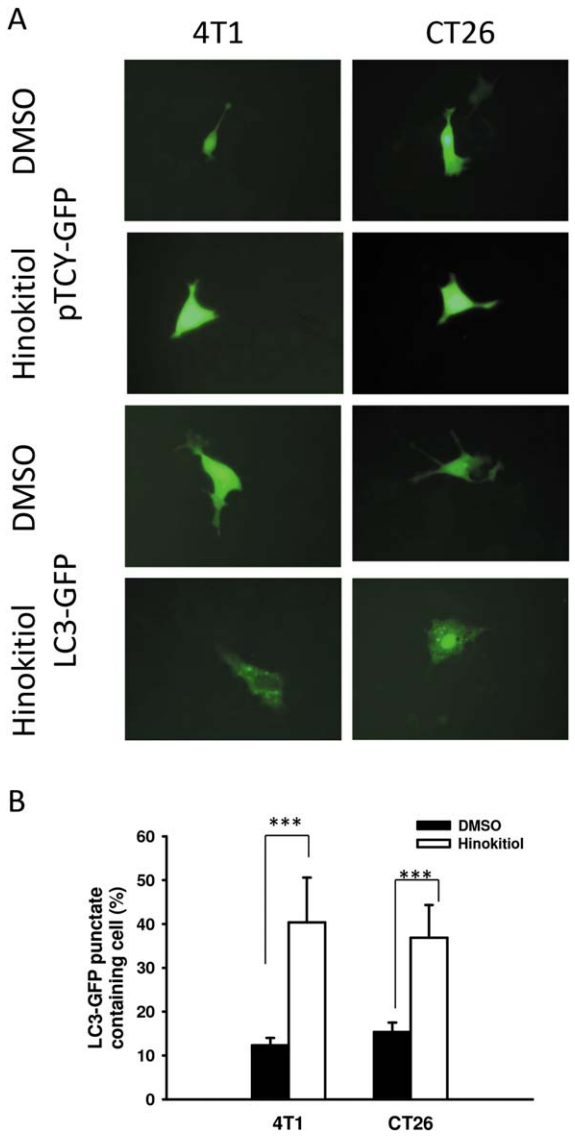

Fig. 3. Hinokitiol-induced autophagy in tumor cells. (**A**) 4T1 and CT26 cells were transfected with plasmids encoding GFP-LC3 or GFP and then treated with hinokitiol (1.25 μM) for 2 h. The cells containing GFP-LC3 puncta were visualized by fluorescence microscopy. (**B**) Percentages of cells containing autophagosomes. ***, $P < 0.001$. Data are expressed as the mean ± SD of hexaplicate determinations. Each experiment was repeated three times with similar results. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

examine the effect of hinokitiol on cell death, cells were incubated with different concentrations of hinokitiol and then analyzed for viability. As a positive control, cells were treated with rapamycin. Treatment with hinokitiol significantly reduced the viability of 4T1 and CT26 cells compared with untreated cells. The half-maximal inhibitory concentrations (IC50) of hinokitiol were 2.1718 and 2.2598 μM in 4T1 and CT26 cells, respectively. We also observed that hinokitiol-induced tumor cell death in a dose-dependent manner in 4T1 [Fig. 1(A)] and CT26 [Fig. 1(B)] cells *in vitro*. Taken together, these results indicate that hinokitiol can induce tumor cell death.

Hinokitiol-Induced Autophagy-Related Cell Death

The induction of tumor death was correlated with hinokitiol treatment in the tumor cells. We next investigated whether autophagy contributes to hinokitiol-induced cell death in tumor cells using a rescue experiment with an autophagy inhibitor. Cells were treated with the autophagy inhibitor 3-MA and hinokitiol (1.25 μM) and were assessed in a cell

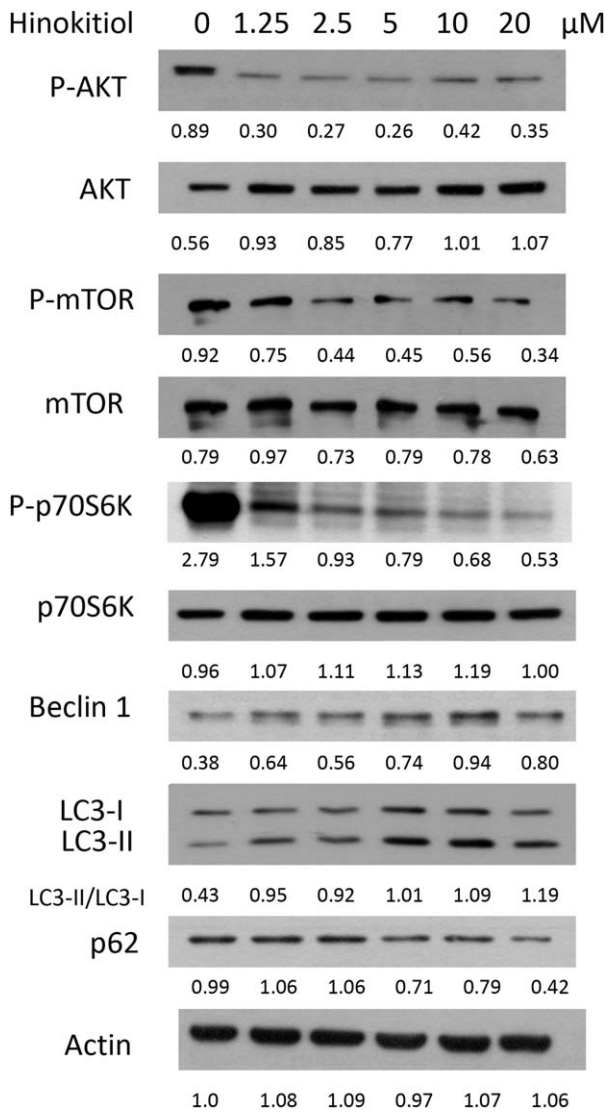

Fig. 4. Hinokitiol-induced autophagic signaling in 4T1 cells. The 4T1 cells were treated with various concentrations of hinokitiol for 2 h. The expression levels of AKT/mTOR proteins and autophagic markers in cells were determined by immunoblot analysis. Inset values indicate protein expression normalized to β-actin.

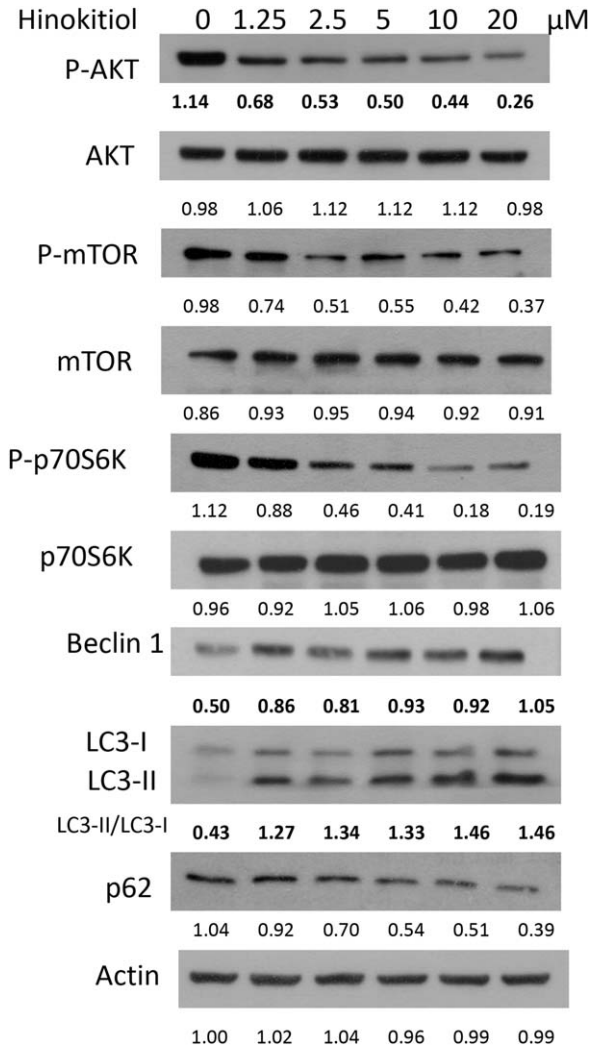

Fig. 5. Hinokitiol-induced autophagic signaling in CT26 cells. The CT26 cells were treated with various concentrations of hinokitiol for 2 h. The expression levels of AKT/mTOR proteins and autophagic markers in cells were determined by immunoblot analysis. Inset values indicate protein expression normalized to β-actin.

viability assay. Treatment with 3-MA could partially protect both 4T1 and CT26 cells from hinokitiol-induced cell death [Fig. 2(A,B)], suggesting that 3-MA protected cells from autophagy-related cell death. Immunoblot analysis revealed that treatment with hinokitiol in tumor cells enhanced the conversion of LC3-I to LC3-II [Fig. 2(C,D)], and the addition of 3-MA reduced this conversion in cells treated with hinokitiol. During the autophagic process, LC3 is concentrated in autophagosome membranes, and the punctate fluorescence produced by GFP-fused LC3 (GFP-LC3) is considered a good indicator of autophagy. As shown in Figure 3(A), cells treated with DMSO showed diffuse cytoplasmic distribution of green fluorescence, whereas significant punctate fluorescence of GFP-LC3 was observed in hinokitiol-treated cells. Hinokitiol did not induce punctate fluorescence of GFP in cells transfected with GFP control vectors [Fig. 3(A)]. The percentage of cells with GFP-LC3 puncta was significantly increased in the cells treated with hinokitiol compared with the DMSO group [Fig. 3(B)]. These results demonstrate that LC3 is concentrated in autophagosomes after hinokitiol treatment.

Hinokitiol-Induced Autophagy Through the AKT/mTOR Pathway

Autophagy induction is associated with various signaling pathways. The above findings prompted us to further explore the detailed mechanism underlying the autophagic effects of hinokitiol in tumor cells. The AKT/mTOR/p70S6K signaling pathway negatively regulates autophagy (Yo et al., 2009). We therefore examined whether the AKT/mTOR/p70S6K signaling pathway participates in hinokitiol-induced autophagy. In a dose-dependent manner (Figs. 4 and 5), treatment with hinokitiol decreased the phosphorylation of AKT, mTOR, and p70S6K, indicating the downregulation of the AKT/mTOR/p70S6K pathway by hinokitiol in 4T1 cells (Fig. 4). Furthermore, very similar results were observed upon treatment of CT26 cells with hinokitiol (Fig. 5). Modulation of beclin 1 expression can affect the induction of autophagy (Pattingre and Levine 2006). Figures 4 and 5 also show that treatment of tumor cells with hinokitiol dramatically increased the expression of beclin1 and enhanced the conversion of LC3-1 to LC3-II, which indicates autophagic induction. p62 binds directly to LC3 proteins via a specific sequence motif, and the protein is itself degraded by autophagy. As shown in Figures 4 and 5, treatment of 4T1 and CT26 cells with hinokitiol dramatically decreased the expression of p62. These results suggested that hinokitiol-induced autophagy in both 4T1 and CT26 cells. Treatment of 4T1 and CT26 cells with hinokitiol for 24 h [Fig. 6(A)] or 48 h [Fig. 6(B)] also induced increased expression levels of beclin 1 and LC3-II and decreased expression of p62. Taken together, these results indicate that induction of autophagy in tumor cells by hinokitiol was associated with downregulation of the AKT/mTOR/p70S6K pathway.

Hinokitiol-Induced Autophagy Via Downregulation of the AKT Signaling Pathway

We found that hinokitiol-induced autophagy by reducing AKT phosphorylation. The AKT/mTOR/p70S6K signaling pathway was activated by transfecting constitutively active AKT plasmid. The suppressive effect of hinokitiol on the AKT/mTOR/p70S6K signaling pathway was relieved by transfecting constitutively active AKT into 4T1 [Fig. 7(A)] and CT26 [Fig. 7(B)] cells. Transfection of constitutively active AKT plasmid reduced the expression of beclin 1 and the conversion of LC3-I to LC3-II after hinokitiol treatment in comparison with the vector-only control transfection. The

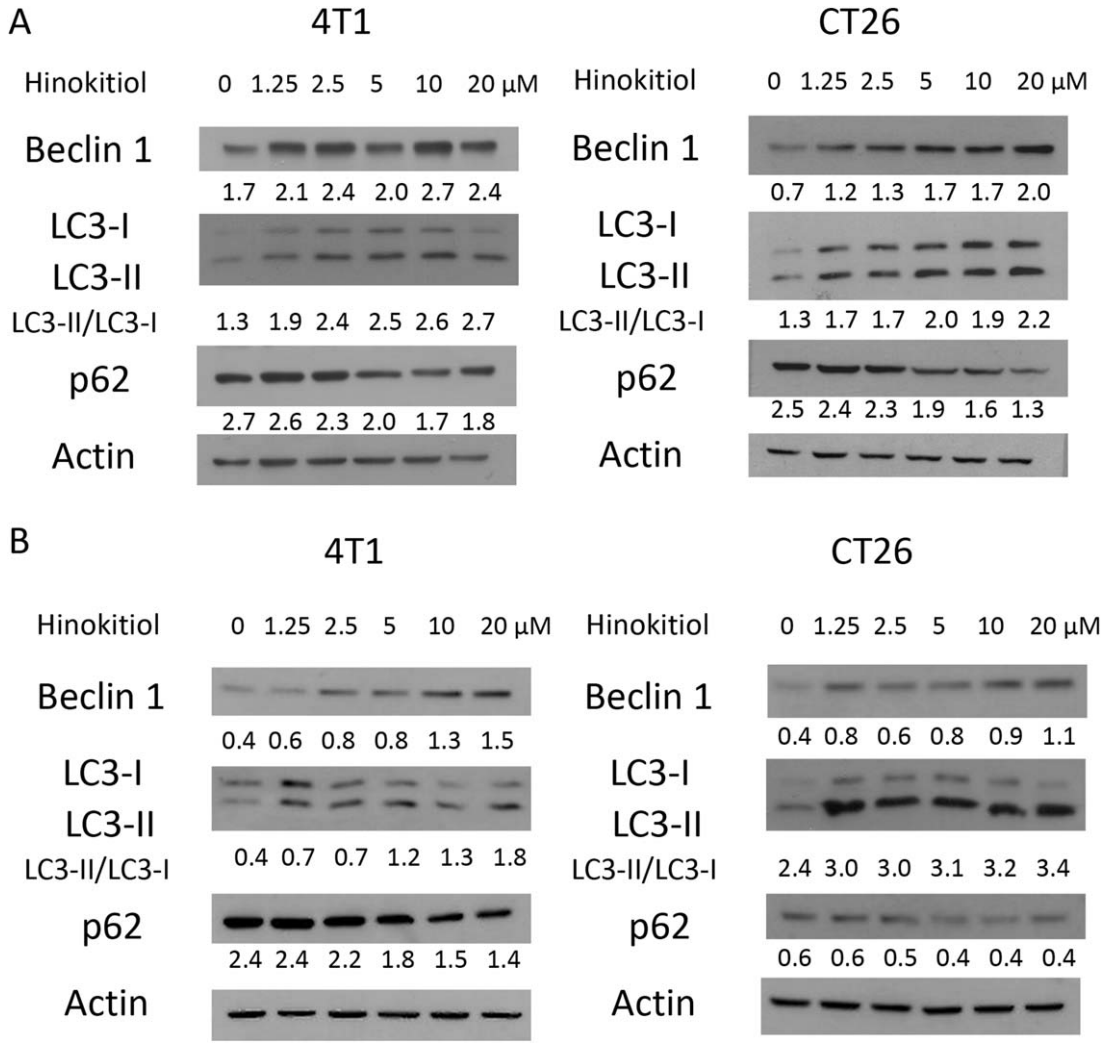

Fig. 6. Hinokitiol-induced the expression of autophagic markers in 4T1 and CT26 cells. 4T1 and CT26 cells were treated with various concentrations of hinokitiol for (**A**) 24 h or (**B**) 48 h. The expression levels of autophagic markers (beclin-1, LC3, and p62) were assessed by immunoblot analysis. Inset values indicate protein expression normalized to β-actin.

hinokitiol-induced cell death was also reduced by transfection with the constitutively active AKT plasmid [Fig. 7(C,D)]. These results suggest that downregulation of AKT is required for hinokitiol-induced autophagy in 4T1 and CT26 cells. Our results show that hinokitiol suppressed the phosphorylation of AKT and decreased mTOR/p70S6K signaling, followed by the upregulation of beclin 1/LC3-II expression. Hinokitiol inhibits tumor cell growth through modulating autophagy and the AKT/mTOR signaling pathway (Fig. 8).

DISCUSSION

We investigated the antitumor activity of hinokitiol in murine tumor models. Autophagy is a multifaceted and evolutionarily conserved process, and alterations in autophagic signaling pathways are frequently found in tumor cells (Lu et al., 2012). The AKT/mTOR/p70S6K pathway is known to be a negative regulator of autophagy. We observed that the levels of phosphorylated AKT, mTOR, and p70S6K were significantly decreased in hinokitiol-treated tumor cells compared with control cells (Fig. 8). These results indicated that hinokitiol can induce autophagic activities in CT26 and 4T1 cells.

Autophagy plays a very important role in maintaining cellular homeostasis. Autophagy is considered to have opposing roles (both promotion and suppression) in tumor cells. Autophagy can provide energy for tumor cells under stress conditions, thus acting as a tumor promoter. However, autophagy also has a tumor-suppressing role (Zhu et al., 2014). The protein beclin 1 has multiple functions, including antitumor activity, that have been linked with autophagy (Sun et al., 2011). Herein, hinokitiol dramatically increased the expression of beclin 1 in tumor cells.

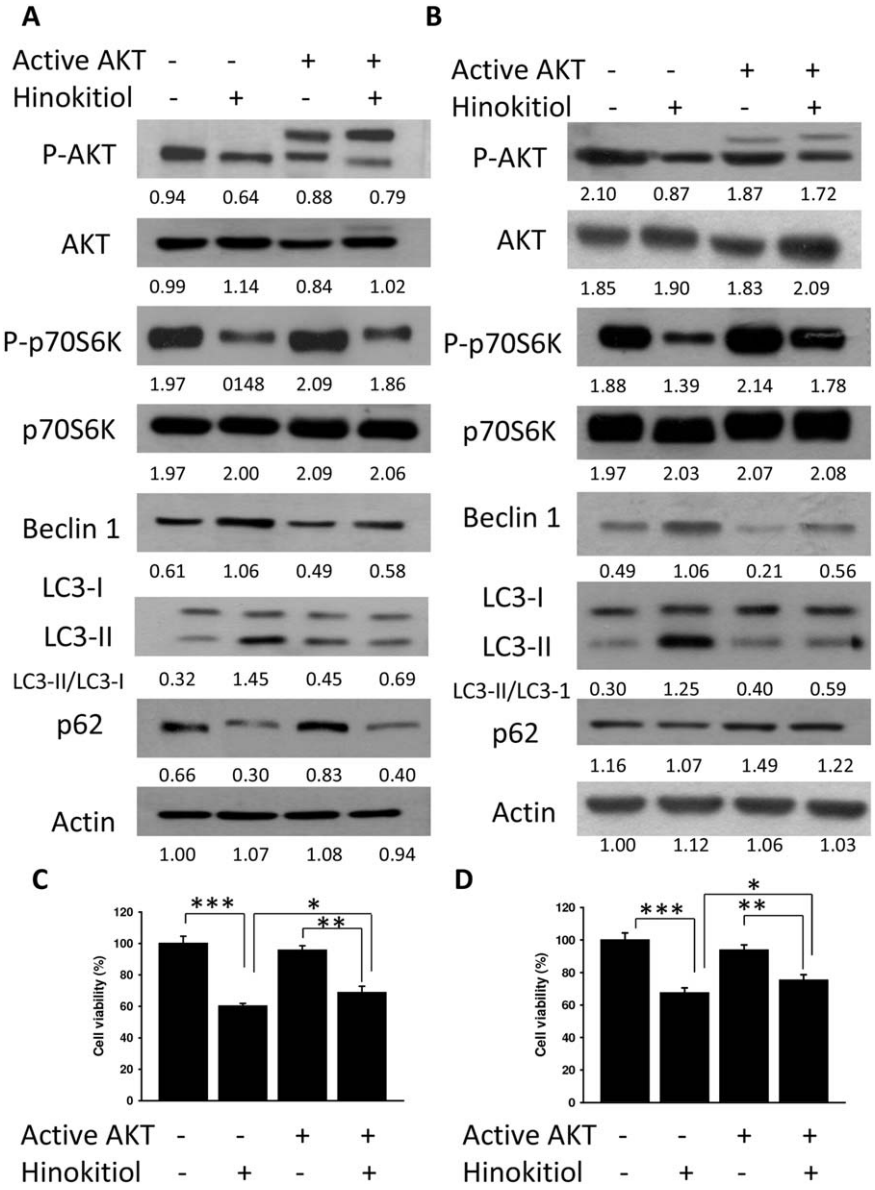

Fig. 7. Effect of hinokitiol on AKT phosphorylation and autophagic signaling. The (**A**) 4T1 and (**B**) CT26 cells were transfected with control or constitutively active AKT plasmids and treated with hinokitiol. The expression levels of AKT/mTOR proteins and autophagic markers were determined by immunoblot analysis. Inset values indicate protein expression normalized to β-actin. The (**C**) 4T1and (**B**) CT26 cells transfected with control or constitutively active AKT plasmids were treated with hinokitiol. Cell were harvested and stained with trypan blue for viability assays. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$. Data are expressed as the mean ± SD of hexaplicate determinations. Each experiment was repeated three times with similar results.

Hinokitiol-induced cell death of tumor cells was associated with the induction and processing of the autophagy marker LC3 (Figs. 4 and 5). Hinokitiol-induced LC3-II conversion was blocked by 3-MA. However, when autophagy is inhibited by 3-MA, hinokitiol can induce tumor cell death through the activation of caspase 3 (Lee et al., 2014). Although autophagy and apoptosis are distinct processes, their signaling pathways are interconnected through various crosstalk mechanisms (Eisenberg-Lerner et al., 2009). The relationship between autophagy and apoptosis is complex and varies among cell types and stresses. Occasionally, autophagy and apoptosis occur simultaneously after stress; at other times, only autophagy or apoptosis is observed (Lockshin and Zakeri 2004). Autophagy may co-occur with apoptosis in tumor cells treated with hinokitiol. Furthermore, hinokitiol-induced autophagy may contribute to tumor cell death in part by enhancing apoptosis.

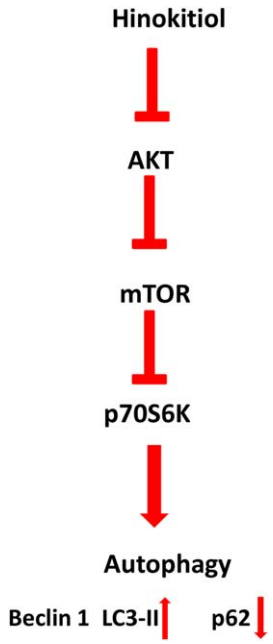

Fig. 8. Hinokitiol induces autophagy by downregulating the AKT/mTOR signaling pathway. Schematic presentation of the signaling pathways involved in hinokitiol-induced autophagy in 4T1 and CT26 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Therefore, this study both evaluates the therapeutic efficacy of hinokitiol for cancer treatment and elucidates the cellular mechanisms underlying antitumor activities mediated by hinokitiol.

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