The Natural Diterpenoid Isoforretin A Inhibits Thioredoxin-1 and Triggers Potent ROS-Mediated Antitumor Effects

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Abstract

Aberrant expression of thioredoxin 1 (Trx1) plays an important role in cancer initiation and progression and has gained attention as an anticancer drug target. Here we report that the recently discovered natural diterpenoid isoforretin A (IsoA) significantly inhibits Trx1 activity and mediates anticancer effects in multiple preclinical settings. The inhibitory effect of IsoA was antagonized by free radical scavengers polyethylene glycol-catalase, polyethylene glycol superoxide dismutase, thiol-based antioxidants N-acetylcysteine and glutathione. Mass spectrometry analysis revealed that the mechanism of action was based on direct conjugation of IsoA to the Cys32/Cys35 residues of Trx1. This conjugation event attenuated reversible thiol reduction of Trx1, leading to ROS accumulation and a broader degradation of thiol redox homeostasis in cancer cells. Extending these in vitro findings, we documented that IsoA administration inhibited the growth of HepG2 tumors in a murine xenograft model of hepatocellular carcinoma. Taken together, our findings highlight IsoA as a potent bioactive inhibitor of Trx1 and a candidate anticancer natural product.

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Introduction

Thioredoxin 1 (Trx1; approved symbol: TXN; synonyms: TRX) is a critical antioxidant protein and participates in a wide range of cellular processes, such as cell proliferation, apoptosis, and aging (1, 2). Trx1 contains the active site Cys-Gly-Pro-Cys, which reduces the target proteins by cysteine thiol-disulfide exchange (3). Upon oxidation, the two conserved cysteine residues in the active site form a disulfide bond, and the oxidized Trx (Trx-S-S) is then recycled to the reduced form Trx-(SH)2 through the action of thioredoxin reductase and NADPH (4).

Acumulating evidence has suggested that Trx1 is extensively involved in tumor initiation and progression. Trx1 is overexpressed in many malignant cell lines and in a wide variety of human tumors compared with normal corresponding tissues (5–12). Overexpression of Trx1 can promote tumor cell proliferation and help tumor cells evade apoptosis through binding to apoptosis signal regulating kinase-1 (ASK1; ref. 13) and the tumor suppressor PTEN (14). Besides, Trx1 stimulates sustained angio genesis in tumors by increasing hypoxia-inducible factor 1α level and enhancing VEGF function (15, 16). Moreover, overexpression of Trx1 promotes cancer metastasis, and clinically is correlated to poor prognosis (10–12, 17, 18). These actions evidently make Trx1 a promising target for cancer therapy.

So far, several Trx1-specific inhibitors have been identified, such as semisynthetic unsymmetrical 2-imidazolyl disulfides, which interact with Trx1 as its substrates (3, 19). Of note, 1-methylpropyl 2-imidazolyl disulfide (PX12) has been in clinical trials, but little success has been observed (20, 21). Currently, no natural compounds have shown specific inhibitory effect on Trx1 by binding to Trx1. Therefore, it is of great value to explore novel natural Trx1-specific inhibitors for effective cancer therapy.

Isoodon forrestii var. forrestii is a traditional Chinese medicinal herb of the Isoodon species distributed in the Southwest China and used for centuries in China. The Isoodon plants are rich in ent kaurane diterpenoids, which attract considerable interest for their well-known anticancer activities (22–27). IsoA is a novel ent kaurane constituent isolated from the leaves of I. forrestii var. forrestii. The anticancer activity of IsoA has not been fully characterized.

In this study, we report that IsoA inhibited the growth of cancer cells both in vitro and in vivo. Mechanically, IsoA suppressed Trx1 activity through covalently binding to the Cys32/Cys35 residues of the activation sites of Trx1 and triggered ROS accumulation, resulting in DNA damage and apoptosis in cancer cells. Our work suggested, for the first time, that IsoA, as a Trx1 inhibitor, is
selectively cytotoxic to cancer cells and has potential to be a novel agent for cancer therapy.

Materials and Methods

Reagents

IsoA was isolated from the leaves of *I. forrestii* var. *forrestii* according to Supplementary Methods. Its structure was characterized by one-dimensional nuclear magnetic resonance (NMR) spectrometer, 2D NMR analysis, and single-crystal X-ray diffraction experiment (Supplementary Materials). IsoA (99% or higher purity) was dissolved in dimethylsulfoxide as a 40 mmol/L stock solution and stored at −20°C. Primary antibodies against Trx1, γH2AX, 8-oxoguanine (8-oxoG), GAPDH, and nitrotyrosine were purchased from Santa Cruz Biotechnology; goat anti-mouse IgG and goat anti-rabbit IgG antibodies from LI-COR Biosciences; benzylxycarboxyl-Val-Ala-Asp fluoromethylketone (Z-VAL-FMK) from Selleck; 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) from Invitrogen; 2’,7’-dichlorofluorescein (DCF), NAC, GSH, PEG-SOD, PEG-Catalase, and other chemical reagents from Sigma-Aldrich.

Cell lines and cultures

Human fetal lung fibroblasts HFL1, hepatocellular carcinoma (HepG2, BEL7402, and QGY7701), breast cancer (MCF7 and MDA-MB-231), cervical carcinoma (HeLa), lung cancer (A549), and colon cancer (Caco2) cell lines were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology in June 2013. Human mammary epithelial cell line MCF 10A was kindly provided by Stem Cell Bank, Chinese Academy of Sciences in November 2015. Human osteosarcoma (U2OS), melanoma (A375), and normal hepatic (LO2) cell lines were purchased from Cell Bank of Kunming Institute of Zoology, Chinese Academy of Sciences in June 2013. All the cell lines were kept within 10 passages and preserved in liquid N2 after receipt. The used cells were resuscitated within 1 month. Cell lines were authenticated by the above cell banks through short tandem repeat (STR) analysis. HepG2, MCF7, MDA-MB-231, and Caco2 cells were cultured in MEM supplemented with 10% FBS. HeLa, A549, U2OS, A375, and LO2 cells were cultured in DMEM supplemented with 10% FBS. BEL7402 and QGY7701 cells were cultured in RPMI1640 containing 10% FBS. HFL1 cells were grown in F12K medium supplemented with 10% FBS. MCF 10A cells were cultured with MEGM Kit (Lonza/Clonetics, CC-3150) supplemented with cholera toxin (100 ng/mL) and 10% FBS.

Cell proliferation and apoptosis analysis

Cell proliferation and viability were determined by MTT assay and Trypan blue exclusion. Apoptotic cells were stained with Hoechst 33258, and also quantified using a FACScan laser flow cytometer (Guava easyCyte HT; Millipore) after staining with Annexin V and 7-AAD.

ROS determination

The level of cellular ROS was analyzed using DCFH-DA as a fluorescent probe. Cells were incubated with 10 μmol/L DCFH-DA for 30 minutes at 37°C in a 5% CO2 humidified environment. The labeled cells were washed with PBS for three times. To quantify ROS, cells were harvested and the DCFH-DA fluorescence was measured using a FACScan laser flow cytometer (Guava easyCyte HT; Millipore).

Assessment of GSH levels

The intracellular levels of GSH were measured using GSH and GSSG Assay Kit (Beyotime). According to the manufacturer’s instructions, the IsoA-treated cells were collected and homogenized. The protein concentrations were quantified using the BCA method. The total GSH levels were carefully measured by the enzymatic recycling method using glutathione reductase and 5’, 5’-dithio-bis (2-nitrobenzoic acid). The sulfhydryl group of GSH reacts with DTNB and produces a yellow-colored 5-thio-2-nitrobenzoic acid, which has an absorbance at 405 to 414 nm. Oxidized glutathione (GSSG) levels were accomplished firstly by first derivatizing GSH with 2-vinylpyridine. The concentrations of reduced GSH were calculated by subtracting the GSSG levels from the total GSH (GSH = total GSH − 2 × GSSG). The intracellular levels of GSH were determined on the basis of cellular protein concentrations.

Western blot analysis

For Western blot analysis, cells were lysed with RIPA to extract total proteins. Equal amounts of proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore). Membranes were blocked with 5% nonfat milk at room temperature, and probed with primary antibodies at 4°C overnight, followed by IRDye-conjugated secondary antibodies. The membranes were scanned with an Odyssey infrared fluorescent scanner (LI-COR Biosciences).

8-Oxoguanine quantification by flow cytometry

The IsoA-treated cells were fixed in 1% paraformaldehyde and permeabilized by 0.5% Triton X-100 dissolved in PBS, followed by incubating with a FITC-conjugated 8-oxoguanine (8-oxoG) antibody at room temperature. Then, the cells were stained with propidium iodide to determine DNA content. Fluorescence was analyzed using a FACScan laser flow cytometer (Guava easyCyte HT; Millipore).

Trx1 activity analysis

For in vivo Trx1 activity, cells or tissues were lysed in the lysis buffer [20 mmol/L HEPES (pH 7.9), 300 mmol/L NaCl, 100 mmol/L KCl, 10 mmol/L EDTA, and 0.1% Nonidet P-40] containing protease and phosphatase inhibitors cocktail (Pierce). The activity of Trx1 was measured according to the method described previously (28).

For in vitro Trx1 activity, this assay was performed using a modified Thioredoxin Activity Fluorescent Assay Kit (Camyen Chemical Company). The samples containing 0.02 μmol/L hTrx1 (the final concentration in the assay) were prepared according to the manufacturer’s instructions. Next, 2 μL of IsoA at various concentrations (0.195–100 μmol/L) was added to each sample, followed by addition of 5 μL of β-NADPH to all samples and incubation at 37°C for 30 minutes. Finally, the fluorescent substrate was added, and the Trx1 activity was recorded as the emission at 518 nm after 488 nm excitation for 30 to 60 minutes, with a Thermo Scientific Varioskan Flash fluorescent microplate reader.

Determination of glutaredoxin activity in vitro

The in vitro Grx activity assay was performed using a modified Fluorescent Glutaredoxin Assay Kit (Camyen Chemical Company). The samples containing 1.5 mmol/L of hGrx-1 (the final...
concentration in the assay) were prepared according to the manufacturer’s instructions. Next, 2 μL of IsoA at various concentrations (0.195–100 μmol/L) was added to each sample, followed by adding 10 μL of fluorescent substrate to each well, and recording the emission at 545 nm after excitation at 520 nm for 15 to 30 minutes with a Thermo Scientific Varioskan Flash fluorescent microplate reader.

**Covalent docking**

The crystal structure of human Trx1 was obtained from the protein data bank (PDB ID: 4PUF_C). The protein structure was prepared with Protein Preparation Tool (ProPrep) in the Schrödinger 2014-4 suit software. Hydrogen atoms were added at pH 7.0 by the PROPKA tool in Maestro with optimized hydrogen-bond network. OPLS_2005 force field was used for restrained minimization with converge heavy atoms to 0.30 Å. The ligand IsoA was sketched in Maestro and subjected to Ligand Preparation Tool (LigPrep) using OPLS_2005 at pH 7.0 to generate low-energy conformation. Covalent docking was performed using Glide Docking module. The reactive residues Cys32 and Cys35 were supposed to form a covalent bond with the ketene of IsoA through Michael addition reaction.

**MS/MS analysis**

A stock solution (5 mg/mL) of human Trx1 protein (Sino Biological Inc.) was prepared in HPLC-grade water. Next, 4 μL of IsoA (40 μmol/L stock solution) was incubated with 140 μL of human Trx1 stock solution in 25 mmol/L Tris-HCl buffer solution (pH 7.4) for 2 hours at room temperature. In this reaction, the final concentrations of IsoA and Trx1 were about 80 μmol/L and 291 μmol/L, respectively. The samples were reduced according to the methods of Shimadzu Biotech Proteome Kit, trypsin digested by MonoSpin Trypsin, and desalted using a MonoSpin C18 column, before being analyzed using MALDI 7090 (SHIMADZU).

**RNA interference**

Trx1-specific siRNA and scramble siRNA duplexes were transfected to HepG2 cells using Lipofectamine 2000 (Invitrogen). The siRNA sequences for Trx1 were SiTrx1-a# 5'-CUCCAGGU-GAUAAACUCUUGUTT-3', SiTrx1-b# 5'-GAUCAAGCCUGUIURICUUCU-CAUUG-3'. The scramble siRNA (negative control, NC) sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. The knockdown efficiency was assessed by Western blotting, and the Trx1 activity was determined by insulin reduction assay. Cell viability of Trx1 knockdown cells after treating with IsoA was measured by MTT assay.

**Murine models**

All animal experiments were conducted under protocols approved by the Animal Care and Use Committee of Jiangsu Province Academy of Traditional Chinese Medicine. Nude BALB/c mice (13 ± 2 g) were raised in air-conditioned pathogen-free environment. HepG2 cells (2 × 10^6) were injected subcutaneously into the right flank of nude mice. When tumors became palpable, the mice were randomized into two groups (n = 6, per group), and treated with IsoA (15 mg/kg) or vehicle control for 14 days. The body weight and tumor size were measured every 3 days. Tumor volumes were calculated according to following formula: \( V = \frac{4}{3} \pi a^2 b \), where a is the smallest diameter and b is the diameter perpendicular to a. At the end of the experiments, all animals were sacrificed and the tumors were excised, weighed, snap-frozen in liquid nitrogen, and stored at −80°C, or fixed in 4% paraformaldehyde for further analysis.

To explore the toxicity of IsoA to main organs, nude BALB/c mice (13 ± 2 g) were treated with IsoA (15 mg/kg) or vehicle control for 14 days (n = 5, per group). At the end of the experiments, all animals were anesthetized using isoflurane (3%) inhalation, and blood samples were collected from the retro-orbital plexus. Complete blood counts were done on Auto Hematology Analyzer BC-5380 (Mindray) and plasma biochemical indices were analyzed on Cobas C311 (Roche Diagnostics).

**Immunohistochemical staining and in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay**

The immunostaining assay was performed on tumor specimens that were fixed in 4% paraformaldehyde and embedded with
Tissue sections were deparaffinized and rehydrated, followed by antigen retrieval and endogenous peroxidase blocking. Primary antibodies against 8-oxoG and nitrotyrosine were added and incubated overnight at room temperature. Immuno-signals were detected by the two-stage peroxidase-based EnVision (Dako) method. Apoptosis index in the tumor samples was assessed by in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis using the One Step TUNEL Apoptosis Assay Kit (Beyotime).

Statistical analysis
The difference between two different treatments was assessed by unpaired Student t test using PRISM software. ANOVA was used to compare multiple treatment groups and the nontreatment group. The intensity of the immune-reactive bands in Western blots was quantified by ImageJ software (NIH, Bethesda, MD). P < 0.05 was considered as statistically significant.

Results
IsoA preferably induced apoptosis in cancer cells
IsoA was obtained as colorless crystal needles. On the basis of meticulous one-dimensional (1D) NMR, 2D NMR analyses, and single-crystal X-ray diffraction using anomalous scattering of CuKα radiation data (CCDC 1063025), the absolute configuration of IsoA was assigned and described according to the following nomenclature: (2S, 3R, 5S, 6S, 8S, 9S, 10R, 11R, 12R, 13R)-6-hydroxy-2,3,11,12-tetraacethoxy-ent-kaur-16(17)-en-15-one (Fig. 1A). The detailed structural information of IsoA was illustrated in the Supplementary Information (Supplementary Figs. S1–S7; Supplementary Table S1).

To investigate the antitumor potential of IsoA, 10 human cancer cell lines, including hepatic, breast, lung, colon, and cervical carcinoma, osteosarcoma, melanoma cells, along with nontumor human hepatic cell line LO2, fetal lung fibroblasts HFL1, and mammary epithelial cell line MCF 10A were tested for...
the growth-inhibitory effect of ISOA. Compared with the nonmalignant cells, ISOA displayed a preferential antiproliferative activity against cancer cells, with IC_{50} values of <30 μmol/L (Fig. 1B; Supplementary Table S2). By clonogenic assay, we further found that ISOA drastically inhibited the colony formation of all tested cancer cells at 10 μmol/L (Supplementary Fig. S8).

Of all the tested cancer cell lines, HepG2 cells were the most sensitive to ISOA, with an IC_{50} value of 15.83 ± 1.10 μmol/L. Thus, this cancer cell line was chosen as a model to further investigate the anticancer activity and the underlying mechanisms of ISOA. MTT assay demonstrated that ISOA reduced the viability of HepG2 cells in a concentration-dependent manner, but did not obviously alter the viability of LO2 cells even at 40 μmol/L (Fig. 1C). This was further verified by Trypan blue exclusion assay (Fig. 1D). Hoechst staining revealed that ISOA was able to induce chromatin condensation and fragmentation (Fig. 1E), suggesting induction of apoptosis. Flow cytometry analysis further showed that the pan-caspase inhibitor Z-VAD-FMK significantly attenuated ISOA-induced apoptosis (Fig. 1F). These data indicated that the ISOA-induced apoptosis was at least partially mediated by the activation of caspase cascade.

### ISOA-induced oxidative stress in cancer cells

Induction of ROS has been reported to preferably induce cancer cell apoptosis (29, 30). Next, we therefore investigated the effects of ISOA on the induction of ROS using a fluorescent probe DCFH-DA. As shown in Fig. 2A and B, the induction of ROS was detected in HepG2 cells after exposure to ISOA. Similar results were obtained from another hepatocellular carcinoma cell line BEL7402 (Fig. 2C and D). To rule out the changes in ester cleavage, uptake, or efflux of DCFH-DA, we further detected ISOA-induced ROS in HepG2 cells using the oxidation insensitive analogue DCF as a control. ISOA treatment caused a remarkable shift in the fluorescence signal in cells loaded with DCFH-DA, but not with DCF (Supplementary Fig. S9). These results suggested that induction of ROS might play a critical role in inhibiting cancer cell growth.

Because GSH is an important antioxidant in cells to defend oxidant damage and regulate redox homeostasis (31), we next examined the association between intracellular GSH levels and the ISOA-induced ROS elevation. As shown in Fig. 2E, ISOA treatment of HepG2 cells resulted in a swift depletion of GSH (Fig. 2F). Using a fluorescent antibody that recognizes 8-oxoG, we characterized and quantified DNA damage by flow cytometry and quantified the DNA damage by flow cytometry and quantified (Fig. 2G). As shown in Fig. 2H and I, ISOA-induced ROS elevation caused a remarkable shift in the fluorescence signal in cells loaded with DCFH-DA, but not with DCF (Supplementary Fig. S9). These results suggested that induction of ROS might play a critical role in inhibiting cancer cell growth.

Because GSH is an important antioxidant in cells to defend oxidant damage and regulate redox homeostasis (31), we next examined the association between intracellular GSH levels and the ISOA-induced ROS elevation. As shown in Fig. 2E, ISOA treatment of HepG2 cells resulted in a swift depletion of GSH compared with that of the control cells. Furthermore, treatment of HepG2 cells with ISOA for 24 hours also led to a dramatic depletion of reduced GSH (Fig. 2F). The main cytotoxicity of excessive ROS is through DNA damage by base oxidation and double-strand breaks (DSB). Using a fluorescent antibody that recognizes 8-oxoG, we characterized and quantified DNA damage by flow cytometry. As shown in Fig. 2C, a significant increase of 8-oxoG was detected in ISOA-treated HepG2 cells. To examine whether the DNA damage involves DSBs, we performed Western blot analysis for the phosphorylated form of H2AX (γH2AX), a specific marker for DSBs (32). As shown in Fig. 2H and I, ISOA induced a dose- and time-dependent expression of γH2AX in HepG2 cells. Taken together, our results suggested that ROS accumulation might be a general mechanism of ISOA in inhibiting cancer cell growth, and ISOA triggered both DSBs and oxidative DNA lesions in cancer cells.

**Figure 3.** ROS scavengers attenuate ISOA’s activity in HepG2 cells. A-D, HepG2 cells were pretreated with NAC (A), GSH (B), PEG-Catalase (C), and PEG-SOD (D) at indicated concentrations for 30 minutes, and then incubated with indicated concentrations of ISOA for 24 hours. The cell viability was determined by MTT assay. E, HepG2 cells were pretreated with 2 μmol/L of NAC for 30 minutes and then incubated with 15 μmol/L of ISOA for 16 hours, followed by apoptosis assay using Annexin V staining and flow cytometry. F, Quantification of Annexin V staining results. G, HepG2 cells were pretreated with 2 μmol/L of NAC for 30 minutes and then incubated with 15 μmol/L of ISOA for 1 hour. ROS levels were measured by flow cytometry and quantified. H, HepG2 cells were pretreated with 1,000 U of PEG-Catalase for 30 minutes, followed by incubating with 15 μmol/L of ISOA for 16 hours, and cell apoptosis was analyzed by flow cytometry and quantified. I, HepG2 cells were pretreated with 1,000 U of PEG-Catalase for 30 minutes, followed by incubating with 15 μmol/L of ISOA for 1 hour, and ROS levels were analyzed by flow cytometry and quantified.
Antioxidant alleviated IsoA-induced ROS accumulation and apoptosis

To further understand the contribution of ROS to IsoA-induced cell growth inhibition and apoptosis, we investigated whether the thiol-based antioxidant agents NAC or GSH could antagonize IsoA. For this, HepG2 cells were pretreated with NAC or GSH for 30 minutes, and then exposed to IsoA (0–30 μmol/L) for 24 hours, followed by MTT assay. NAC (2 mmol/L) or GSH (2 mmol/L) markedly attenuated the inhibitory effect of IsoA on cell viability (Fig. 3A and B). To further clarify whether ROS or some other oxidants were induced, we evaluated the effect of the powerful free radical scavenger PEG-Catalase or PEG-SOD on IsoA-induced cytotoxicity. The results showed that PEG-Catalase or PEG-SOD also antagonized IsoA-induced cytotoxicity in HepG2 cells (Fig. 3C and D). Our additional experiments further demonstrated that NAC and PEG-Catalase abrogated the IsoA-induced apoptosis and intracellular accumulation of ROS in HepG2 cells (Fig. 3E–H). Collectively, our results demonstrated that the IsoA-induced apoptosis was primarily induced by oxidative stress.

Mitochondria are known to be the major place of intracellular ROS generation during electron transport flow. To determine whether IsoA induction of ROS was through disrupting the oxidative metabolism of mitochondria, we generated A549 Rho0 cells, which lacked mitochondrial function due to depleting mitochondrial DNA. IsoA treatment induced similar levels of ROS in both normal A549 and A549 Rho0 cells (Supplementary Fig. S10A). In line with this, IsoA treatment also resulted in similar reduction of cell viability in both A549 and A549 Rho0 cells (Supplementary Fig. S10B). These results suggested that mitochondria should not be the main site of IsoA-induced ROS formation.

IsoA inhibited Trx1 activity

Chemical structure analysis revealed that IsoA contained an α, β-unsaturated carbonyl group, which can readily interact with thiols to form covalent adducts. We first evaluated whether IsoA could inhibit the activity of thioredoxin 1 (Trx1), a major intracellular thiol that is responsible for reducing protein disulfide bonds. As shown in Fig. 4A, IsoA inhibited Trx1 activity in a dose-dependent manner. The in vitro enzyme activity assay revealed that IsoA (0.02 μmol/L) inhibited Trx1 activity by 50% (Fig. 4B). To further confirm this in a cellular context, we treated HepG2 cells with IsoA at various concentrations for 8 hours and measured Trx1 activity. As shown in Fig. 4C, IsoA significantly reduced Trx1 activity in a concentration-dependent manner. We also determined Trx1 and thioredoxin reductase (TrxR) levels in HepG2 cells treated with IsoA by Western blotting (Fig. 4D). The results showed that IsoA downregulated Trx1 expression but did not affect TrxR expression, suggesting that the reduction in Trx1 activity was due to the inhibition of Trx1 expression.

To further investigate the role of Trx1 in IsoA-induced apoptosis, we silenced Trx1 expression in HepG2 cells using siRNA and measured the effect on IsoA-induced cell viability. As shown in Fig. 4E, silencing Trx1 expression using specific siRNA significantly suppressed IsoA-induced cell death. These results suggested that Trx1 might be a key player in IsoA-induced apoptosis.

In conclusion, our findings provide new insights into the mechanism by which IsoA induces apoptosis in HepG2 cells. We demonstrated that IsoA-induced ROS accumulation and mitochondrial dysfunction contribute to cell death, and that Trx1 is a critical target for IsoA-induced apoptosis.
with biological molecules by forming covalent bonds with free thiol of cysteine or by acting as an electrophilic center in redox reactions. Hence, we speculated that IsoA might be a novel inhibitor of Trx1. For this, the levels of Trx1 in all cell lines were detected by Western blotting. As shown in Supplementary Fig. S11, Trx1 was extensively overexpressed in cancer cells relative to nonmalignant cells, which was consistent with previous reports (5–10). Next, we investigated the inhibition potency of IsoA toward Trx1 using an in vitro Trx1 activity fluorescent assay. Our results demonstrated that IsoA effectively inhibited Trx1 activity with an IC₅₀ of approximately 5.177 μmol/L, whereas it had little inhibitory effect on Grx (Fig. 4A).

To investigate the specificity of IsoA for Trx1, we analyzed the in vitro–inhibitory effects of IsoA on a spectrum of thiol-containing enzymes including glutathione reductase, thioredoxin reductase, glutathione-S-transferase, and protein disulfide isomerases. The results showed that IsoA inhibited thioredoxin reductase activity with an IC₅₀ of approximately 5.177 μmol/L, whereas it had little inhibitory effect on Grx (Fig. 4A). To investigate the specificity of IsoA for Trx1, we analyzed the in vitro–inhibitory effects of IsoA on a spectrum of thiol-containing enzymes including glutathione reductase, thioredoxin reductase, glutathione-S-transferase, and protein disulfide isomerases. The results showed that IsoA inhibited thioredoxin reductase activity with an IC₅₀ of approximately 5.177 μmol/L, whereas it had little inhibitory effect on Grx (Fig. 4A).

Trx1 interacts with ASK1, and inhibits its kinase activity and ASK1–mediated apoptosis (13). ROS releases Trx1 partly through oxidizing Trx1 and activates ASK1/JNK death signaling cascade (33). To determine whether IsoA-mediated cell apoptosis is associated with the activation of ASK1 signaling, we examined the effect of IsoA on the phosphorylation levels of ASK1 and JNK. The Western blot analysis results showed thatIsoA indeed increased the phosphorylation levels of ASK1 and JNK (Supplementary Fig. S13), suggesting that the activation of ASK1/JNK may contribute to IsoA-induced cell apoptosis.

To assess whether the effects of IsoA are Trx1 dependent, we used siRNAs to knockdown Trx1 expression. Trx1 protein level was reduced in the Trx1 knockdown cells (Fig. 4E and F), which rendered cancer cells more sensitive to IsoA (Fig. 4G). These results indicated that Trx1 levels influence cell sensitivity to IsoA.
IsoA covalently conjugated to Trx1 at Cys32 and Cys35 residues

To investigate whether IsoA directly interacts with the catalytic site of Trx1, we predicted the binding mode between IsoA and Trx1 through molecular docking. As shown in Fig. 5A and B, the docking poses of IsoA were almost identical to each other. The free sulfhydryl of reactive residues Cys32 and Cys35 acted as a nucleophile to attack α, β-unsaturated carbonyl group of IsoA, forming strong covalent bond between the receptor and the ligand through Michael addition in each complex. Meanwhile, the hydrogen bond formed between the acetyl of IsoA and Met74 also contributed to the stable binding interaction in both complexes.

To further confirm whether IsoA binds to Trx1 directly, we incubated IsoA with Trx1 protein in vitro, and examined the products by MS. We detected two major components at m/z 1624.79 and m/z 2694.12, respectively. The molecular weight of IsoA was 534.6. The component at m/z 1624.79 represented the peptide LVVVDFSATWCGPCK, corresponding to the 22 to 36 residues of Trx1. The molecular weight of this peptide plus two IsoA molecules was 2694.12, equal to the molecular weight of another component at m/z 2694.12, indicating that a covalent reaction occurred between IsoA and Trx1 at the ratio of 2:1 (Fig. 5C). Further MS/MS analysis demonstrated that IsoA covalently conjugated to both Cys32 and Cys35 residues of Trx1 together (Fig. 5D).

In vivo antitumor efficacy of IsoA in a xenograft mouse model

We next evaluated the in vivo antitumor efficacy of IsoA. In the xenograft model, HepG2 cells were inoculated subcutaneously into the nude mice. The mice were then treated by intraperitoneal injection with vehicle or IsoA (15 mg/kg/d) for 14 days. We found that treatment with IsoA did not show significant toxicity on the basis of stable body weights (Fig. 6A). However, treatment...
with IsoA significantly inhibited the growth of HepG2 xenograft (Fig. 6B) and reduced the weights of tumors compared with the vehicle-treated group (Fig. 6C). The DNA damage marker 8-oxoG and protein oxidative marker nitrotyrosine (Fig. 6D) were obviously increased in IsoA-treated tumors, suggesting that IsoA induced oxidative stress in the xenografts. Further analysis revealed that there was a significant increase of TUNEL-positive cells in the xenografts treated with IsoA, compared with the vehicle control (Fig. 6E). Besides, IsoA significantly suppressed the activity of Trx1 and depleted the GSH levels in the tumors (Fig. 6F), consistent with our in vitro observations. These results demonstrated that IsoA exhibits potent antitumor activity in vivo.

To explore the safety of IsoA further, we screened its toxicity to bone marrow (blood counts), liver (aspartate aminotransferase, AST; alanine aminotransferase, ALT; alkaline phosphatase, ALP), and kidney (creatinine, and blood urea nitrogen, BUN) in mice. All the indices of routine blood test including red cell count, white blood cell (WBC) count, lymphocyte count, platelet count, and hemoglobin level remained in the normal ranges after IsoA treatment (Fig. 7A). There were no significant differences in blood biochemical parameters (ALT, AST, ALP, BUN, and creatinine) between IsoA-treated and the control groups (Fig. 7B–F). In addition, after collecting blood samples for hematology, the vital organs (liver, spleen, and kidney) were collected, fixed in formalin, and processed for hematoxylin and eosin (H&E) staining. Histopathologic evaluation did not reveal any significant differences between the vehicle and IsoA-treated groups (Supplementary Fig. S14).

**Discussion**

In this study, we evaluated anticancer potentials of IsoA, a key component and a novel ent-kaurane diterpenoid from *I. forrestii* var. *forrestii*. Our data established IsoA as a Trx1 inhibitor, which inhibited tumor cell growth both in vitro and in vivo. The inhibitory effect of IsoA on Trx1 in cancer cells was demonstrated with the following lines of evidence. First, IsoA induced a rapid increase of ROS in cancer cells, and the collapse of redox homeostasis may be responsible for IsoA's anticancer activity. This result suggested that IsoA regulated cellular antioxidant systems. Second, IsoA suppressed the activity of Trx1 and depleted the GSH levels in the tumors (Fig. 6F), consistent with our in vitro observations. These results demonstrated that IsoA exhibits potent antitumor activity in vivo.

To explore the safety of IsoA further, we screened its toxicity to bone marrow (blood counts), liver (aspartate aminotransferase, AST; alanine aminotransferase, ALT; alkaline phosphatase, ALP), and kidney (creatinine, and blood urea nitrogen, BUN) in mice. All the indices of routine blood test including red cell count, white blood cell (WBC) count, lymphocyte count, platelet count, and hemoglobin level remained in the normal ranges after IsoA treatment (Fig. 7A). There were no significant differences in blood biochemical parameters (ALT, AST, ALP, BUN, and creatinine) between IsoA-treated and the control groups (Fig. 7B–F). In addition, after collecting blood samples for hematology, the vital organs (liver, spleen, and kidney) were collected, fixed in formalin, and processed for hematoxylin and eosin (H&E) staining. Histopathologic evaluation did not reveal any significant differences between the vehicle and IsoA-treated groups (Supplementary Fig. S14).

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In this study, we evaluated anticancer potentials of IsoA, a key component and a novel ent-kaurane diterpenoid from *I. forrestii* var. *forrestii*. Our data established IsoA as a Trx1 inhibitor, which inhibited tumor cell growth both in vitro and in vivo.
modification of cysteine residues of Trx1 may be a direct and effective way to suppress its activation.

As IsoA showed stronger suppressive effect against Trx1 than several other thiol-containing enzymes (glutaredoxin, glutathione reductase, thioredoxin reductase, glutathione-S-transferase, and protein disulfide isomerase) tested (Fig. 4A; Supplementary Fig. S12), we propose that IsoA is a relatively specific inhibitor of Trx1. The results that IsoA triggered ROS and activated ASK1/JNK cascade associated with apoptosis (Supplementary Fig. S13) and knockdown of Trx1 sensitized the cytotoxicity of IsoA (Fig. 4F) also demonstrated a critical role of Trx1 in IsoA-mediated apoptosis of cancer cells. Currently, we could not rule out the possibility that IsoA may interact with other thiol-containing enzymes or proteins. Nevertheless, at least, Trx1 is one of the important targets for IsoA. Accumulating evidence clearly showed that the reactivity of thiol-containing proteins was not determined solely by their CXXC motif, but also by several residues spatially close to the active sites (41, 42). The formation of a hydrogen bond or a salt bridge in the active site has a dramatic effect on the function of the protein. In our study, besides the Cys32/35 in the active sites, molecular docking showed that IsoA also formed a hydrogen bond with Met74, which also contributed to the stable binding interaction in both complexes (Fig. 5A and B). Consequently, we reasoned that the relatively specific inhibitory effect of IsoA on Trx1 might attribute to the extended active-site motif as well as residues distant in sequence but spatially close to the active site [Cys- Gly-Pro-Cys] of Trx1. Further research is needed to explore the mechanism by which IsoA had a relatively higher affinity with Trx1.

Recently, numerous ROS-inducing agents have been described to selectively kill cancer cells but spare normal cells (29–31, 43–45). In this work, IsoA induced an intracellular burst of ROS in cancer cells (Fig. 2A–D). Antioxidants NAC, GSH, PEG-SOD, or PEG-Catalase markedly abolished IsoA-induced growth inhibition, ROS generation, and apoptosis (Fig. 3). Therefore, ROS elevation may play a central role in mediating antitumor activity of IsoA through blocking Trx1. Consistent with the ROS threshold theory, our findings suggest that IsoA may be a novel Trx1 inhibitor with minimal toxicity to normal cells.

In conclusion, here we present evidence that IsoA is a novel Trx1 inhibitor through covalently binding to its catalytic sites Cys32 and Cys35 and inhibits tumor cell growth both in vitro and in vivo. The selective cytotoxic activity of IsoA to cancer cells can be explored further to develop novel antitumor agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Sun, W. Wang, J. Chen, Y. Yang, W. Lu, J. Pu
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