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PYDDT, a novel phase 2 enzymes inducer, activates Keap1–Nrf2 pathway via depleting the cellular level of glutathione

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ABSTRACT

Keap1–Nrf2 pathway has emerged as a regulator for the endogenous antioxidant response, which is critical in defending cells against carcinogenesis. Herein, we demonstrated that depleting the cellular level of glutathione (GSH) by a novel electrophilic agent 2-(pro-1-ynyl)-5-(5,6-dihydroxypenta-1,3-diynyl) thiophene (PYDDT) could activate Keap1–Nrf2 pathway. In above process, it was found that Keap1 was modified by *S*-glutathionylation, an important post-translational modification of protein cysteines with critical roles in oxidative stress and signal transduction. We concluded from our findings that conjugation with intracellular GSH by PYDDT might lead to Keap1 *S*-glutathionylation and was a key event involved in its Nrf2 inducing activity.

1. Introduction

2-(Pro-1-ynyl)-5-(5,6-dihydroxypenta-1,3-diynyl) thiophene (PYDDT) is a natural alkynol group-substituted thiophene isolated from the roots of *Echinops grijsii*, a traditional Chinese medicine used to clear heat, detoxicate, expel miasma and stimulate milk secretion for a long history (Jin et al., 2008). The chemical structure of PYDDT indicates that it is an electrophilic compound (Fig. 1), which is quite novel among known ones. There were few previous reports about the bioactivity of PYDDT, only Biswas et al. (2007) reported that it possessed anti-amoebic activity.

Kelch-like ECH-associated protein 1 (Keap1) is a ~70 kD protein that serves as a sensor for electrophile-induced activation of nuclear factor erythroid-2 related factor 2 (Nrf2), a transcription factor that mediates gene induction of protective phase 2 detoxifying enzymes, such as heme oxygenase 1 (HO-1), NAD(P)H: quinone oxidoreductase1(NQO1) and glutathione-*S*-transferase (GST) (Hong and Sporn, 1997). Under nonstressed conditions, Keap1 acts as a negative regulator of Nrf2 by proteasomal degradation of Nrf2 through a Keap1-Cul3-Rbx-dependent mechanism in which Keap1 serves as the substrate adaptor subunit in the E3 ubiquitin ligase (Kobayashi et al., 2006). Modifications of critical reactive thiol groups in Keap1 result in dissociation of the Keap1–Nrf2 complex and translocation of Nrf2 into the nucleus, where it binds to the 5′-upstream regulatory antioxidant response element (ARE) regions of detoxification genes and accelerates the expressions of phase 2 detoxifying enzymes (Itoh et al., 1999; Dinkova-Kostova et al., 2002; Eggler et al., 2005). There are three functional domains containing in Keap1: the Kelch repeat domain that binds the Neh2 domain of Nrf2 (McMahon et al., 2006), the BTB dimerization region that is toward the N-terminus, and the central linker region (CLR) that conjoins the former two domains.

S-glutathionylation is a reversible post-translational modification with critical roles in oxidative stress and signal transduction. Upon *S*-glutathionylation, protein cysteine residues are modified by the addition of the glutathione (GSH), an important low-molecular-mass thiol within most cell types (Dalle-Donne et al., 2009). Until now, a variety of proteins have been identified to be potentially regulated by reversible *S*-glutathionylation. In many investigations *S*-glutathionylation is characterized as inhibitory, for example, nuclear factor kappa B (NF-κB) (Pineda-Molina et al., 2001; Qanungo et al., 2007), creatine kinase (Reddy et al., 2000), actin-Cys374 (Dalle-Donne et al., 2003a,b; Wang et al., 2001,

Abbreviations: AHH, aryl hydrocarbon hydroylase; ARE, antioxidant response element; carboxy-DCFDA, 5-(and-6)-darboxy-2′,7′-dichlorofluorescin diacetate; CLR, central linker region; DMSO, dimethyl sulfoxide; DTNB, 5′,5′-dithio-bis(2-nitrobenzoic acid); ECL, enhanced chemiluminescence; GSH, glutathione; GST, glutathione-*S*-transferase; HO-1, heme oxygenase 1; HRP, horseradish peroxidase; IKK, IκB Kinase; Keap1, Kelch-like ECH-associated protein 1; LC–MS, liquid chromatography–tandem mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; NOQ1, NAD(P)H: quinone oxidoreductase1; Nrf2, nuclear factor erythroid-2 related factor 2; PMSF, phenylmethylsulphonyl fluoride; PYDDT, 2-(pro-1-ynyl)-5-(5,6-dihydroxypenta-1,3-diynyl) thiophene; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; TNB, 2-nitro-5-thiobenzoic acid; UV, ultraviolet.

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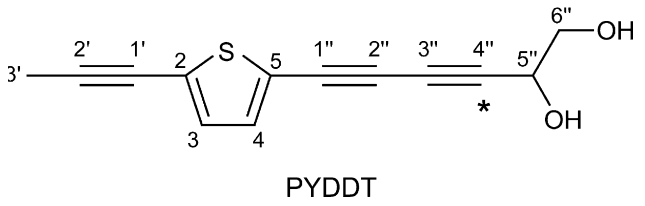

Fig. 1. Chemical structure of PYDDT. Asterisk represents electron-deficient center.

2003) and IκB Kinase (IKK) (Reynaert et al., 2006). Likewise, there are some cases where *S*-glutathionylation represents an activated function, for example, carbonic anhydrase III phosphatase-Cys186 (Cabiscol and Levine, 1996), HIV-1 protease-Cys67 (Davis et al., 1996; Davis et al., 1997), matrix metalloproteinase (Okamoto et al., 2001) and hRas-Cys118 (Adachi et al., 2004). The exact intracellular mechanisms of *S*-glutathionylation have not yet been clearly established. One of potential mechanisms of *S*-glutathionylation is "thiol-disulfide exchange", which depends on the intracellular GSH/GSSG ratio and the specific oxidation potential for the formation of the disulfide (Gilbert, 1995). In this theory, driving conversion of protein-SH to protein-SSG needs declining of intracellular GSH/GSSG ratio. In addition, sulfenic acid intermediates, sulfenylamide intermediates, thiyl radical intermediates, thiosulfinate intermediates and *S*-nitrosylated intermediates are other potential mechanisms of protein-SSG formation that are yet to be identified (Mieyal et al., 2008).

Previous studies have demonstrated that depleting the cellular level of GSH by electrophilic compounds (e.g. sulforaphane) associated with the stimulation of Keap1–Nrf2 pathway and the induction of the phase 2 detoxifying enzymes including GSTs and NQO1 (Kim et al., 2003). However, the exact mechanism between the depletion of cellular GSH and the induction of phase 2 detoxifying enzymes is not very clear. In this paper, we report for the first time that after depleting the cellular level of GSH by PYDDT, Keap1 was modified by *S*-glutathionylation and the Nrf2 expression increased markedly, in the hope of providing molecular theoretical basis for the possible application of PYDDT in the future.

2. Materials and methods

2.1. Materials and chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). 5-(and-6)-darboxy-2′,7′-dichlorofluorescin diacetate (carboxy-DCFDA) was purchased from Invitrogen (Carlsbad, CA, USA). Stock solution carboxy-DCFDA (15 mM) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. All the antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GSH was purchased from Bio Basic Inc. HPLC-grade acetonitrile (Merck, Darmstadt, Germany) and formic acid (Tedia, Fairfield, OH, USA) were utilized for the HPLC analysis. Deionized water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Isolate PYDDT from the dichloromethane extract of *E. grijsii*

The roots of *E. grijsii* Hance. were collected in Bozhou, north of Anhui Province, People's Republic of China, in June 2006. The plant material was identified by Dr. Xian Li, who is a professor of Pharmacognosy in Shenyang Pharmaceutical University and works on plant taxonomy and natural product chemistry. A voucher specimen (No. EGH060703) has been deposited in the herbarium of School of Pharmaceutical Sciences, Zhejiang University. The air-dried roots (15 kg) were extracted with 95% ethanol at 95 °C for three times to give a crude ethanol extract. The residue was dissolved in H_2O and then extracted successively with petroleum ether and dichloromethane. The dichloromethane fraction (140 g) was subjected to column chromatography over a silica gel (200–300 mesh, 1.2 kg) and eluted with petroleum ether (60–90 °C)/acetic ether (1:0, 200:1, 100:1, 50:1, 30:1, 20:1, 10:1, 5:1, 1:1, 0:1 (v/v)) to obtain 10 fractions (Fr. A–J). Fr. I was further separated by preparative HPLC using acetonitrile/H_2O as the mobile phase (40% acetonitrile maintained for 10 min and increased to 80% in 20 min, flow rate 10 mL/min) to yield PYDDT (123.2 mg, t_R 22.4 min). The preparative HPLC experiments were performed on Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) using column Zorbax SB-C_{18} (21.2 mm × 250 mm, 10 μm). The ^{1}H NMR (500 MHz, $CDCl_3$), ^{13}C NMR (125 MHz, $CDCl_3$), HSQC and HMBC spectra, see Fig. S1.

2.3. Cell culture

Hepa 1c1c7 murine hepatoma cells and c1 cells were obtained from the American Type Culture Collection (ATCC). Hepa 1c1c7 cells were maintained in α-minimum essential medium supplemented with 0.1% penicillin-streptomycin, 10% fetal bovine serum (GIBCO, NY, USA). C1 cells were maintained in α-minimum essential medium supplemented with 0.1% penicillin-streptomycin, 10% heat-inactivated fetal bovine serum. Both the cells were incubated in 5% CO_2 at 37 °C.

2.4. Crystal violet assay for determining cell viability

The cytotoxicities of PYDDT were determined on Hepa 1c1c7 and c1 cells using the crystal violet assay with some minor modifications (Ishiyama et al., 1996). Briefly, Hepa lclc7 or c1 cells were seeded in 96-well plates at a density of 2×10^4 cells/well. After 12 h, different concentrations of PYDDT were added into each well for an additional 24 h. Then decant the media and add 180 μL of 0.2% crystal violet in 2% ethanol solution to each well. After incubation for 10 min, the plates were rinsed for 2 min with water. The bound dye was solubilized by incubation with 200 μL of 0.5% SDS in 50% ethanol solution at 37 °C for 5 min. The absorption of crystal violet was measured at 550 nm, and the IC_{50} values were determined.

2.5. NQO1 induction assay

Induction of NQO1 activity was assayed using Hepa 1c1c7 and c1 cells as described previously with some minor modifications (Cheng et al., 2009). Briefly, Hepa lclc7 or c1 cells were seeded in 96-well plates at a density of 2×10^4 cells/well. After 12 h, different concentrations of PYDDT were added into each well in the presence or absence of GSH for an additional 24 h. Then the medium was decanted and the cells were incubated with 50 μL of 0.8% digitonin and 2 mM EDTA solution (pH 7.8) at 37 °C for 10 min. Then 200 μL of a mixed solution containing bovine serum albumin (0.67 mg/mL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.72 mM), 1.5% Tween 20, 0.5 M Tris–HCl, 5 μM FAD, 150 mM glucose-6-phosphate, 2 units/mL glucose-6-phosphate dehydrogenase, 50 mM NADP and 50 mM menadione were added into each well. After incubation for 5 min, the plates were scanned at 550 nm. The specific activity of NQO1 was determined by measuring NADPH-dependent menadione-mediated reduction of MTT to blue formazan.

2.6. Western blot analysis

After treatment, Hepa 1c1c7 cells were lysed in 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 containing 1 mM phenylmethylsulphonyl fluoride (PMSF) at 4 °C for 30 min and then centrifuged at 14,000 rpm for 10 min to obtain the total protein. Nuclear extracts were obtained using KEYGEN Nuclear Extraction Kit according to the manufacturer's protocol. For western blot analysis, 50 μg of protein from nuclear extracts or total protein lysates were used and separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with primary antibodies and then incubated with a horseradish peroxidase (HRP) conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence (ECL) western blotting detection reagents (Millipore, Billerica, MA, USA). To investigate *S*-glutathionylation of the proteins, cell lysates were separated under nonreducing conditions (without DTT in loading buffer).

2.7. Immunofluorescence

Hepa 1c1c7 cells were plated in 6-well plates at a density of 10^5 cells/well overnight and then treated with DMSO vehicle or PYDDT (20 μM) for 2 h. Cells were washed twice with PBS and fixed in 4% paraformaldehyde at room temperature for 0.5 h. The samples were then washed twice with PBS and permeabilized with 1% Triton X-100 for 5 min. After washing twice with PBS, the cells were incubated with the appropriate antibody (1:50 diluted in PBS) overnight at 4 °C. After washing three times with PBS, the cells were then incubated with FITC-labeled goat anti-rabbit IgG secondary antibody or Cy3-labeled goat anti-mouse IgG secondary antibody (both 1:200 diluted in PBS) at room temperature for 1 h. Cells were then washed three times in PBS and stained with DAPI (1:1000 diluted in PBS), then imaged with Leica CRT6500 fluorescence microscope.

2.8. Immunoprecipitation

After treatment, Hepa 1c1c7 cells were harvested and lysed in 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 5 μg/ml leupeptin and 1% Triton X-100 at 4 °C for 10 min and then centrifuged at 14,000 rpm for 10 min to obtain total protein. Aliquots (400 μg) of proteins were precleared by adding 1 μg of the appropriate normal immunoglobulin G and 20 μL of protein A + G-agarose conjugate at 4 °C for