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Bioassay-guided isolation of dehydrocostus lactone from *Saussurea lappa*: A new targeted cytosolic thioredoxin reductase anticancer agent

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ABSTRACT

In a screen for mammalian thioredoxin reductases inhibitors, an MeOH extract from the roots of *Saussurea lappa* C.B. Clarke (Compositae) inhibited the activity of cytosolic thioredoxin reductase (TrxR1). Bioassay-guided separation of the extract led to the isolation of a new TrxR1 inhibitor, dehydrocostus lactone (DHC), a guaiane-type sesquiterpene. The content of DHC in the extract was determined to be 0.4%. DHC inhibited human cervical carcinoma HeLa cells with an IC_{50} of ~12.00 μM but displayed less cytotoxicity to human immortalized normal liver cells L02. We observed that DHC killed HeLa cells through induction of apoptosis. DHC inhibited the activity of TrxR1 in HeLa cells, which elicited an accumulation of reactive oxygen species (ROS) in cells and a collapse of the intracellular redox equilibrium and eventually induced apoptosis of HeLa cells.

1. Introduction

Cancer, a complex disease, has become one of the most serious threats to human health [1,2]. Although modern medical technology has rapidly advanced over the last several decades, no cure has been identified for almost any cancer around the world [3,4]. Recently, considerable interest has been focused on targeting enzymes as an approach for cancer chemotherapy [5–7]. The mammalian thioredoxin reductases (TrxRs), NADPH-dependent and FAD-containing disulfide reductases, which have a unique active site of penultimate selenocysteine residue at the C-terminus [8–10], have attracted the interest of researchers [11–13]. TrxR1 is omnipresent in all living cells and is expressed at high levels in cancer cells [14–17]. TrxR1 is critical for cellular redox regulation, tumor proliferation, apoptosis and metastasis [18,19]. In recent years, accumulating evidence has shown that TrxR1 is a promising target for the development of anticancer agents [20–22].

Natural products could provide unlimited opportunities for new TrxR inhibitors thanks to the matchless availability of chemical diversity [23–25]. A variety of small molecule TrxR1 inhibitors have been reported, including diterpenoids [26], polyphenols and quinones [27–30]. In our ongoing search for new mammalian TrxR inhibitors, we found that the MeOH extract from the roots of *S. lappa* inhibited the activity of TrxR1. The roots of *S. lappa* have been widely used in traditional medicine in Asia for the treatment of cancer [31]. This use prompted us to investigate its active components. The MeOH extract was fractioned with petroleum ether, EtOAc and *n*-BuOH. The petroleum ether and EtOAc fractions were more active to TrxR1 than the *n*-BuOH part. Previous studies on this species have reported that dehydrocostus lactone (DHC) (Fig. 1) is one of the major active components [32–35]. We determined the content of DHC in the two fractions by HPLC quantitative analysis, and the content was found to be 24.6% and 7.0% for petroleum ether and EtOAc, respectively. DHC was obtained from the effective parts by chromatography over silica gel, and the structure was elucidated

Abbreviations: TrxR1, cytosolic thioredoxin reductase; DHC, dehydrocostus lactone; ROS, reactive oxygen species; PAGE, SDS–polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DTNB, 5, 5′-dithiobis-(2-nitrobenzoic acid); PMSF, phenylmethyl-sulfonyl fluoride; FBS, fetal calf serum; EDTA, ethylene diamine tetraacetic acid; PBS, phosphate-buffered saline; DTT, DL-dithiothreitol.

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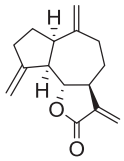

Dehydrocostus lactone (DHC)

Fig. 1. Chemical structure of dehydrocostus lactone.

through comprehensive analysis of spectroscopic data. DHC showed persuasive cytotoxicity on HeLa cells with an IC_{50} of ~12.00 μM. The high levels of DHC and its potent anticancer activity led us to investigate its interaction with TrxR1. In this study, we report that DHC caused TrxR1 inhibition, ROS accumulation in HeLa cells, and eventually induced apoptosis of HeLa cells. DHC also inhibited the activity of pure TrxR1 with an IC_{50} value of ~2.24 μM. Targeting TrxR1 with DHC revealed a new molecular mechanism underlying the cellular action of DHC and sheds light on a new aspect of DHC as an anticancer agent.

2. Materials and methods

2.1. Chemical

2.1.1. General experimental procedures

Column chromatography: silica gel (200–300 mesh, Qingdao Marine Chemical Factory, China). TLC: silica gel GF 254 plates (10–40 μm, Qingdao Marine Chemical Factory, China). Semi-preparative HPLC was carried out on a Waters 1525 binary pump system with a Waters 2489 detector using an X Bridge C 18 ODS-A (250 × 4.6 mm, 5 μm) column. HRESIMS Spectra: Bruker Daltonics APEX II 47e Spectrometer. ^{1}H NMR: Bruker Avance III-400 (400 MHz) spectrometer; ^{13}C NMR: Bruker Avance III-400 (100 MHz) spectrometer; recorded in $CDCl_3$; δ ppm relative to TMS; J values in Hz.

2.1.2. Plant material

The roots of *S. lappa* were purchased from Yellow River Chinese Medicine Market, Lanzhou city, Gansu Province, China, in October 2010.

2.1.3. Dehydrocostus lactone

Crystalline solid, $C_{15}H_{18}O_2$, $[\alpha]^{20}_D = -20$ ($CHCl_3$, c 0.10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.19 (1H, d, J = 3.2 Hz, H-13a), 5.46 (1H, d, J = 3.2 Hz, H-13b), 5.23 (1H, brs, H-14a), 5.03 (1H, brs, H-14b), 4.90 (1H, brs, H-15a), 4.78 (1H, brs, H-15b), 3.93 (1H, t, J = 9.1 Hz, H-6), 2.85 (3H, m, H-1, H-5, H-7), 2.46 (3H, m, H-3, H-9α), 2.27 (2H, m, H-8β, H-9β), 1.92 (2H, m, H-2), 1.46 (1H, m, H-8α); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2 (C-12), 151.2 (C-4), 149.2 (C-10), 139.7 (C-11), 120.1 (C-13), 112.6 (C-15), 109.5 (C-14), 85.2 (C-6), 52.0 (C-5), 47.5 (C-1), 45.0 (C-7), 36.3 (C-9), 32.6 (C-2), 30.6 (C-8), 30.3 (C-3). HRESIMS m/z 231.1375 $[M + H]^+$.

2.2. Biology

2.2.1. Chemicals and enzymes

Recombinant rat TrxR1 was prepared as described [36] and was a gift from Professor Arne Holmgren at Karolinska Institute, Sweden. Proteins were pure, as judged by Coomassie-stained SDS–polyacrylamide gel electrophoresis (PAGE). Dulbecco's modified Eagle's medium (DMEM), bovine insulin, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), dimethyl sulfoxide (DMSO), Hoechst 33342 and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). *Escherichia coli* Trx was produced as described [37]. NADPH was obtained from Roche (Mannheim, Germany). Fetal bovine serum (FBS) and DTNB were purchased from Sijiqing (Hangzhou, China) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbro-mide (MTT), penicillin, and streptomycin were obtained from Amresco (Solon, OH, USA). Bovine serum albumin, phenylmethyl-sulfonyl fluoride (PMSF), and sodium orthovanadate (V) (Na_3VO_4) were obtained from Beyotime (Nantong, China). A 100 mM solution of DHC was prepared in DMSO and stored at −20 °C. All other reagents were of analytical grade.

2.2.2. Cell cultures

All cells (HeLa, Hep G2, SMMC-7721 and L02) used in this study were provided by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The tested cells were incubated in DMEM supplemented with 10% FBS, 2 mM glutamine, and 100 units/mL penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2.3. Cytotoxicity assay

MTT was used to evaluate cell viability as described [38]. Unless otherwise stated, 8000 cells were incubated with DHC or other agents in triplicate in a 96-well plate for the indicated times at 37 °C in a final volume of 100 μL, using cells incubated only with DMSO as controls. A microplate reader (Thermo Scientific Multiskan GO, Finland) was used to measure absorbance (570 nm).

2.2.4. In vitro TrxR1 activity assays-DTNB assay [37]

A microplate reader was used to determine the TrxR1 activity at room temperature. The TrxR1 (170 nM) was incubated with different concentrations of fractions and DHC (the final volume of the mixture was 50 μL) in a 96-well plate at room temperature. A mixture (50 μL) of Tris–HCl (50 mM, pH 7.5) and EDTA (1 mM) in TE buffer including DTNB (2 mM) and NADPH (200 μM) was added, and a linear increase in absorbance at 412 nm during the initial 3 min was recorded. In the control experiments, the same amounts of DMSO were added, and the activity was recorded as the percentage of the control.

2.2.5. Determination of TrxR1 activity in HeLa cells

After cells (70–80% confluent) were incubated with different concentrations of DHC for 12 and 24 h, they were harvested and washed twice by phosphate-buffered saline (PBS). Total cellular proteins were extracted with RIPA buffer (50 mM Tris–HCl, pH 7.5, 0.5% deoxycholate, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, and 1 mM PMSF) for 30 min on ice. The Bradford procedure was used to quantify the total protein content. TrxR1 activity in cell lysates was evaluated using the endpoint insulin reduction assay [39]. In general, the cell extract (containing 20 μg of total proteins) was incubated in a final mixture of 50 μL volume (100 mM Tris–HCl, pH 7.6, 0.3 mM insulin, 660 μM NADPH, 3 mM EDTA, 15 μM *E. coli* Trx) at 37 °C for 30 min. The reaction was terminated by adding 200 μL of 1 mM DTNB in 6 M guanidine hydrochloride, pH 8.0. The absorbance was determined at 412 nm. In the control experiments, the same amounts of DMSO were added, and the activity was recorded as the percentage of the control.

2.2.6. Imaging of cellular TrxR1 activity by TRFS-green

TRFS-green is a specific TrxR fluorogenic probe that can be used to image TrxR activity in live cells [40], and it has been applied to image TrxR activity in different cells [41]. HeLa cells were incubated with the indicated concentrations of DHC for 8 h, then continually