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Steroids from *Ganoderma sinense* as new natural inhibitors of cancer-associated mutant IDH1

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ABSTRACT

Isocitrate dehydrogenase (IDH) is one of the key enzymes in the tricarboxylic acid cycle, and IDH mutations have been associated with many cancers, including glioblastoma, sarcoma, acute myeloid leukemia, *etc*. Three natural steroids **1**–**3** from *Ganoderma sinense*, a unique and rare edible-medicinal fungi in China, were found as potential IDH1 inhibitors by virtual ligand screening method. Among the three compounds, **3** showed the highest binding affinity to IDH1 with significant calculated binding free energy. Enzymatic kinetics demonstrated that **3** inhibited mutant enzyme in a noncompetitive manner. The half effective concentration of **3** for reducing the concentration of D-2HG in HT1080 cells was 35.97 μM. The levels of histone H3K9me3 methylation in HT1080 cells were reduced by treating with **3**. Furthermore, knockdown of mutant IDH1 in HT1080 cells decreased the anti-proliferative sensitivity to **3**. In short, our findings highlight that compound **3** may have clinical potential in tumor therapies as an effective inhibitor of mutant IDH1.

1. Introduction

Isocitrate dehydrogenase (IDH) is a family of enzymes that catalyzes the oxidative decarboxylation of isocitrate (ICT) to α-ketoglutarate (α-KG) using either NADP or NAD as a cofactor, which is one of the key reactions in tricarboxylic acid cycle [1]. There are three IDH isoenzymes in humans, with IDH1 located in cytoplasm, IDH2 and 3 in mitochondria [2,3]. IDH1 and IDH2 with a single-point mutation are highly associated with cancer risk.

Recently, mutation of arginine 132 in IDH1 has been reported to correlate with certain brain tumors, including approximately 80% of grade II-III gliomas and secondary glioblastomas in humans [4,5]. Besides brain tumors, IDH1 mutations have also been detected in other cancers, such as acute myeloid leukemia [6], colorectal cancer [7], and prostate cancer [8]. Mutated IDH2 (more frequently at Arg140 and less frequently at Arg172) is found in 12.1% of patients with normal acute myeloid anemia [9].

These somatic mutations are most commonly occurred at a key arginine residue belonging to the catalytic triad found in the enzyme's active site IDH1R132 [1]. These mutations confer a neomorphic enzymatic activity: NADPH dependent reduction of α-KG to D-2-hydroxyglutarate (D-2HG) [9]. D-2HG has been confirmed to be a competitive inhibitor of α-KG-dependent dioxygenases, including histone (such as H3K9me3) and DNA demethylases, which play a key role in regulating the epigenetic state of cells [10], therefore, as a result of mutations in IDH, high cellular concentration of D-2HG may cause global methylation of histone and DNA, and finally lead to tumorigenesis [11]. In summary, these findings suggested mutant IDH1 as an oncogene and a compelling drug target for new therapies for glioma and AML patients.

Because of the potential of IDH1 inhibitors as anticancer therapeutics, a number of research groups have investigated the structures and mechanisms of IDH inhibitors. For example, AGI-5198 as a selective IDH1R132H inhibitor induced demethylation of histone H3K9me3 and expression of genes associated with gliogenic differentiation [12]. A crystal structure of AGI-6780 combined with IDH2/R140Q revealed that the inhibitor bound to the dimer interface in an allosteric manner [13].

Abbreviations: IDH1, isocitrate dehydrogenase; MST, microscale thermophoresis; CCK8, cell counting Kit-8; IPTG, isopropyl *β-D*-1-thiogalactopyranoside; PMSF, phenylmethanesulfonyluoride; PVDF, polyvinylidene; H&E, hematoxylin-eosin

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Natural products (NPs) have played a highly significant role in anticancer drug discovery and development [14]. *Ganoderma sinense* (Chinese name: Lingzhi), a unique and rare edible-medicinal fungi in China for improving human health, has already been used as a folk medicine for thousands of years. The mushrooms of *Ganoderma* showed various biological functions, such as anti-inflammation [15,16], immune regulation [17,18], hepatoprotection [19], antitumor [20–22], *etc*. And polysaccharides, triterpenes, sterols, farnesyl phenols, and some other compounds have been proved to be the main active constituents [23–28].

In this work, we aimed to identify natural IDH1 inhibitors from the in-house NP library through virtual ligand screening, enzyme, and cell-based experiments [29]. Interestingly, one of the natural steroids (**3**) from the adjuvant medicine for cancer, *Ganoderma sinense*, was confirmed as a novel inhibitor of mutant IDH1, which could reduce the cellular levels of D-2HG, showing dramatically on-target activity.

2. Materials and method

2.1. Compounds

(22E,24R)-Ergosta-7,9(11),22-triene-3β,5α,6β-triol (**1**), ergosta-4,6,8(14),22-tetraen- 3-one (**2**), cyathisterol (**3**) were previously obtained from the fruit body of *Ganoderma sinense* in our laboratory [27]. These compounds were dissolved in DMSO to make a stock solution, aliquoted and stored at −20 °C. The DMSO concentration was kept below 0.05% in all cell cultures used and did not exert any detectable effect on cell growth or death.

2.2. Reagents and antibodies

IDH1R132C mutant HT1080 fibrosarcoma cells, L929 and LO2 cells were obtained from the American Type Culture Collection (ATCC, USA). The antibody against H3K9me3 was obtained from Cell Signaling Technology (Beverly, MA), β-actin and horseradish peroxidase (HRP) conjugated secondary antibody from rabbit were purchased from Wuxi UcallM Biotechnology Co., Ltd (China). All other common chemicals, solvents and reagents were of highest grade available from various commercial sources.

2.3. Molecular docking and virtual ligand screening

The in-house natural product database included more than 1000 compounds isolated from plants and fungi in our group. They were saved as mol2 files, and then used as the input for docking. The docking was performed by using ICM 3.8.1 modeling software on an Intel i7 4960 processor (MolSoft LLC, San Diego, CA). Ligand binding pocket residues were selected by using graphical tools in the ICM software, to create the boundaries of the docking search [30]. In the docking calculation, potential energy maps of the receptor were calculated using default parameters. Compounds were imported into ICM and an index file was created. Conformational sampling was based on the Monte Carlo procedure, and finally the lowest-energy and the most favorable orientation of the ligand were selected [31].

2.4. Expression and purification of IDH1, IDH1R132H and IDH1R132C

R132H and R132C mutant IDH1 genes were generated from the wild-type IDH1 plasmid, using Quick-change site-directed mutagenesis kit (Agilent) following the manufacturer's protocol. Correctness of the gene sequences was verified. The IDH1R132H and IDH1R132C were cloned into the pET28a vector (Novagen). The expression construct pET28a-IDH1R132H and pET28a-IDH1R132HC were transformed into *Escherichia coli* strain BL21 (DE) (Invitrogen) and selected on kanamycin plates. The transformed cells were cultivated in Luria-Bertani (LB) media at 37 °C in the presence of kanamycin until the optical density (OD) reached 0.8. Cells were then induced with 0.4 mM IPTG (isopropyl-*β-D*-1-thiogalactopyranoside) for 16 h at 20 °C. The cells harvested by centrifugation were lysed by ultrasonication on ice in a buffer containing 20 mM Tris, pH 8.5, 200 mM NaCl, 5 mM mercaptoethanol, 0.1% TritonX-100, and 5% glycerol. Soluble C-terminally hexa-histidine tagged IDH1R132H and IDHR132C were bound to Ni-agarose affinity resin (Qiagen), washed with buffer A (20 mM Tris, pH 8.8, 200 mM NaCl, and 10 mM imidazole), and eluted with buffer B (20 mM Tris, pH 8.8, 250 mM NaCl, and 150 mM imidazole). The protein was further purified with anion exchange chromatography and size exclusion chromatography.

2.5. Microscale thermophoresis (MST) assay

MST was used to analyze the binding affinity between potential ligands and receptors. Recombinant IDH1R132H was labeled with the Monolith NT™ Protein Labeling Kit RED (Cat#L001) according to the supplied labeling protocol. Labeled IDH1R132H was kept constant at 20 nM, and all tested samples were 1:1 diluted in a 20 mM HEPES (pH 7.4) and 0.05 (v/v)% Tween-20. After 10 min incubation at room temperature, the samples were loaded into Monolith™ standard-treated capillaries and the thermophoresis was measured at 25 °C after 30 min incubation on a Monolith NT.115 instrument (NanoTemper Technologies, München, Germany). Laser power was set to 20% using 30 s on-time. The LED power was set to 100%. The dissociation constant Kd values were fitted by using the NTAnalysis software (NanoTemper Technologies, München, Germany).

2.6. In vitro enzyme inhibition assay

The determination of the activity and inhibition of IDH1R132H and IDH1R132C were based on the initial linear consumption of NADPH in the reaction. The enzyme activity assay was performed in a 96-well microplate by using the purified IDH1 mutant (2 μM) in the buffer containing 4 mM $MgCl_2$, 2 mM α-KG, 100 μM NADPH (> > km for NADPH) in 50 mM HEPES buffer (pH = 7.5). For inhibition assay, firstly, triplicate samples of compounds were incubated with the protein for 5 min before adding α-KG to initiate the reaction. The optical absorbance of each well was monitored every 1 min at 340 nm, where NADPH has the maximum absorption, using a BioTek Synergy HT microplate reader. The activity and inhibition of WT-IDH1 were determined by measuring the production of NADPH. In brief, the enzyme activity assay was performed in a 96-well microplate using the purified IDH1 (43 nM), 4 mM $MgCl_2$, 50 μM sodium (D)-isocitrate, 1 mM NADP + (> > km for NADP) in 50 mM HEPES buffer (pH = 7.5). The reaction can be readily monitored by an increase in optical absorbance at 340 nm. The data were imported into Prism (version 6.02, GraphPad) and the IC_{50} values were calculated by using a standard dose response curve fitting. The initial velocity data were fitted to the Michaelis–Menten equation using Prism 6.02 to obtain the Vmax and km. To determine the mechanism of inhibition, steady-state kinetics data were globally fitted to various inhibition models (competitive, noncompetitive, and uncompetitive modes).

2.7. Cell culture

HT1080, L929 and LO2 cells were cultured in high Glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. Cell cultures were grown and maintained in culture at 37 °C in a humidified tissue culture incubator with 5% CO_2. The cell cultures were performed following the instructions of ATCC.