Glaucocalyxin A exerts anticancer effect on osteosarcoma by inhibiting GLI1 nuclear translocation via regulating PI3K/Akt pathway

Jianwei Zhu¹, Yang Sun¹, Ying Lu¹, Xiubo Jiang¹, Bo Ma¹, Lisha Yu¹, Jie Zhang¹, Xiaochen Dong² and Qi Zhang¹

Abstract
Osteosarcoma, the most common malignant bone tumor with recurring disease or lung metastases, has become one of the leading causes of death in humans. In the current study, we made an investigation on the anticancer effect of glaucocalyxin A, a bioactive ent-kauranoid diterpenoid isolated from Rabdosia japonica var., and unraveled the underlying mechanisms. Here, we found that Glucocalyxin A inhibited the cell viability of numerous osteosarcoma cells. Our results showed that Glaucocalyxin A exerted the pro-apoptotic effect on human osteosarcoma cells, MG-63 and HOS cells. Glaucocalyxin A induced apoptosis by mitochondrial apoptotic pathway through several steps including increasing the Bax/Bcl-2 ratio, triggering the intracellular reactive oxygen species (ROS) generation, reducing mitochondrial membrane potential (MMP), and inducing cleavage of caspase-9 and caspase-3. We demonstrated that Glaucocalyxin A induced apoptosis via inhibiting Five-zinc finger Glis 1 (GLI1) activation by overexpression and knockdown of GLI1 in vitro. We also found that Glaucocalyxin A inhibited GLI1 activation via regulating phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) signaling pathway. We further confirmed our findings by using PI3K activator and inhibitor to verify the inhibitory effect of Glaucocalyxin A on PI3K/Akt/GLI1 pathway. Moreover, our in vivo study revealed that glaucocalyxin A possessed a remarkable antitumor effect with no toxicity in the xenograft model inoculated with HOS tumor through the same mechanisms as in vitro. In conclusion, our results suggested that Glaucocalyxin A induced apoptosis in osteosarcoma by inhibiting nuclear translocation of GLI1 via regulating PI3K/Akt signaling pathway. Thus, Glaucocalyxin A might be a potential candidate for human osteosarcoma in the future.

Introduction
Osteosarcoma, a prevailing primary bone cancer among adolescents and young adults, has become a high risk for death in humans. Although there are lower-grade variants, most of them are high-grade malignancies for lung metastases at a high propensity. Recently, the standard treatment consists of surgical resection and chemotherapy leading to nearly 60% of patients with local extremity disease and 20–30% of patients with primary metastases. Preoperative and postoperative chemotherapy, as well as surgical excision are commonly adopted to treat high-grade osteosarcomas; however, a very limited number of drugs are long-time available for the adverse effect and toxicity.
Therefore, it is urgent to develop novel effective therapeutic agents for osteosarcoma.

Increasing evidence has reported that phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway contributes to cancer initiation and development, such as tumorigenesis, inhibition of apoptosis, proliferation, and chemoresistance. PI3K/Akt pathway can enhance the tolerance of cells to hypoxia and nutritional deficiencies through the inhibition of apoptosis, so it is related to the development of breast cancer, lung cancer, melanoma, lymphoma, and other human tumors. PI3K could catalytically induce the production of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane, leading to the recruitment and activation of the downstream targets, such as the serine-threonine protein kinase Akt. Akt phosphorylation plays a crucial role in the anti-apoptotic pathway. Akt can be activated by insulin-like growth factor 1 (IGF1) and prevents PTEN-mediated apoptosis. Akt activation also plays an anti-apoptotic role by phosphorylating the downstream target proteins, such as Bcl-2 and caspase-3 and then prevent apoptosis. The downstream proteins of PI3K/Akt pathway mainly regulate apoptosis on the outer mitochondrial membrane and control the initiation of mitochondrial outer membrane permeabilization. Moreover, PI3K/Akt pathway is frequently hyperactivated in osteosarcoma. Inhibiting PI3K/Akt signaling pathway leads to increased apoptotic cells in osteosarcoma via downregulation of the inhibitor of apoptosis protein and activation of caspase-9 and caspase-3. Therefore, targeting PI3K/Akt pathway has commanded a great deal of recent attention for the development of anticancer agents.

Hedgehog signaling pathway has an essential impact on the formation of most tissues and organs in mammals, such as cell growth and survival, cell fate determination and organ morphogenesis, and it is closely related to the development of human tumors. The intracellular factors involved in Hedgehog signaling transduction include transcription factor Cubitus Interruptus (CI)/five-zinc finger Gli5 (GLI2). GLI (GLI1 and GLI2), as a crucial transcription factor in the Hedgehog signaling pathway, regulates the transcription of multiple downstream target genes and promote tumor progression. Studies from many laboratories have found the activation of GLI in a variety of human cancer, including basal cell carcinomas, medulloblastomas, leukemia, gastrointestinal, lung, ovarian, breast, and prostate cancers. It is thus believed that targeted inhibition of GLI may be effective in the treatment and prevention of human cancer. It has been documented that GLI enabled to promote the development of osteosarcoma. The nuclear translocation of GLI can induce the expression of various context-specific genes, for example, encoding the D-type cyclins, c-MYC (also called MYC), BCL2 and SNAIL (also called SNAI1), which respectively regulated cellular differentiation, proliferation, and survival. The GLI1/Bcl-2 pathway is related to anti-apoptosis, with accompanying of the caspase cascade deregulation. Non-canonical GLI1 activation is regulated by PI3K/Akt signaling pathway and inhibiting PI3K/Akt/GLI1 pathway can induce apoptosis and suppress the growth of renal cell carcinoma in vitro and in vivo. Additionally, recent study has reported that PI3K/Akt leads to the activation of GLI1 in human esophageal adenocarcinoma cells OE19 in vitro. Therefore, chemotherapy targeting PI3K/Akt pathway can inhibit GLI activation to prevent cancer.

Glaucocalyxin A, an ent-kaurenoid diterpene from Rabdosia japonica var., is known to possess numerous biological activities including inhibition of platelet aggregation, immunosuppressive activity, antioxidative and DNA damage protective activity, and cytotoxic activity. It has been documented that Glaucocalyxin A induced apoptosis in human leukemia HL-60 cells and human breast cancer cells. Moreover, Glaucocalyxin A suppressed cell proliferation and promoted apoptosis in a dose-dependent manner in human-derived malignant glioma U87MG cells. However, the anticancer effect of Glaucocalyxin A on osteosarcoma has not been reported till now and the potential mechanisms still remain unclear. In the present study we investigated the anticancer effect of Glaucocalyxin A on human osteosarcoma and the underlying mechanisms. We demonstrated that Glaucocalyxin A exerted a dramatic pro-apoptotic effect by inhibiting GLI1 activation via regulating PI3K/Akt signaling pathway in vitro and in vivo. Our findings indicated that Glaucocalyxin A might have an attractive advantage to be a promising and effective candidate for human osteosarcoma in the future.

Results
Glaucocalyxin A induced apoptosis in human osteosarcoma cells
The chemical structure of Glaucocalyxin A is shown in Fig. 1a. We evaluated the inhibitory effect of Glaucocalyxin A on the cell viability of human osteosarcoma cells including HOS, Saos-2, U-2OS, and MG-63 cells after different concentrations of Glaucocalyxin A for 24 and 48 h. After treatment with Glaucocalyxin A for 24 h, the IC_{50} (the concentration of drug inhibiting 50% of cells) values of HOS, Saos-2, U-2OS, and MG-63 cells were 10.65, 14.14, 15.69, and 9.519 μM, respectively (Fig. 1b). After treatment for 48 h the IC_{50} values were 7.015, 7.316, 8.364, and 5.296 μM, respectively (Fig. 1b). CCK8 assay was also used to assess the cell viability of these four cell lines after treatment with Glaucocalyxin A for 24 and 48 h (Fig. 1c). The CCK8 results further confirmed that MG-63 and HOS cell lines were more susceptible to Glaucocalyxin A; thus we chose these two cell lines in the following
Fig. 1 (See legend on next page.)
osteosarcoma cells. Glaucocalyxin A induced typical apoptosis in human (Supplementary Fig. 1a and b). Our data demonstrated that caspase inhibitor Z-VAD-FMK reversed the pro-apoptotic dependent apoptosis. The results showed that the pan-

demonstrate that the cell death was primarily caspase-

Moreover, we added pan-caspase inhibitor Z-VAD-FMK to

RGF

To observe the morphological changes of HOS and MG-63
cells in the presence of Glaucocalyxin A for 24 h, we used
DAPI staining assay to test if Glaucocalyxin A induced
apoptosis in MG-63 and pHOS cells. The result showed
that the control cells remained round shaped, whereas cells
treated with Glaucocalyxin A presented morphological
features of apoptotic chromatin condensation and DNA
fragmentation in a dose-dependent manner (Fig. 1g). These
data suggested that Glaucocalyxin A had a selectively inhibitory
effect on osteosarcoma cells but not on non-
transformed cells.

As shown in Fig. 1f, glaucocalyxin A-treated MG-63 cells
came round and broke into fragments. The degree of
sloughing of cells was correlated with drug concentrations.
To observe the morphological changes of HOS and MG-63
cells in the presence of Glaucocalyxin A for 24 h, we used
DAPI staining assay to test if Glaucocalyxin A induced
apoptosis in MG-63 and pHOS cells. The result showed
that the control cells remained round shaped, whereas cells
treated with Glaucocalyxin A presented morphological
features of apoptotic chromatin condensation and DNA
fragmentation in a dose-dependent manner (Fig. 1g). These
results suggested that the inhibitory effect of Glaucocalyxin
A on the growth of osteosarcoma cells might be attributed
to inducing apoptosis. Annexin V/PI staining assay was
used to confirm the pro-apoptotic effect of Glaucocalyxin
A. The apoptotic rates of HOS and MG-63 cells were sig-
ificantly increased by Glaucocalyxin A in a dose-
dependent manner, compared with the control group
(Fig. 1h). The quantitative analysis for the percentage of
apoptotic cells showed that Glaucocalyxin A remarkably
induced apoptosis in HOS and MG-63 cells (Fig. 1f).
Moreover, we added pan-caspase inhibitor Z-VAD-FMK to
demonstrate that the cell death was primarily caspase-
dependent apoptosis. The results showed that the pan-
caspase inhibitor Z-VAD-FMK reversed the pro-apoptotic
effects of Glaucocalyxin A on HOS and MG-63 cells
(Supplementary Fig. 1a and b). Our data demonstrated that
Glaucocalyxin A induced typical apoptosis in human
osteosarcoma cells.

Glaucocalyxin A induced mitochondria-mediated
apoptosis in HOS and MG-63 cells

The mitochondrial function is important for cell survi-
val. The change of MMP (ΔΨm) is a hallmark of
mitochondrial dysfunction in early apoptosis. To further
investigate Glaucocalyxin A-induced apoptosis, MMP
(ΔΨm) was detected by flow cytometry. The value of
average cell MMP dramatically decreased after the

treatment with glaucocalyxin A (Fig. 2a). The results
suggested that Glaucocalyxin A induced apoptosis by
aggravating the mitochondrial dysfunction in osteo-
sarcoma cells.

Oxidative stress is an important factor causing mito-
chondrial dysfunction. Moreover, reactive oxygen spec-
ies (ROS) play a key role in cell apoptosis. Therefore,
we examined the effect of Glaucocalyxin A on ROS
generation in HOS and MG-63 cells. The results showed
that Glaucocalyxin A increased the generation of ROS in a
concentration-dependent manner (Fig. 2b), suggesting
that Glaucocalyxin A triggered the generation of intra-
cellular ROS. We used N-acetylcysteine (NAC), an ROS
scavenger, to examine the effect of Glaucocalyxin A on
the generation of ROS. The results revealed that the
level of ROS triggered by Glaucocalyxin A was alleviated
by NAC (Fig. 2c). To further determine the kind of
ROS overproduced by Glaucocalyxin A, we measured
intracellular superoxide anion (O2−) and hydrogen
peroxide (H2O2) levels in osteosarcoma cells. Similar
with ROS, intracellular O2− level could be elevated by
Glaucocalyxin A and reversed by NAC (Fig. 2d), whereas
H2O2 level remained unchanged (Fig. 2e). These results
manifested that mainly ROS triggered by glaucocalyxin A
was O2−.

We detected the levels of the apoptosis-related proteins
such as Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3 by
western blot. After treatment with Glaucocalyxin A for 24
h, the protein expression of the apoptotic protein Bax
increased while the protein expression of the anti-
apoptotic protein Bcl-2 decreased in a concentration-
dependent manner (Fig. 2f). The ratio of Bax/Bcl-2 is an
indicator of mitochondrial apoptotic pathway. Our results
revealed that the ratio of Bax/Bcl-2 was markedly
increased by Glaucocalyxin A (Fig. 2g). Caspase-9 and
caspase-3 cleavage were remarkably activated after Glau-
cocalyxin A treatment (Fig. 2f, h). Besides, the mRNA
levels of Bcl-2 and Bax were also regulated by Glaucoca-
lyxin A accordingly (Fig. 2i). All these findings indicated
that Glaucocalyxin A induced mitochondrial apoptosis in
osteosarcoma cells.
Fig. 2 (See legend on next page.)
Glucocalyxin A induced apoptosis by inhibiting GLI1 nuclear translocation in human osteosarcoma cells

GLI1 is in charge of regulating normal physiological activities and many diseases such as cancer. GLI1 activation is crucial in several stages of tumorigenesis. When it is inhibited, the number of apoptotic cells significantly increased.18,26,27 The activation of GLI1 promoted the development of osteosarcoma.37,38 Here we investigated the inhibitory effect of Glucocalyxin A on the activation of GLI1 in human osteosarcoma cells. Western blot analysis showed that Glucocalyxin A decreased the nuclear expression of GLI1, while it increased the cytoplasmic expression of GLI1 in a concentration-dependent manner (Fig. 3a, b). This result was further confirmed by the immunofluorescence staining. The result showed that 10 μM Glucocalyxin A inhibited the nuclear translocation of GLI1 (Fig. 3c). We further used GLI1 plasmid to confirm the effect of Glucocalyxin A on apoptosis in vitro. Western blot analysis showed that GLI1 protein was overexpressed by GLI1 plasmid (Fig. 3d, e). GLI1 plasmid significantly attenuated Glucocalyxin A-induced apoptosis in MG-63 and HOS cells (Fig. 3f). Moreover, we detected the effects of Glucocalyxin A on the protein expression of Bax, Bcl-2, cleaved caspase-9, and cleaved caspase-3 after transfection with GLI1 plasmid. In the presence of GLI1 plasmid, the effects of Glucocalyxin A on the protein expression of apoptosis-related proteins were obviously reversed (Fig. 3g–i). We also used GLI1 siRNA to confirm the effect of Glucocalyxin A on apoptosis in vitro. As expected, GLI1 siRNA reduced GLI1 protein efficiently (Supplementary Fig. 2a and b). After transfection with GLI1 siRNA, Glucocalyxin A hardly had effect on apoptosis (Supplementary Fig. 2c and d) and the protein expression of apoptosis-related proteins in osteosarcoma cells (Supplementary Fig. 2e–h). These results suggested that Glucocalyxin A induced apoptosis by inhibiting nuclear translocation of GLI1.

Glucocalyxin A induced apoptosis via inhibiting GLI1 nuclear translocation by regulating PI3K/Akt signaling pathway in human osteosarcoma cells

It has been reported that PI3K/Akt signaling pathway plays a key role in osteosarcoma progression.39 We investigated the effect of Glucocalyxin A on PI3K/Akt pathway. Our results showed that the protein expression of PI3K and p-Akt in HOS and MG-63 cells was decreased by Glucocalyxin A. The protein expression of Akt remained constant after treatment with Glucocalyxin A (Fig. 4a, b). We further used an activator and an inhibitor of PI3K/Akt signaling pathway, IGF-1 and LY294002, to determine whether Glucocalyxin A inhibited PI3K/Akt pathway. The inhibitory effect of Glucocalyxin A on PI3K/Akt signaling pathway was reversed by the treatment with 20 ng/ml IGF-1 (Fig. 4c, d). These results indicated that Glucocalyxin A inhibited PI3K/Akt signaling pathway.

Recent report has demonstrated that PI3K/Akt pathway can regulate the activation of GLI. We further used IGF-1 and LY294002 to confirm whether Glucocalyxin A inhibited GLI1 activation by regulating PI3K/Akt pathway. The results showed that 10 μM glaucocalyxin A inhibited the nuclear translocation of GLI1; however, this effect was also withdrawn by IGF-1 (Fig. 4e, f). Moreover, there was no significant difference between the inhibitory effect of Glucocalyxin A and LY294002 on PI3K/Akt signaling pathway and the nuclear translocation of GLI1 (Fig. 4e, f).

We continued to examine whether Glucocalyxin A induced apoptosis by regulating PI3K/Akt pathway using IGF-1 and LY294002. The pro-apoptotic effect of Glucocalyxin A was reversed by the treatment with 20 ng/ml IGF-1 (Fig. 4g). Moreover, the ratio of Bax/Bcl-2 and the cleavage of caspase-9 and caspase-3 increased by Glucocalyxin A was withdrawn by 20 ng/ml IGF-1 (Fig. 4h–i). There was no significant difference between the pro-apoptotic effect of Glucocalyxin A and...
Fig. 3 (See legend on next page.)
translocation through regulating PI3K/Akt signaling pathway in vivo.

Though Glaucoalyx A inhibited the growth of osteosarcoma in vivo, its potential toxicity must be assessed comprehensively. During this study (21 days), we noticed that the body weight of mice in the control and glaucoalyx A groups was not significantly different (Fig. 5i). We also found no obvious change in major organs between the control and Glaucoalyx A-treated groups (Fig. 5k). Moreover, hematological parameters were normal in 80 mg/kg of Glaucoalyx A-treated mice (Table 1). The results indicated that 80 mg/kg of Glaucoalyx A exerted anti-tumor activity without any toxicity in vivo. All our results above demonstrated that Glaucoalyx A inhibited tumor growth by inducing apoptosis via inhibiting GLI1 nuclear translocation through regulating PI3K/Akt pathway in human osteosarcoma.

**Discussion**

Osteosarcoma, the primary bone cancer with high incidence, will always cause death for metastatic disease unless treated by surgery and effective multidrug chemotherapy. It has been reported that high-dose methotrexate, doxorubicin, and cisplatin, with some regimens incorporating ifosfamide, seems to be common methods for treatment. Although we get advances in surgery and targeted therapy for the malignancy nowadays, most patients still have a higher recurrence rate as well as a lower survival rate. Therefore, it is indispensable to develop more effective and less toxic drugs for the treatment of osteosarcoma. In this study, we investigated the anticancer effect of Glaucoalyx A on osteosarcoma and illustrated the underlying mechanisms. Our results demonstrated that Glaucoalyx A induced apoptosis in osteosarcoma by inhibiting GLI1 via regulating PI3K/Akt signaling pathway in vitro and in vivo.

Apoptosis, a typical programmed cell death with distinct biochemical and genetic pathways in normal tissues,
Fig. 4 (See legend on next page.)
upregulating Bcl-2, has been implicated in the initiation of apoptosis in the end. In the present study, the apoptotic member of the Bcl-2 family, forms ion channels in the normal balance between cell survival and cell death in mammals. In tumors, apoptosis is caused by caspases through targeting cysteine aspartyl proteases. Bax, the pro-apoptotic member of the Bcl-2 family, forms ion channels directly causing mitochondria to release cytochrome c, which induces apoptosis in the end. In the present study, the apoptotic rates of HOS and MG-63 cells were both remarkably increased by Glaucocalyxin A, suggesting that Glaucocalyxin A induced apoptosis in osteosarcoma. We also found that Glaucocalyxin A induced mitochondrial apoptosis by increasing Bax/Bcl-2 ratio, loss of mitochondrial membrane potential (ΔΨm), triggering ROS generation, and inducing caspase-9 and caspase-3 cleavage in HOS and MG-63 cells. These results demonstrated that Glaucocalyxin A induced mitochondrial apoptosis in osteosarcoma.

The transcription factor GLI is critically important in the Hedgehog signaling pathway. GLI is overexpressed and activated in a variety of cancers, regulating lots of cellular processes, including apoptosis and proliferation. There is a study reported that GLI is highly expressed in lung cancer tissue, but not in normal lung tissue via in situ hybridization. Inhibiting GLI can induce apoptosis in cervical cancer stem cells by modulating the transcription of the target genes. The report has documented that GLI also is a therapeutic option for B-cell chronic lymphocytic leukemia. Moreover, GLI inhibitor has a pro-apoptotic effect on myeloid leukemia cells and hepatocellular carcinoma cells by changing the morphogenesis of apoptosis and activating caspase-3. The activation of GLI1, via gene amplification including upregulating Bcl-2, has been implicated in the initiation and progression of multiple cancers. Bcl-2, an anti-apoptosis oncogene, is reported as a known transcriptional target of GLI, and can be regulated by GLI activation in cancer. The recent study showed that SUFU-mediated suppression of GLI activity was controlled by a BH3 sequence-dependent interaction between SUFU and three prosurvival Bcl-2 family members such as Bcl-2 and Bcl-xL. In other models, GANT61 inhibition of GLI transcription has been shown to inhibit several cell activities including Bcl-2. Moreover, it has been reported that GLI1 maintained cell survival by binding the promoter regions and facilitating transcription of Bcl-2 genes. Cyclopamine blocked the growth of colorectal cancer SW116 cells by modulating target Bcl-2 family genes of GLI1 including Bcl-2 and Bax in vitro. Therefore, targeting GLI activation may be an attractive strategy for the cancer treatment. It is also reported that GLI signaling is active and regulates the target genes in osteosarcoma cells. Inhibition of GLI is capable to prevent the progression of osteosarcoma. In our study, we demonstrated that Glaucocalyxin A inhibited the nuclear translocation of GLI in osteosarcoma cells HOS and MG-63.

The activation of PI3K/Akt pathway can promote the development of various human cancers such as breast cancer, lung cancer, melanoma, and lymphoma. PI3K/Akt pathway plays a crucial role in multiple processes of cancer, such as apoptosis, proliferation, metastasis, by modulating many downstream transcription factors. It has been demonstrated that inhibiting PI3K/Akt pathway can induce dramatic apoptosis of osteosarcoma. Increasing evidence has suggested that GLI protein can be modulated directly and indirectly by proliferative and oncogenic inputs, in addition or independent of upstream Hedgehog signaling. There is a recent study reporting the regulatory effect of PI3K/Akt pathway on GLI
Fig. 5 (See legend on next page.)
Table 1  Effects of Glaucocalyxin A on hematology indices in nude mice

| Hematological parameters                  | Control          | Glaucocalyxin A 20 mg/kg | Glaucocalyxin A 40 mg/kg | Glaucocalyxin A 80 mg/kg | Standard |
|------------------------------------------|------------------|--------------------------|--------------------------|--------------------------|----------|
| White blood cells (×10³/ml)              | 7.11 ± 0.81      | 5.80 ± 0.65              | 6.94 ± 0.50              | 6.79 ± 0.98              | 4.5–9    |
| Red blood cells (×10⁶/blood cell)        | 8.71 ± 0.43      | 8.79 ± 0.43              | 8.96 ± 0.35              | 9.04 ± 0.17              | 7.5–9.66 |
| Hemoglobin (g/dl)                        | 14.42 ± 0.59     | 14.02 ± 0.60             | 14.08 ± 0.43             | 13.87 ± 0.30             | 12.8–16.1|
| Hematocrit (%)                           | 43.02 ± 2.41     | 43.83 ± 3.24             | 44.63 ± 2.40             | 44.25 ± 0.71             | 34–50    |
| Mean corpuscular volume (fl)             | 44.98 ± 1.04     | 46.40 ± 3.38             | 45.77 ± 2.82             | 44.90 ± 1.40             | 41–60    |
| Mean corpuscular hemoglobin (pg)         | 14.43 ± 0.27     | 14.98 ± 0.36             | 14.60 ± 0.43             | 14.92 ± 0.39             | 13–19    |
| Lymphocytes (%)                          | 56.78 ± 2.38     | 62.00 ± 3.43             | 56.00 ± 1.97             | 69.85 ± 6.04             | 49–82    |
| Monocytes (%)                            | 3.58 ± 0.32      | 3.13 ± 0.83              | 2.52 ± 0.32              | 4.12 ± 0.54              | 2–8      |
| Eosinophils (%)                          | 2.17 ± 0.43      | 2.03 ± 0.26              | 2.57 ± 0.25              | 2.30 ± 0.30              | 0–3      |
| Basophils (%)                            | 0.52 ± 0.18      | 0.57 ± 0.15              | 0.62 ± 0.29              | 0.55 ± 0.13              | 0–3      |
| Platelet                                 | 618±17 ± 138.49  | 673.67 ± 62.80           | 381.33 ± 52.70           | 259.50 ± 48.83           | 115–1037 |

Each data point represents the mean ± SD of six mice.
P< 0.05 versus basal levels of the control group.

activation, demonstrating that GL11 and GL12 were activated by PI3K/Akt pathway. PI3K-Akt cascade is reported to maintain the stabilization of GL11 protein as well. Thus, our significant attention was focused on the nonclassical GL11 activation regulated by PI3K/Akt pathway. Our results showed that Glaucocalyxin A reduced the protein expression of PI3K and phosphorylated-Akt in HOS and MG-63 cells after treatment with Glaucocalyxin A. The inhibitory effect of Glaucocalyxin A on the activation of GL1 was confirmed by PI3K activator (IGF-1) and a PI3K inhibitor (LY294002). Furthermore, Glaucocalyxin A-induced apoptosis was reversed by PI3K activator (IGF-1). The effect of Glaucocalyxin A on the expression of apoptosis-related proteins was also withdrawn by IGF-1. These results demonstrated that Glaucocalyxin A induced apoptosis by inhibiting nuclear translocation of GLI through modulating PI3K/Akt signaling pathway.

The in vitro study showed that Glaucocalyxin A induced mitochondrial apoptosis pathway with increasing Bax/Bcl-2 ratio, reducing MMP, activation of caspase-9 and caspase-3. The results also indicated that apoptosis induced by Glaucocalyxin A in osteosarcoma cells was not cell specific. Moreover, our study first demonstrated that Glaucocalyxin A possessed the pro-apoptotic effect by inhibiting GLI via regulating PI3K/Akt pathway in osteosarcoma. Our further study documented that Glaucocalyxin A exerted the antitumor effect in vivo through
inducing apoptosis in tumors. The standard toxicology studies suggested that Glaucocalyxin A may exhibit anticancer effect without obvious toxicity in vivo.

In conclusion, as a natural compound from Chinese herb medicine, Glaucocalyxin A induced apoptosis and inhibited tumor growth by inhibiting nuclear translocation of GLI1 via regulating PI3K/Akt signaling pathway in osteosarcoma cells and in xenograft tumor model. Therefore, our present study suggested that glaucocalyxin A might be a promising agent against human osteosarcoma for its good anticancer efficiency and high safety.

**Materials and methods**

**Materials**

Glaucocalyxin A is a white amorphous powder with purity of more than 98%, the molecular formula is C20,H28,O4 with a molecular weight of 332.437 Da, which was purchased from Cheng Du Purechem-Standard Co., Ltd (Sichuan Province, China). Glaucocalyxin A was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) and DMSO-treated cells were used as a vehicle control. Penicillin, streptomycin, minimum essential medium and McCoy’s 5A (Modified) medium were obtained from Thermo Fisher Scientific (USA). Fetal bovine serum (FBS) was provided by Gibco Life Technologies (New York, USA). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide] obtained from Sigma Aldrich (USA). MTT solution (5 mg/ml in PBS) was added and incubated for 4 h. Supernatants were removed and 100 μl DMSO was added at about 25 °C to dissolve formazan crystals. The optical absorbance was recorded at 492 nm by a Thermo Multiskan Mk3 Microplate Reader. The cell growth inhibitory effects were calculated by the following equation: cell viability (%) = (Atreatment/Acontrol) × 100%. A cell growth inhibition curve was generated by plotting cell growth inhibition against drug concentration, and the half-maximal inhibitory concentration (IC50) was determined using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

**CCK8 assay**

The CCK-8 detection kit (Beyotime Institute of Biotechnology, Nantong, China) was used to measure cell viability according to the manufacturer’s protocol. Cells were seeded onto 96-well plates (5×103 cells per well). After 24 h, cells were treated with different concentrations of Glaucocalyxin A. The cells were cultured respectively for 24 and 48 h. Subsequently, CCK-8 solution was added to each well, and incubated at 37 °C for an additional 3 h. The viable cells were counted by absorbance measurements with a monochromator microplate reader at a wavelength of 450 nm. The optical density value was reported as the percentage of cell viability in relation to the control group (set as 100%).

**Annexin V-FITC/ propidium iodide staining**

The apoptosis was analyzed using Annexin V-FITC/ propidium iodide (PI) dual staining. HOS and MG-63 osteosarcoma cells were harvested after treatment with Glaucocalyxin A at concentrations of 2.5, 5, and 10 μM for 24 h and stained with Annexin V-FITC/PI Cell Apoptosis Detection Kit (KeyGen Biotech, Nanjing, China) according to the manufacturer’s protocol. The apoptosis rates of the cells were then analyzed by a flow cytometer (BD Biosciences, San Jose, CA, USA).

**Cell morphological assessment**

HOS and MG-63 cells were plated onto six-well plates and treated with 2.5, 5, and 10 μM Glaucocalyxin A for 24 h. The cultured cells were observed by the inverted light microscope (Nikon, Chiyodaku, Tokyo, Japan). Cell nucleus was visualized after DNA staining with the fluorescent dye 4′-6-Diamidino-2-phenylindole (DAPI)
Cells were incubated in the dark for 10 min and washed with PBS twice. The nuclear morphology was observed using fluorescence microscope (Nikon, Chiyodaku, Tokyo, Japan).

Western blot analysis
Osteosarcoma cells were treated with 2.5, 5, and 10 μM Glucocalyxin A for 24 h, and lysed in RIPA Lysis Buffer (Beyotime Institute of Biotechnology, China). The lysates were then centrifuged at 12,000 rpm for 15 min at 4 °C. The concentrations of the total proteins were determined using the BCA assay by Varioskan spectrofluorometer (Beyotime Institute of Biotechnology, China). The protein was separated with 12% SDS-PAGE gel, transferred onto the PVDF membranes (Millipore, Billerica, MA) and then incubated with specific antibodies overnight at 4 °C followed by incubation with secondary antibodies (Cell Signaling Technology, USA) for 60 min at room temperature. The protein bands were detected using Bioshine ChemiQ series 4800 Mini System (Bioshine, Shanghai, China).

Quantitative real-time PCR analysis
Total RNA was isolated using the TriPure solution (Takara Bio, Inc., Otsu, Shiga, Japan) after glucocalyxin A treatment, and then cDNA templates were generated by reverse transcription reaction using Primerscript reverse transcriptase (Takara Bio, Inc.) according to the manufacturer’s instructions. Then, the cDNAs were used as templates for determining the expression of related genes by quantitative real-time PCR. Each assay was done in triplicate.

Measurement of reactive oxygen species and superoxide anions (O2−) level
The generation of intracellular ROS and superoxide anions (O2−) level were detected using fluorescent dye 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) (KeyGen Biotechnology, China), DHE (S0063; Beyotime Institute of Biotechnology, Nantong, China), respectively, according to the manufacturer’s protocols. The samples were pretreated with or without 5 mM NAC (S0077; Beyotime Institute of Biotechnology, Nantong, China) for 2 h before cells were treated with 2.5, 5, and 10 μM of Glaucocalyxin A for 24 h. Cells were collected and incubated with the corresponding dye in serum-free medium in 5% CO2 at 37 °C for 20 min. After washing by serum-free medium twice, the fluorescence intensity was measured by FACSCalibur flow cytometry (Becton–Dickinson) at Ex./Em. −488/525 nm.

Hydrogen peroxide (H2O2) assay
The content of H2O2 in treated cells was analyzed with a hydrogen peroxide assay kit (S0038; Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer’s instructions. In brief, cells were harvested, lysed, and centrifuged at 12,000 × g for 5 min. Then, test tubes containing 50 μl of supernatants and 100 μl of test solution were placed at room temperature for 30 min, and were measured immediately at a wavelength of 560 nm.

Measurement of mitochondrial membrane potential
Quantitative changes of MMP were determined by flow cytometry using 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) Apoptosis Detection Kit (Beyotime Institute of Biotechnology, China). Briefly, after harvesting glucocalyxin A-treated cells, they were incubated with JC-1 for 20 min at 37 °C. Then the cells were washed with cold buffer, resuspended and analyzed by flow cytometry (FACSCalibur, Becton-Dickinson).

Immunofluorescence
HOS and MG-63 cells were treated with Glucocalyxin A (10 μM) for 24 h and then harvested. The cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton* X-100. Then cells were incubated with primary antibody at 37 °C for 1 h and overnight at 4 °C, and added the secondary antibody (Cell Signaling Technology, USA) at 25 °C for 1 h. The cells were washed with PBS, incubated with DAPI staining solution for 5 min. After washing with PBS, samples were observed with a confocal laser scanning microscope (Fluoview FV1000, Olympus, Tokyo, Japan).

Transfection of GLI1 plasmid, GLI1 siRNA, and PI3K siRNA
HOS and MG-63 cells were plated in six-well plates with fresh medium. GLI1-plasmid, GLI1 siRNA, and PI3K siRNA transfections were performed according to the manufacturer’s instructions of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After that, cells were exposed to Glucocalyxin A and harvested for further experiments.

Antitumor effects in nude mice
Male BALB/c nude mice (35–40-day-old), weighing 18–22 g, were purchased from the Comparative Medicine Centre of Yangzhou University. The animal study was carried out according to National Institutes of Health regulations and approved by the Institutional Animal Care and Use Committee. The mice were maintained in a pathogen-free environment (21 ± 2 °C and 45 ± 10% humidity) on a 12 h light and 12 h dark cycle with food and water supplied freely during the entire experiment. On day 1, 5×106 HOS cells suspended in 100 μl PBS were subcutaneously inoculated in the right flank of each nude mice. After 10–12 days, when tumor sizes reached around 80–150 mm³, nude mice with similar tumor volume were randomly assigned to four groups (with six...
nude mice/group). Glucosalyxin A (20, 40, 80 mg/kg) groups received intraperitoneal injection of 20, 40, 80 mg/kg/2 days respectively. The control group was administered saline. Tumor volume (TV) was measured daily to observe dynamic changes in tumor growth and calculated according to the formula: $TV (\text{mm}^3) = 0.5 \times d^2 \times D$, where $d$ and $D$ are the shortest and the longest diameters, respectively. At the end of 21 days, all nude mice were sacrificed, and the tumor tissues were removed and measured. The major organs of the mice were removed for the toxicity assessment.

**TUNEL assay**

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to analyze the apoptosis induction in the tumor tissues. It was carried out on xenograft murine model treated as previously described using an in situ cell death detection kit following the manufacturer’s protocol. The slides were photographed under an Olympus FV1000 confocal microscope.

**Immunohistochemistry**

The protein expression of Bax, Bcl-2, p-Akt, GLI1, PI3K of the tumor tissues was assessed as described in the previous study.

**Statistical analysis**

All data were shown as mean ± standard deviation (SD) from at least three independent experiments, each in triplicate samples for individual treatment or dosage. Statistical analyses were performed using one-way ANOVA analysis of variance with Dunnett’s test. All comparisons are made relative to untreated controls and significance of difference is indicated as *$P < 0.05$ and **$P < 0.01$.

**Acknowledgements**

This work was supported by the National Natural Science Foundation of China (No. 81373478 and No. 81703556), the Natural Science Foundation of Jiangsu Province (No. BK20171024), the Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture, Key University Science Research Project of Jiangsu Province (15KJA430006), QingLan Project and Program for Innovative Research Team in University of Jiangsu Province. We would like to add the No. of the fund as the Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture (No. XTD1819).

**Authors’ contributions**

Y.S., Q.Z., and X.D. conceived and designed the experiments; J.W.Z. performed the experiments; interpreted the data and prepared the figures; Y.S. wrote the manuscript; Y.L. and X.J. analyzed the data and wrote the manuscript; J.W.Z., X.J., Y.L., I.Y. helped perform the experiments; B.M. and J.Z. helped in writing the manuscript. All authors read and approved the final manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.
24. Yang, L., Xie, G., Fan, Q. & Xie, J. Activation of the hedgehog-signaling pathway in human cancer and the clinical implications. Oncogene 29, 469–481 (2010).

25. Scales, S. J. & de Sauvage, F. J. Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. Trends Pharmacol. Sci. 30, 303–312 (2009).

26. Chan, L. H. et al. Hedgehog signaling induces osteosarcoma development through Yap1 and H19 overexpression. Oncogene 33, 4857–4866 (2014).

27. Amakye, D., Jagani, Z. & Dorsch, M. Unraveling the therapeutic potential of the Hedgehog pathway in cancer. Nat. Med. 19, 1410–1422 (2013).

28. Lin, Z. X. et al. Suppression of GLI sensitizes medulloblastoma cells to mitochondria-mediated apoptosis. J. Cancer Res. Clin. 142, 2469–2478 (2016).

29. Zhou, J. C. et al. Non-canonical GLI1/2 activation by PI3K/AKT signaling in renal cell carcinoma: a novel potential therapeutic target. Cancer Lett. 370, 313–323 (2016).

30. Kebenko, M. et al. EbrB2 signaling activates the Hedgehog pathway via PI3K-Akt in human esophageal adenocarcinoma. Identification of novel targets for concerted therapy concepts. Cell Signal. 27, 373–381 (2015).

31. Gao, L. W., Zhang, J. A., Yang, W. H., Wang, B. & Wang, J. W. Glaucocalyx A induces apoptosis in human leukemia HL-60 cells through mitochondria-mediated death pathway. Toxicol. Vitr. 25, 51–63 (2011).

32. Xiao, X. et al. Glaucocalyx A, a negative Akt regulator, specifically induces apoptosis in human brain glioblastoma U87MG cells. Acta Biochim. Biophys. Sin. 45, 946–952 (2013).

33. Li, M., Jiang, X. G., Gu, Z. L. & Zhang, Z. B. Glaucocalyx A activates FasL and induces apoptosis through activation of the JNK pathway in human breast cancer cells. Asian Pac. J. Cancer Prev. 14, 5805–5810 (2013).

34. Jo, S. et al. Myrctin induces apoptosis of human anaplastic thyroid cancer cells via mitochondria dysfunction. Anticancer Res. 37, 1705–1710 (2016).

35. Liu, H. et al. Gilbendamidine, a diabetic drug, prevents acute radiation-induced liver injury of mice via up-regulating intracellular ROS and subsequently activating Akt-NF-kappa B pathway. Oncotarget 8, 40568–40582 (2017).

36. Forman, H. J. et al. Even free radicals should follow some rules: a guide to free radical research terminology and methodology. Free Radic. Bio. Med. 78, 233–235 (2015).

37. Lo, W. H. et al. Involvement and targeted intervention of dysregulated Hedgehog signaling in osteosarcoma. Cancer-Am. Cancer Soc. 120, 537–547 (2014).

38. Scheibler, R. D., Old, L. J. & Smyth, M. J. Cancer immuneediting: integrating immune's roles in cancer suppression and promotion. Science 331, 1565–1570 (2011).

39. Shao, X. J. et al. The down-regulation of microRNA-497 contributes to cell growth and cisplatin resistance through PI3K/Akt pathway in osteosarcoma. Cell Physiol. Biochem. Int J. Exp. Cell Physiol. Biochem. Pharmacol. 36, 2051–2062 (2015).

40. Kudawara, I. et al. Neoadjuvant and adjuvant chemotherapy with high-dose ifosfamide, doxorubicin, cisplatin and high-dose methotrexate in non-metastatic osteosarcoma of the extremities: a phase II trial in Japan. J. Chemother. 25, 41–48 (2011).

41. Hassan, M., Watari, H., AbulAlmaaty, A., Ohba, Y. & Sakurai, N. Apoptosis and molecular targeting therapy in cancer. Biomed. Res. Int. 2014, 150845 (2014).

42. Cotter, T. G. Apoptosis and cancer: the genesis of a research field. Nat. Rev. Cancer 9, 501–507 (2009).

43. Kerr, J. F., Wyllie, A. H. & Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer. 26, 239–257 (1972).

44. Cyrn, V. & Yuan, J. Proteases to die for. Genes Dev. 12, 1551–1570 (1998).

45. Tomberson, N. A. & Lazebnik, Y. Caspases: enemies within. Science 281, 1312–1316 (1998).

46. Antonsson, B. et al. Inhibition of Bax channel-forming activity by Bcl-2. Science 277, 370–372 (1997).

47. Indran, I. R., Tufo, G., Penaizs, S. & Brenner, C. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochim. Biophys. Acta 1807, 755–745 (2011).

48. Teiglind, S. & Toftgard, R. Hedgehog beyond medulloblastoma and basal cell carcinoma. Biochim. Biophys. Acta 1805, 181–208 (2010).

49. Rovida, E. & Stecca, B. Mitogen-activated protein kinases and Hedgehog-GLI signaling in cancer: a crosstalk providing therapeutic opportunities? Semin. Cancer Biol. 35, 154–167 (2015).

50. Chi, S. M. et al. Activation of the hedgehog pathway in a subset of lung cancers. Cancer Lett. 244, 53–60 (2006).

51. Nayak, A. et al. Nanoparticle-induced apoptotic death in cervical cancer stem cells through the inhibition of hedgehog-GLI1 cascade: role of GLI-1. Sci. Rep.-UK 6, 20600 (2016).

52. Desch, P. et al. Inhibition of GLI, but not Smoothened, induces apoptosis in chronic lymphocytic leukemia cells. Oncogene 29, 4885–4895 (2010).

53. Pan, D. et al. GlI inhibitor GANT61 causes apoptosis in myeloid leukemia cells and acts in synergy with cytarabine. Leuk. Res. 36, 742–748 (2012).

54. Chen, X. L. et al. GLI-1 siRNA induced apoptosis in HuH7 cells. World J. Gastroenterol. 14, 582–589 (2008).

55. Katoh Y, Katoh M. Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. Current molecular medicine 9, 873–886 (2009).

56. Bigelow RL, et al. Transcriptional regulation of bc-2 mediated by the sonic hedgehog signaling pathway through gli-1. The Journal of biological chemistry 279, 1197–1205 (2004).

57. Wu JY, et al. Cyclopamine blocked the growth of colorectal cancer SW116 cells by modulating some target genes of Gl1 in vitro. Hippo-gastroenterology 58, 1511–1518 (2011).

58. Wu, X. et al. Extra-mitochondrial prosurvival BCL-2 proteins regulate gene transcription by inhibiting the SUFU tumour suppressor. Nature cell biology 19, 1226–1236 (2017).

59. Gonnissen A, Isebaert S, Haustermans K. Targeting the Hedgehog signaling pathway in cancer beyond Smoothened. Oncotarget 6, 13899–13913 (2015).

60. Shahi, M. H., Holt, R. & Rehnb, R. B. Blocking signaling at the level of GLI regulates downstream gene expression and inhibits proliferation of canine osteosarcoma cells. PloS ONE 9, e96593 (2014).

61. Yu, H. G. et al. Phosphoinositide 3-kinase/Akt pathway plays an important role in chemoresistance of gastric cancer cells against etoposide and doxorubicin induced cell death. Int. J. Cancer 122, 433–443 (2008).

62. Xu, X., Wang, B. & Xu, Y. Expression of ftsuk oxidase in human osteosarcoma and its clinical significance: a tumor suppressive role of LOX in human osteosarcoma cells. Int. J. Oncol. 43, 1578–1586 (2013).

63. Tedesco, I. et al. Deacetylholated red wine induces autophagic and apoptotic cell death in an osteosarcoma cell line. Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. 60, 377–384 (2013).

64. Pandolfi, S., Stecca B. Cooperative integration between HEDGEHOG-GLI signaling and other oncogenic pathways: implications for cancer therapy. Exp. Rev. Mol. Med. 17, e5 (2015).

65. Katoh, Y. & Katoh M. Integrative genomic analyses onGLI1: positive regulation and acts in synergy with rapamycin. Leuk. Res. 36, 1410–1417 (2012).

66. Sun, Y. et al. Wogonoside protects against dextran sulfate sodium-induced experimental colitis in mice by inhibiting NF-kappa B and NLRP3 inflammasome activation. Biochem. Pharmacol. 94, 142–154 (2015).