Bergenin exerts antitumor effects by promoting survivin degradation

Xiaoying Li
The Third Xiangya Hospital of Central South University
Yu Gan
The Third Xiangya Hospital of Central South University
Shuangze Han
The Third Xiangya Hospital of Central South University
Jinzhuang Liao
The Third Xiangya Hospital of Central South University
Li Zhou
Central South University
Wei Li (weililx@csu.edu.cn)
The Third Xiangya Hospital of Central South University

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Abstract

Survivin is overexpressed in multiple human malignancies and correlated with poor prognosis. In this study, we found that survivin is highly expressed in non-small cell lung cancer (NSCLC) tissues, and depletion of survivin inhibits tumorigenesis of NSCLC cells. Moreover, our results showed that bergenin, a natural product, exhibits antitumor activity against NSCLC cells. Bergenin suppresses cell viability and colony formation in A549, H1299 and H460 cells, and reduces the protein level of survivin dose-dependently. The mechanism study revealed that bergenin inhibits the phosphorylation of survivin by regulating Akt/Wee1/CDK1 signaling pathway, thereby enhancing the interaction between survivin and E3 ligase Fbxl7. In addition, bergenin enhances the expression of cleaved-PARP and -caspase 3, which promotes apoptosis of NSCLC cells. The in vivo study showed that the tumor volume, weight, and the expression of Ki67 and survivin were decreased obviously in bergenin treated group. Overall, our data indicate that bergenin has a significant antitumor activity and is a promising agent with therapeutic potential for NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) is the predominant subtype of lung cancer, accounting for 80–85%, and remains the primary cause of cancer-related mortality worldwide[1,2]. NSCLC can be subdivided into adenocarcinoma (ADC), squamous cell carcinoma (SCC), large cell carcinoma (LCC), and mixed histology[3]. Recently, people have made growing efforts to improve detection methods to screen high-risk groups to achieve early diagnosis. However, only a small proportion of NSCLC patients (< 20%) are diagnosed at an early stage due to the lack of specific symptoms, and most of them (47%) are still diagnosed in the advanced stages[4]. The treatment methods of NSCLC include traditional surgery, radiotherapy/chemotherapy, targeted therapy, and immunotherapy[5,6]. Although monotherapy or combination therapy has improved patients' quality of life and overall survival, patients are still at risk of tumor recurrence and progression. Continuing pre/clinical trials aimed to develop new drugs to improve the management of NSCLC is in urgent demand.

Survivin, a member of the inhibitors of apoptosis proteins (IAPs), plays a crucial role in regulating cell division and apoptosis. It is found in different subcellular localization (nucleus, cytoplasm, mitochondria and extracellular) with multiple functions. Nuclear survivin participates in the formation of the chromosome passenger complex (CPC) and regulates cell division, while cytoplasmic and mitochondrial expression is considered to function as apoptotic suppressor[7,8]; the extracellular pool of survivin is secreted by tumor cells in the form of membrane vesicles and absorbed by surrounding cells, which is related to increase therapeutic resistance and promote tumor development[9]. Survivin is not observed in normal adult tissues but is highly expressed in embryonic tissues, cancer cells, and cancer stem cells such as lung cancer, bladder cancer, ovarian cancer, breast cancer and pancreatic ductal adenocarcinoma (PDAC), and this expression is associated with aggressiveness and progression of cancer[10–13]. Recently,

survivin has been served as a universal tumor-associated antigen and a biomarker of cancer progression. Therefore, targeting survivin is emerging as a promising therapeutic strategy in cancer.

Here, we investigated the antitumor effect of bergenin and revealed the underlying mechanism. The natural product bergenin may be a potential survivin inhibitor and an antitumor candidate for NSCLC therapy.

Materials And Methods

Cell culture and reagents

The immortalized lung epithelial cells NL20 and MRC5 and human NSCLC A549, H1299 and H460 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cells were grown in a humidified incubator at 37°C with 5% CO_2 and subjected to mycoplasma analysis routinely. The cell culture medium of RPMI-1640 and Fetal Bovine Serum (FBS) were obtained from Invitrogen (Grand Island, NY). The natural products bergenin, MG 132, and cycloheximide (CHX) were obtained from Selleck Chemicals (Houston, TX). Transfection reagent lipofectamine 2000 was purchased from Thermo Fisher Scientific (Waltham, MA). Antibodies against survivin, cleaved-caspase 3, cleaved-PARP, cleaved-caspase 3, cytochrome C, Bax, VDAC1, p-Survivin Thr34, p-Akt Ser473, p-Wee1 Ser642, p-CDK1 Tyr15 and p-CDK1 Thr161, ubiquitin, Flag-tag, HA-tag, α-Tubulin and β-actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The anti-ki 67 and FbxL 7 antibodies were products of Abcam (Cambridge, United Kingdom).

MTS Assay.

The MTS assay, used to analyze cell viability, was performed as described previously[14]. Briefly, human NSCLC cells were seeded into the 96-well plates (2×10^3 cells/well) and maintained overnight. Cells were incubated with various concentrations of bergenin for 24, 48, and 72 h. Then cell viability was analyzed according to the standard protocol after adding the MTS reagent (#G3580, Madison, WI) to the cell culture medium.

Anchorage-independent cell growth.

The Anchorage-independent cell growth assay was performed as described previously[15]. Briefly, Eagle's basal medium consists of 0.6% agar, 10% FBS and various concentration of bergenin, which were loaded in a 6-well plate as an agar base. Human NSCLC cells were resuspended at a concentration of 8000 cells/ml and inoculated in 6-well plates containing 0.6% Basal Medium Eagle (0.3% agar, 10% FBS, and different doses of bergenin. Colonies were counted after maintaining for 2 weeks.

Clinical tissue sample collections.

A total of 39 cases of NSCLC tissues and matched adjacent non-tumor tissues were collected from 39 patients in the department of pathology, The Third Xiangya Hospital of Central South University, Changsha, Hunan, China. All the patients signed written informed consent and did not receive treatment before surgery.

Western blotting.

The cells were treated with bergenin and lysed in RIRA buffer (#89901, Thermo Fisher Scientific) at 4℃ for around 30min followed by centrifugation at 12,000 × rpm for 10min to collect supernatant as the whole-cell extract (WCE). Then the BCA protein assay kit (#23228, Thermo Fisher Scientific) was used to detect the WCE concentration. A total of 20ug WCE was subjected to SDS-PAGE gels and transferred to the PVDF membrane. Subsequently, the membranes are blocked with 5% non-fat milk for 1 h and incubated with primary and second antibodies. The ECL reagent (#34579, Thermo Fisher Scientific) was used to visualize the target protein expression.

Co-immunoprecipitation (Co-IP).

Total proteins from NSCLC cells cultured in 10 cm culture dishes were used for the Co-IP assay. Briefly, primary antibodies against HA or Flag were added to each cell lysate and incubated overnight at 4℃ with rotation. Protein A/G agarose beads were added and incubated with mild rotation at 4°C for 2 h to bind to the primary antibody. The agarose beads were washed with PBS and cell lysis buffer to wash off unbound antibodies. The agarose beads were resuspended in 20 µl 1x SDS-PAGE loading buffer and boiled and centrifuged to collect the supernatant for western blot analysis.

Generation of survivin knockdown stable cell lines.

To generate survivin knockdown stable cells, 293T cells were co-transfected with pLKO.1-shsurvivin lentivirus plasmids (TRCN0000073718, TRCN0000073721, Millipore Sigma), PSPAX2 and PMD2-G. The supernatant containing viral was collected at 72 h after transfection. NSCLS cells were grown at 70%-80% confluence and infected with the lentivirus and polybrene (5 µg/ml). The infected cells were cultured with fresh medium containing puromycin (1µg/ml) and maintained for 1 week for colony selection.

In vivo tumor growth.

The in vivo tumorigenesis was approved by the Institutional Animal Care and Use Committee (IACUC) of the Third Xiangya Hospital of Central South University (Changsha, China). A549 and H1299 cells (2×10^6) were injected into the right flank of 6-week-old athymic nude mice (n = 5). Tumor growth was monitored, and volume was measured every 2 days. The tumor-bearing mice were randomly divided into two groups (n = 5) when tumor volume reached around 100 mm^3. The treatment group was initiated bergenin (30 mg/kg) via intraperitoneal injection every two days, and the control group was administrated the vehicle control. The mice were euthanized with CO_2 (3 L/min) for 5 min at the endpoint. Tumor volume was calculated following the formula: length × width × width/2.

Immunohistochemical staining (IHC).

The IHC staining was performed as described previously[16]. Tumor tissues obtained from clinical samples and xenograft tumors were subjected to IHC analysis. Briefly, the tissue sections were deparaffinized and rehydrated. The antigen retrieval was performed subsequently by submerging into sodium citrate buffer (10 mM, pH 6.0) and boiled for 10 min. Then the tissue slides were treated with 3% H_2O_2 in methanol for 10 min, washed with PBS and blocked with 10% goat serum albumin, and incubated with primary antibodies overnight at 4°C. After hybridization with secondary antibodies at room temperature, the target protein was visualized using the DAB substrate and counterstained by hematoxylin.

Statistical analysis.

GraphPad Prism 5 (GraphPad 5.0, San Diego, CA, USA) software was used for statistical analysis. Data were performed from at least three independent determinations and represented as mean ± sd. Statistical comparisons between different groups were analyzed by Student's t-test or ANOVA. The Kaplan-Meier method was applied to estimate survival functions and the log-rank test was used to compare survival differences between groups. A probability value of $p < 0.05$ was used as the criterion for statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001).

Results

Survivin is overexpressed in NSCLC tumor tissues.

We first examined survivin protein expression in NSCLC tissues and adjacent tissues by immunohistochemistry (IHC). The result showed that survivin expression in tumor tissues is higher than in adjacent tissue (Fig. 1A). The Kaplan-Meier survival analyses revealed that the high level of survivin is correlated with worse overall survival (OS), first progression survival (FP), and post-progression survival (PPS) of NSCLC patients (Fig. 1B). To determine the oncogenic role of survivin, we generated survivin knockdown stable cell lines in A549 and H1299 cells. The MTS assay and the colony formation assay revealed that knockdown survivin inhibited cell viability and anchorage-independent cell growth significantly (Fig. 1C-D). We further conducted the athymic nude mouse xenograft models. We found that depletion of survivin robustly inhibited tumor growth. The tumor volume and weight were significantly reduced in the A549-shSurvivin xenografts (Fig. 1E-F). Consistently, the IHC data showed that the population of Ki67 positive cells was attenuated obviously in shsurvivin-expression xenograft tumors (Fig. 1G). These results indicated that survivin is overexpressed in NSCLC tissues and may play an important role in NSCLC tumorigenesis.

Bergenin inhibits the viability of NSCLC cells and exhibits little cytotoxicity.

Earlier studies have reported that natural plant extract bergenin (Fig. 2A) has several pharmacological activities, such as anti-inflammatory, hypolipidimic, antitumor and antimalarial, etc[17]. To determine the antitumor effect of bergenin on NSCLC cells, A549, H1299 and H460 cells were treated with different

concentrations of bergenin. The MTS and colony formation data revealed that bergenin significantly inhibited the cell viability and colony formation dose-dependently (Fig. 2B-D). Exposure to bergenin (40 μM) for 48 h decreased the cell viability of all tested NSCLC cells by over 50%. Meanwhile, the anchorage-independent growth assay showed that the colony formation reduced over 75% of A549 and H1299 cells treated with 20 uM bergenin, and 40uM bergenin decreased the colony number by over 90% of all three cell lines. Moreover, to explore the effect of bergenin on non-tumor cells, the immortalized non-tumor lung tissue cells NL20 and MRC5 were treated with bergenin. The results showed that the cell viability did not decrease significantly even if the concentration of bergenin reached 160 uM (Fig. 2E). These results indicate that bergenin has antitumor effect against NSCLC cells and is well-tolerated in immortalized non-tumor cells.

Bergenin facilitates Fbxl7-mediated survivin ubiquitination and degradation in NSCLC cells.

To determine whether the antitumor effect of bergenin in NSCLC cells is through the regulation of survivin, we then detected the expression level of survivin in bergenin-treated NSCLC cells by immunoblotting (IB) assay. The data showed that survivin decreased in A549 and H1299 cells dose-dependently (Fig. 3A). Moreover, treatment with MG132, a proteasome inhibitor, restored the protein level of survivin in bergenin-treated 549 and H1299 cells (Fig. 3B-C). In addition, bergenin reduced the half-life of survivin substantiall (Fig. 3D), indicating that bergenin might affect the stability of survivin protein. We next used the co-IP analysis to investigate the ubiquitination of survivin in bergenin-treated cells. The result showed that bergenin promoted survivin ubiquitination obviously (Fig. 3E) and enhanced the interaction between survivin and the E3 ligase Fbxl7 (Fig. 3F). To determine whether E3 ligase Fbxl7 is indispensable for survivin ubiquitination in bergenin-treated NSCLC cells, we transfected siRNA to silence Fbxl7 in A549 cells and found that knockdown of Fbxl7 impaired bergenin-induced survivin ubiquitination (Fig. 3G). Furthermore, double mutation of the residues K90/91, two ubiquitination sites required for survivin degradation, compromised Fbxl7-induced survivin ubiquitination in A549 cells (Fig. 3H). Meanwhile, the survivin K90/91 mutant reduced bergenin-induced survivin ubiquitination significantly (Fig. 3I). These results imply that bergenin facilitates survivin ubiquitination and degradation by enhancing the interaction between survivin and Fbxl7.

Bergenin-inhibited Akt/Wee1/CDK1 pathway is required for survivin ubiquitination

Previous studies have shown that protein survivin phosphorylation on Thr34 plays an essential role in its biological function and stability. Therefore, we determined whether bergenin promotes survivin degradation via regulating its phosphorylation. As shown in Fig. 4A, bergenin suppressed survivin Thr34 phosphorylation dose-dependently in A549 and H1299 cells. Moreover, we found that after treating with bergenin, the phosphorylation of Akt (Ser473), the downstream target Wee1 (Ser642), CDK1 (Thr161) (a marker for CDK1 activation), and survivin (Thr34) is decreased dose-dependently. Consistently, CDK1 phosphorylation on Tyr15 is increased (Fig. 4B). These results indicate that Bergenin inhibited the Akt/Wee1/CDK1 signaling. This inhibitory effect was also observed in the experiment of knockdown Akt

with siRNA (Fig. 4C). Moreover, transfection of Myr-Akt1, a constitutively activated Akt, compromised bergenin-induced reduction of phosphorylation of Wee1 (Ser642), CDK1 (Thr161), and survivin (Thr34) (Fig. 4D). Additionally, a series of experimental analysis data showed that overexpression of Myr-Akt1 rescued cell viability, increased the live cell population, down-regulated the activated cleaved-caspase 3 significantly, and decreased the protein level of cleaved-caspase 3 and -PAPR in bergenin-treated NSCLC cells (Fig. 4E-H). Compared with wild-type (WT) survivin, mutation of survivin Thr34 to Ala34 induced stronger ubiquitination of survivin in bergenin-treated A549 cells (Fig. 4I). Importantly, survivin WT, but not the T34A mutant, rescued cell viability (Fig. 4J) and increased the population of live cells (Fig. 4H). Collectively, our results demonstrate that inhibition of the Akt-Wee1-CDK1 signal pathway is required for survivin phosphorylation, which promotes survivin ubiquitination and degradation.

Bergenin promotes apoptosis in NSCLC cells.

To examine whether bergenin activates the apoptosis signaling in NSCLC cells, we tested the protein level of cleaved-PARP and -caspase 3 in bergenin-treated A549 and H1299 cells. The IB data showed that bergenin promoted the expression of cleaved-PARP and -caspase 3 dose-dependently (Fig. 5A). Consistently, the enzymatic activity of caspase 3 was significantly enhanced with bergenin treatment (Fig. 5B). Additionally, we examined changes in the intracellular location of apoptosis-related molecules and found that bergenin facilitated the release of cytochrome C from mitochondrial to the cytoplasm. With the increase of bergenin concentration, the protein Bax in the cytoplasm showed a decreasing trend, while that in mitochondria increased (Fig. 5C), suggesting that bergenin can activate the intrinsic apoptotic pathway. We next determined whether the destruction of survivin caused bergenin-induced apoptosis. The results showed that overexpression of survivin restored cell viability (Fig. 5D), compromised the activity of caspase 3 (Fig. 5E), and decreased the protein level of cleaved-caspase 3 and -PARP (Fig. 5F) in bergenin-treated A549 cells. Furthermore, subcellular fractions immunoblotting assay suggested that overexpression of survivin rescued bergenin-promoted intrinsic apoptosis, as the expression level of protein Bax and cytochrome C in cytoplasm and mitochondria were close to the control group without any treatment (Fig. 5G). Our data suggested that bergenin promotes mitochondrial apoptosis in NSCLC cells.

Butein inhibits in vivo tumor growth.

To verify the in vivo anti-tumor effect of bergenin, we performed xenograft mouse models using A549 and H1299 cells. Our data showed that treatment with bergenin significantly delayed the xenograft tumor growth. Compared with the vehicle control group, the tumor volume of the bergenin-treated group was reduced significantly (Fig. 6A-B), and tumor weight decreased by about two-thirds of A549 tumors (Fig. 6C). Consisitently, in the H1299 xenograft tumor model, bergenin exhibited similarly significant antitumor activity (Fig. 6D-6F). Similar to the in vitro findings, the IHC results revealed that bergenin decreased the population of Ki67 positive cells and the number of survivin positive cells in A549-derived tumors (Fig. 6G and 6H). Overall, our data implied that bergenin suppresses in vivo tumor development of NSCLC cells.

Discussion

Accumulating evidence shows that survivin is upregulated in multiple human cancers, and high level of survivin is correlated with the proliferation, invasion, metastasis, recurrence, therapeutic resistance, and poor prognosis in most cancers[18], including NSCLC[19–21]. For instance, it's found that survivin expression level was significantly higher in hepatocellular cancer (HCC) tissue than in normal tissue. And survivin overexpression was found to promot HepG2 cell proliferation, inhibit apotosis and induce invasive ability[22]. The data of a meta-analysis suggested that the level of survivin expression associated with the overall survival (OS) of NSCLC patients was significant and implied positive-survivin expression might be a prognostic factor for NSCLC patients[23]. In the present study, we found that survivin is highly expressed in NSCLC tumor tissues and its expression level is negatively correlated with the prognosis of NSCLC patients. Knockout survivin by sgRNA plasmid transfection inhibited the cell proliferation, colony formation, and tumor growth in vivo. This evidence indicates that survivin plays a crucial role in the tumorigenesis of NSCLC.

Survivin regulation in human cancer cells is complicated and controlled at multiple levels, including transcription, translation, and posttranslational[24]. Transcriptional regulation of survivin mRNA expression includes epigenetic modifications, transcription factors, signaling pathways, etc. Many transcription factors, such as SP1, NF-kB, GATA-1, STAT3, DEC1, and Notch, have emerged as important inducers of survivin expression in various tumors[25]. The signaling pathways of Phosphoinositide 3-Kinase (PI3K)/Akt, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), which are involved to tumor cellular proliferation, differentiation, migration and survival, exist a central role in regulating survivin expression under the action of different stimulators[18]. It has been found that modification of the phosphorylation sites (Thr34, Thr117, Ser20) is closely related to survivin's function. Protein kinase A (PKA) phosphorylates the serine 20 residue to suppress the anti-apoptotic function of survivin; cyclin-dependent kinase 1 (CDK1) phosphorylates the threonine 34, contributing to its stability and promoting interaction with the mitotic spindle[26–28]. Post-translational modifications of ubiquitination and deubiquitination are important to regulate protein expression levels. At present, two E3 ligases (the F-box Protein Fbxl7 and the X-linked inhibitor of apoptosis (XIAP))[29,30] and some deubiquitinases (USP9X, CSN5, USP35)[31–33] have been reported to modulate the expression of survivin. According to the regulatory and functional mechanism of survivin, many drugs targeting survivin have been investigated in clinical and preclinical studies. For example, Piperine prevents the binding of survivin and Smac/DIABLO, making it available for inhibiting IAPs to inhibit the growth of human colon cancer[34]. Tolfenamic acid (TA) downregulates survivin expression and suppresses pancreatic cancer development by inhibiting transcription factors SP1[35]. Xanthohumol inhibits the tumorigenic properties of oral squamous cell carcinoma (OSCC) cells by decreasing survivin phosphorylation on Thr34 and facilitating E3 ligase Fbxl7-mediated ubiquitination, which in turn reduced survivin[36]. In this study, we found that bergenin decreased survivin expression and reduced the protein half-life. Exploring the underlying mechanism revealed that bergenin inhibited survivin Thr34 phosphorylation by regulating the

Akt/Wee1/CDK1 signal pathway, and enhanced the interaction between protein survivin and E3 ligase Fbxl7, which facilitates survivin ubiquitination and degradation. However, it is unclear whether bergenin also acts on other molecules of the IAPs family and other modification sites of survivin. Thus, further investigating the anti-tumor mechanism of bergenin will be meaningful.

Bergenin is reported to have several critical pharmacological activities, including hepatoprotective, anti-inflammatory, anti-oxidation, anti-diabetes, and antitumor effects[37–40]. Previous studies showed that bergenin could attenuate bleomycin-induced pulmonary fibrosis via inhibiting the TGF-β1 signal[41], relieve colitis in vivo through regulating PPARγ/SIRT1/NF-κB-p65 pathway[42], protect acute lung injury by inhibiting NF-κB activation[43], and inhibit human bladder cancer progression via activating the PPARγ signal[44]. In the current preclinical studies, the natural product bergenin is mainly used in non-neoplastic diseases, but its inhibitory effect on malignant tumors is rarely investigated. Our results found that bergenin promoted intrinsic apoptotic pathway by reducing survivin. In NSCLC cells, bergenin inhibited tumor cell proliferation, colony formation, and in vivo tumorigenesis by downregulating the Akt/Wee1/CDK1 signal pathway and reducing survivin stability. These findings indicate that bergenin could be a novel survivin inhibitor that deserves further study for NSCLC treatment.

Conclusion

In summary, we demonstrated that survivin was upregulated in NSCLC tumor tissues and required for maintaining malignant phenotypes of NSCLC cells. Bergenin reduced survivin expression dose-dependently and inhibited the phosphorylation of survivin by suppressing Akt/Wee1/CDK1 signal pathway, which enhanced the interaction between survivin and E3 ligase Fbxl7. This study suggests that bergenin appears to be an effective chemical targeting survivin and may serve as a promising therapeutic candidate for NSCLC.

Statements & Declarations

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Author contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Xiaoying Li], [Yu Gan], [Shuangze Han], [Jinzhuang Liao], [Li Zhou] and [Wei Li]. Writing, review, and/or revision of the manuscript was written by [Xiaoying Li], [Li Zhou] and [Wei Li]. All authors read and approved the final manuscript.

Availability of data and materials

Materials are available upon request.

Ethics approval and consent to participate.

The animal experiments were approved by the Medical Research Animal Ethics Committee, Central South University, China.

Consent for publication

Not applicable.

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Figures

Figure 1

Knockdown of survivin inhibits the malignant phenotype of NSCLC cells. A, IHC staining analysis of survivin in 39 cases of NSCLC tissues and matched adjacent non-tumor tissues. Left, The representative images of survivin IHC staining. Right, Qualification. Scale bar, 40 μm. B, Kaplan-Meier survival analysis for the relationship between survival time and survivin signature in NSCLC was performed by using the online tool (http://kmplot.com/analysis/). OS (left), Overall Survival. FP (middle), First Progression Survival, PPS (right), Post Progression Survival. $p < 0.05$ was considered to be a statistically significant difference. C, Cell viability of A549 (left) and H1299 (right) cells expressing shCtrl or shsurvivin. Top, immunoblotting (IB) analysis of survivin protein level. Bottom, MTS analysis of cell viability. ***$p<0.001$. D, The colony formation of A549 and H1299 cells expressing shCtrl or shsurvivin. ***$p<0.001$. E-G, In vivo tumorigenesis of A549 expressing shCtrl or shsurvivin. Tumor volume (E), tumor weight (F), and IHC staining analysis of the population of Ki67 positive cells (G) of the A549 xenografts. Scale bar, 25 μm. ***$p<0.001$.

Figure 2

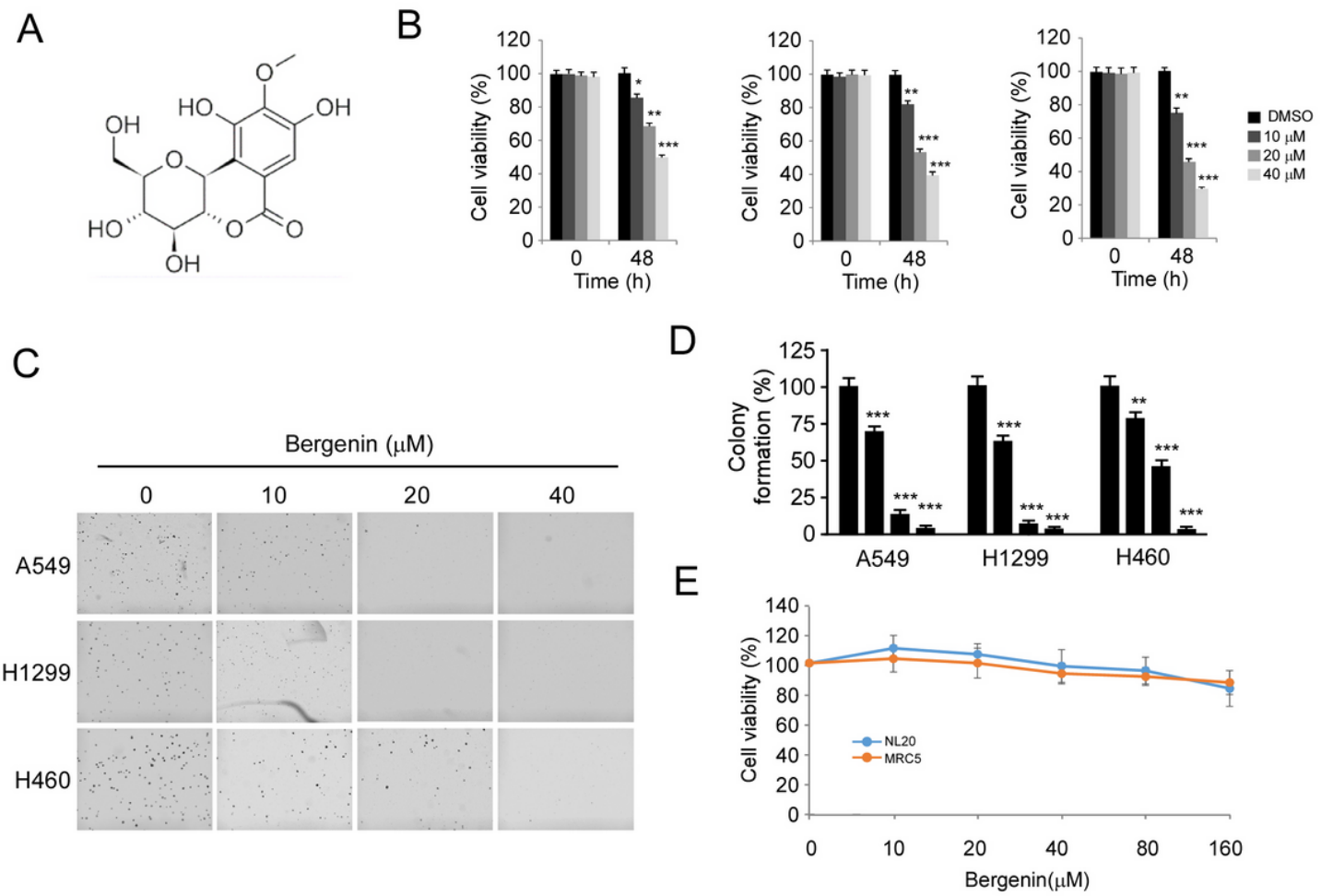

Figure 2

Bergenin suppresses NSCLC cells.

A, The chemical structure of Bergenin. B, MTS assay analysis of the cell viability of A549 (left), H1299 (middle), and H460 (right) cells with bergenin treatment. *p<0.05, **p<0.01, ***p<0.001. C and D, Colony formation assay analysis of the anchroge-independent cell gorwth of A549 (left), H1299 (middle), and H460 (right) cells with bergenin treatment. C, the representative images of colony formation. D, Qualification. **p<0.01, ***p<0.001. E, MTS assay analysis of the effect of Bergenin on immortalized NL20 and MRC5 cells.

Figure 3

Bergenin promotes ubiquitination-mediated survivin degradation.

A, A549 and H1299 cells were treated with bergenin for 48 h. The whole-cell extract (WCE) was subjected to IB analysis. B, A549 and H1299 cells were treated with bergenin for 48 h, and incubated with MG132 (20 μM) for 10 h. The whole-cell extract (WCE) was subjected to IB analysis. C, A549 and H1299 cells were treated with bergenin for 48 h, and incubated with MG132 (20 μM) for various time points. The WCE was subjected to IB analysis. D, A549 cells were treated with bergenin for 48 h, and incubated with CHX for various time points. The WCE was subjected to IB analysis. E, A549 cells were treated with bergenin for 48 h, and incubated with MG132 (20 μM) for 10 h. The WCE was subjected to survivin ubiquitination analysis. F, A549 cells were transfected with various constructs for 24 h, followed by bergenin treated for 48 h, and incubated with MG132 (20 μM) for 10 h. The WCE was subjected to co-IP analysis. G, A549 cells were transfected with siFbxl7 for 24 h and treated with Bergenin for 48 h and incubated with MG132 (20 μM) for 8 h. The WCE was subjected to survivin ubiquitination analysis. H, A549 cells were transfected with various constructs for 48 h and incubated with MG132 (20 μM) for 10 h. The WCE was subjected to survivin ubiquitination analysis. I, A549 cells were transfected with various constructs for 24 h, followed by bergenin treated for 48 h, and incubated with MG132 (20 μM) for 10 h. The WCE was subjected to survivin ubiquitination analysis.

Figure 4

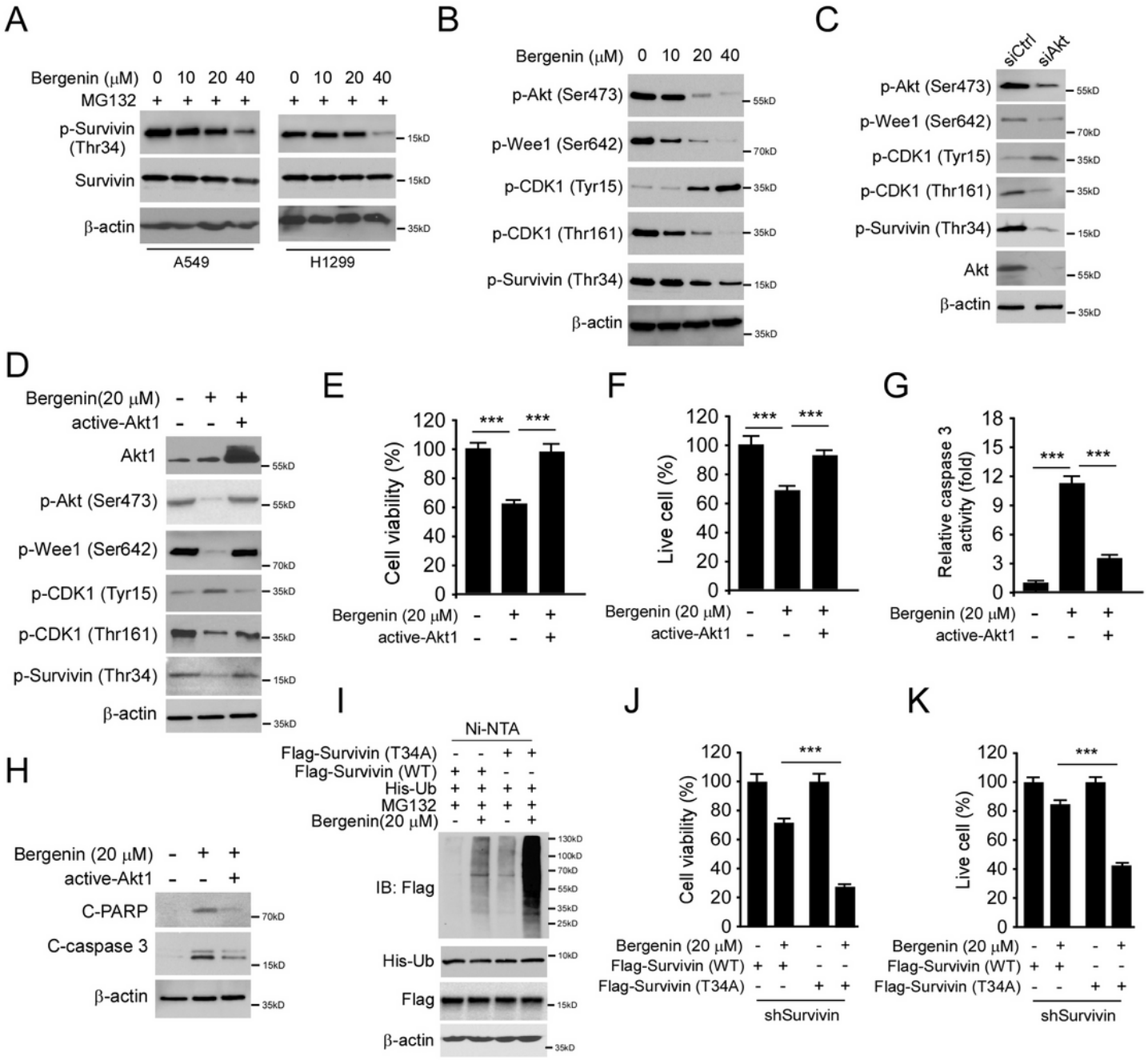

Figure 4

Bergenin reduces survivin Thr34 phosphorylation.

A, A549 and H1299 cells were treated with bergenin for 48 h, followed by MG132 treated for 10 h, the WCE was subjected to IB analysis. B, A549 cells were treated with bergenin for 48 h, the WCE was subjected to IB analysis. C, A549 cells were transfected with siAkt for 48 h, the WCE was subjected to IB

analysis. D-H, A549 cells were transfected with constitutively activated Akt1 for 24 h, followed by bergenin treated for another 48 h, the WCE was subjected to IB analysis (D), cell viability was determined by MTS assay (E). The live cell population was tested by trypan blue exclusion assay (F) and caspase 3 activity was examined by Caspase-3 Assay Kit (G). The protein level of cleaved-PARP (C-PARP) and -caspase 3 (C-caspase 3) was determined by IB analysis (H). ***p<0.001. I, A549 cells were transfected with various constructs for 24 h and treated with bergenin for another 48 h. MG132 was added to the cell culture medium and maintained for 10 h. The WCE was subjected to survivin ubiquitination analysis. J and K, A549 cells expressing shCtrl or shSurvivin were transfected with Flag-survivin WT or T34A mutant, cell viability was examined by MTS assay (J), and the live cell population was tested by trypan blue exclusion assay (K). **p<0.01, ***p<0.001.

Figure 5

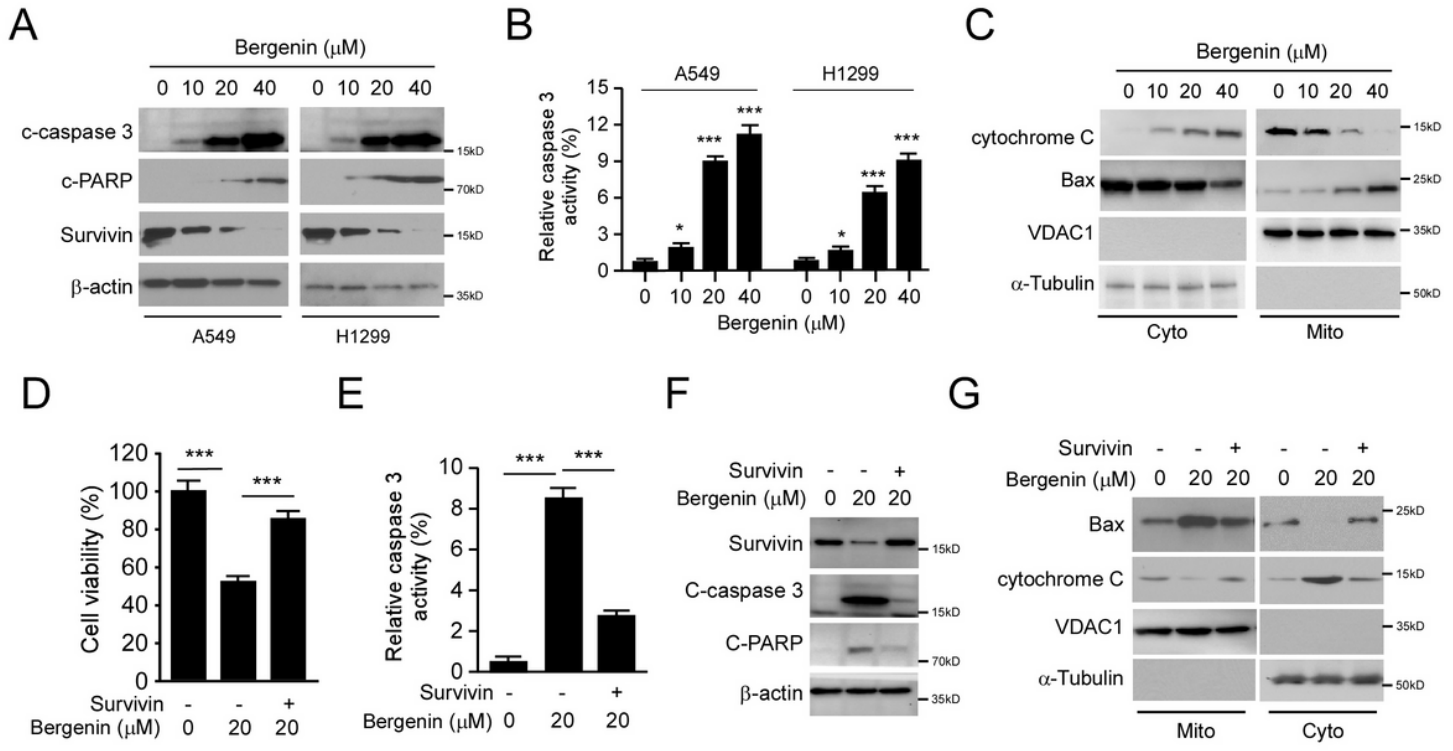

Figure 5

Bergenin promotes apoptosis.

A and B, A549 and H1299 cells were treated with bergenin for 48 h, the WCE was subjected to IB analysis (A), and caspase 3 activity was examined by Caspase-3 Assay Kit (B). *p<0.05, ***p<0.001. C, A549 cells were treated with bergenin for 24 h, and subcellular fractions were isolated and subjected to IB analysis. D-G, A549 cells were transfected with survivin for 24 h, followed by bergenin treated for another 48 h, cell viability was examined by MTS assay (D) and caspase 3 activity was examined by Caspase-3 Assay Kit

(E). The WCE was subjected to IB analysis (F), and subcellular fractions were isolated and subjected to IB analysis (G). ***p<0.001.

Figure 6

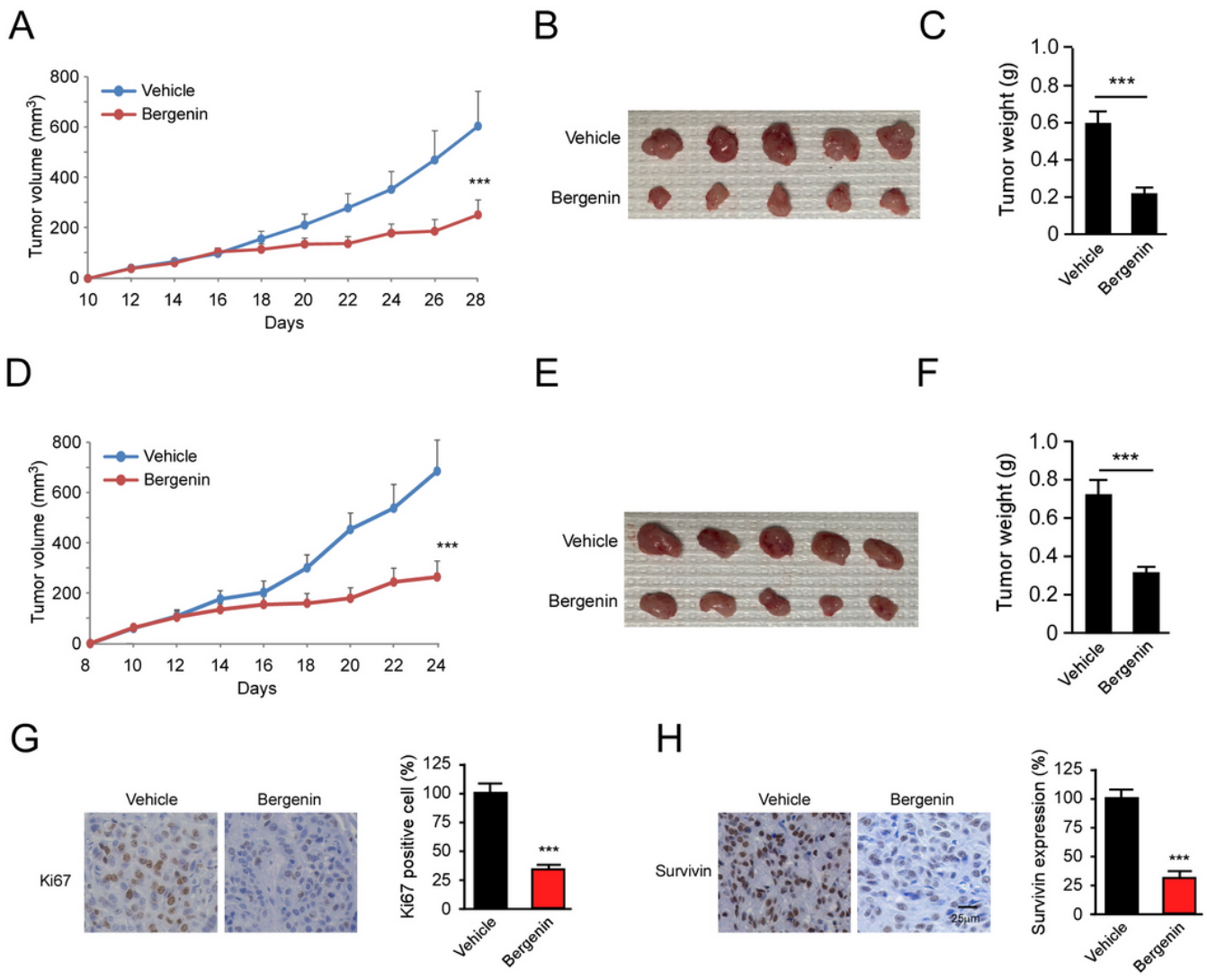

Figure 6

Bergenin suppresses the *in vivo* tumor growth.

A-C, The tumor volume (A), The image of tumor mass (B), and tumor weight (C) of A549-derived xenograft tumors treated with vehicle control or Bergenin. ***p<0.001. D-F, The tumor volume (D), The image of tumor mass (E), and tumor weight (F) of H1299-derived xenograft tumors treated with vehicle control or Bergenin. ***p<0.001. G and H, IHC staining (left) and qualification (right) of Ki67 (G) and survivin (H) in A549-derived xenograft tumors with vehicle or Bergenin treatment. ***p<0.001. Scale bar, 25 μm.