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Renal protection by 3*H*-1,2-dithiole-3-thione against cisplatin through the Nrf2-antioxidant pathway

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ABSTRACT

Cisplatin is commonly used for the treatment of several solid tumors. However, its clinical use is often limited by renal toxicity. The indirect antioxidant 3*H*-1,2-dithiole-3-thione (D3T) has been known to protect cells from oxidative damage by up-regulating the expression of antioxidative genes through the transcription factor NF-E2-related factor 2 (Nrf2) pathway. We hypothesized that D3T treatment may be protective against cisplatin-induced nephrotoxicity by enhancing the antioxidative capacity of renal cells. In cultured murine tubular epithelial cells, D3T facilitates the nuclear accumulation of Nrf2 and the subsequent expression of its target genes such as glutamate cysteine ligase (GCL). Increased GSH pool in D3T-treated renal cells appears to be associated with amelioration of cisplatin-mediated cell death. Protective effects of D3T were also observed in mice. Oral administration of D3T (0.25 mmol/kg) increased the expression of GCL in mouse kidney, which resulted in suppression of cisplatin-mediated increases in blood urea nitrogen and serum creatinine. Histopathological changes representing cisplatin-induced acute renal failure were also effectively ameliorated by D3T treatment. Collectively, these results indicate that pharmacological activation of the Nrf2 pathway might have a beneficial effect on reducing chemotherapy-associated cytotoxic adverse effects.

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum (II)) is known as one of the most effective chemotherapeutic agents for the treatment of tumors. Within the cell, cisplatin is converted into a charged electrophilic compound that can react with nucleophilic molecules including DNA. Cisplatin–DNA adducts can cause the inhibition of transcription, cell-cycle arrest and apoptosis, and are responsible for cancer cell cytotoxicity [1,2]. Combination chemotherapy based on cisplatin is widely used for the treatment of various types of solid tumors including ovarian, testicular, lung, and stomach [3]. However, the clinical utility of cisplatin is often limited by the occurrence of side effects in the renal, neuronal, and auditory systems [3–5]. In particular, cisplatin has been shown to accumulate predominantly in the kidney rather than other organs, and nephrotoxicity is the most prevalent and serious toxicity of cisplatin. The mechanism of renal toxicity caused by cisplatin is not clear; however, there is evidence that cisplatin generates free radicals, inhibits mitochondrial function and sulfhydryl-containing enzymes, and induces DNA damage [6–9]. It has been reported that cisplatin represses the antioxidative capacity of cells by

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Abbreviations: D3T, 3*H*-1,2-dithiole-3-thione; Nqo1, NAD(P)H:quinone oxidoreductase 1; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; BSO, L-buthionine-sulfoxamine; DTNB, 5′,5-dithio-bis(2-nitrobenzoic acid); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BUN, blood urea nitrogen; ARE, antioxidant response element; HO-1, heme oxygenase-1.

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depleting glutathione (GSH), an effect that is strongly associated with renal toxicity [10,11].

Dithiolethiones including 3*H*-1,2-dithiole-3-thione (D3T), anethole dithiolethione (5-(*p*-methoxyphenyl)-1,2-dithiole-3-thione) and oltipraz (4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione) are effective chemopreventive agents, which can protect cells from environmental toxicants in animal and, in the cases of the latter two, human studies. The major mechanism underlying this protective effect is the activation of the Nrf2 pathway and subsequent induction of the target genes for this transcription factor [12–14]. Nrf2, a member of the NF-E2-related factor family, is anchored in the cytoplasm by Kelch-like ECH associated protein 1 (Keap1) under basal conditions. Recent studies showed that Keap1 is also involved in proteasome-mediated degradation of Nrf2 in quiescent cells by acting as an adaptor protein of Cul3-based E3 ubiquitin ligase complex [15,16]. Activation of Nrf2 is mediated by divergent stimuli including oxidative stress and exogenous chemicals. These stimulants cause dissociation of Nrf2 from Keap1 and lead Nrf2 to translocate into the nucleus where it can bind to the Antioxidant Response Element (ARE), a *cis*-acting element on the promoter of multiple cytoprotective genes. Among Nrf2-target genes, glutamate cysteine ligase (GCL) plays a crucial role in protection of cells from oxidative damage via synthesizing GSH [17,18]. The GCL is a heterodimeric enzyme that is composed of a catalytic subunit (GCLC) and a modulatory subunit (GCLM) [19]. GSH is largely responsible for detoxication of cisplatin. Administration of GSH or GSH ester to animals protects the kidney from cisplatin-mediated toxicity, while depletion of GSH by using buthionine sulfoximine (BSO) enhances the renal toxicity of cisplatin [20,21]. Another protective factor against cisplatin is the metallothioneins (MTs), which are cysteine-rich metal binding proteins. It has been reported that pre-administration of MT-increasing metals could suppress cisplatin-mediated renal toxicity [22,23].

Based on above knowledge, it can be hypothesized that pharmacological activators of Nrf2 may protect renal cells from cisplatin-mediated cytotoxicity. Several direct antioxidant therapies have been applied to reduce the degree of cisplatin adverse effect, however indirect antioxidants, which are modulating transcription factors governing the expression of antioxidant genes, have not been tried yet. Here we report that D3T effectively activates the Nrf2 system and leads to increase in total GSH. Pre-treatment with D3T protected renal tubular epithelial cells from cisplatin-mediated cell death and ameliorated kidney dysfunction by cisplatin in mice. Our results suggest that conjunctive use of pharmacological activators of Nrf2 might be beneficial in reducing side effects of cisplatin-based chemotherapy.

2. Material and methods

2.1. Materials

D3T was provided by Dr. Thomas Curphey (Dartmouth Medical School, NH, USA). Cisplatin, glutathione reductase, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and β-NADPH were purchased from Sigma (St. Louis, MO, USA). Antibodies against Nrf2 and lamin B, and the secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Bio-Rad (Hercules, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco Inc. (Solon, OH, USA).

2.2. Cell culture and treatment

TCMK-1 cells (murine kidney epithelial cells) were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in Minimum Essential Medium with Earle's BSS (HyClone, Logan, Utah, USA) containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% penicillin/streptomycin (HyClone). Human ovarian cancer SK-OV cells were obtained from Korean Cell Line Bank (Kwanak-gu, Seoul, South Korea) and maintained in RPMI-1640 medium (HyClone) with L-glutamine, HEPES, 10% fetal bovine serum, and 1% penicillin/streptomycin.

2.3. Animals and treatment

Male mice (ICR, 8 weeks old) were obtained from Orient Bio. (Gyeonggi-do, South Korea) and were fed AIN-76A semi-purified diet (Central Lab. Animal Inc., Seoul, South Korea) for 1 week before starting treatments. Mice were treated with vehicle or D3T (0.25 mmol/kg) by *gavage* in a suspension consisting of olive oil (0.5 mL/kg), glycerol (1.25 mL/kg), and β-cyclodextrin (0.25 mmol/kg). Cisplatin in PBS was administered to mice by intraperitoneal injection (10, 20, and 40 mg/kg). For the measurement of renal levels of GSH and gene expression patterns, mice were sacrificed 24 h after treatment with D3T. For the measurement of the renal toxicity of cisplatin, mice were pre-treated with vehicle or D3T (0.25 mmol/kg) at day 1 and cisplatin administration was followed at day 2. For the repeated administration of D3T, mice were treated with an additional dose of D3T (0.25 mmol/kg) at day 3. Mice were sacrificed at day 5, which is 72 h after cisplatin administration. Blood and kidneys were collected for measurements of renal function and RNA extraction and GSH quantification, respectively. Animal experiment was performed according to the institutional guidelines for the Care and Use of Laboratory Animals as adopted by the Unites Sates National Institute of Health and the Yeungnam University Animal Care and Use Committee.

2.4. Total RNA extraction and RT-PCR analysis

Total RNA was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, kidneys from D3T or cisplatin-treated mice were homogenized in a Trizol reagent using a T25 Ultra-turax (IKA, Staufen, Germany), and RNA extraction was performed. The reverse transcriptase reaction was performed using 200 ng of total RNA in reaction mixture of nuclease free water, 10× PCR buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl), 50 mM $MgCl_2$, 100 mM dNTPs, 0.5 μg/μL oligo $(dT)_{12–18}$ (Promega, Madison, WI, USA), and 200 U/μL Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). PCR amplification of each gene was carried out using a Thermal Cycler (MyCycler, Bio-Rad) and amplification conditions were 27–30 cycles of 40 s at 95 °C, 30 s at 56 °C and 40 s at 72 °C. Primers were synthesized by Integrated DNA Technology (Coralville,

Iowa, USA) or Bioneer (Daejeon, South Korea): GCLC, 5′-ATGATGCCAACGAGTCTGAC-3′ and 5′-CGCCTTTGCAGATGTCTTTC-3′; GCLM, 5′-AGGAGCTTCGGGACTGTATT-3′ and 5′-TGGGCTTCAATGTCAGGGAT-3′; NQO1, 5′-ATCCTTCCGAGTCATCTCTA-3′ and 5′-CAACGAATCTTGAATGGAGG-3′; glutathione reductase 1 (GR1), 5′-GGCATGATAAGGTACTGAGA-3′ and 5′-TTCGTCTACTAGGATGTGGC-3′; β-actin, 5′-GCAGAAGGAGATTACTGCTC-3′ and 5′-CTAGAAGCACTTGCGGTGCA-3′; MT-1, 5′-TGGACCCCAACGCTCCTG-3′ and 5′-GCACAGCACGTGCACTTG-3′. Generated PCR products were resolved on 1.2% agarose gels and the images were captured by using a Visi Doc-It™ Imaging System (UVP, Upland, CA). Image intensities were quantified using a Image J software (National Institute of Mental Health, Bethesda, MD, USA).

2.5. Preparation of nuclear extract

TCMK-1 cells were incubated with D3T for 6 h and nuclear proteins were extracted as described previously [24]. Briefly, crude nuclear fractions were obtained by lysing the cells with homogenization buffer (2 M sucrose, 1 M HEPES, 2 M $MgCl_2$, 2 M KCl, 30% glycerol, 0.5 M EDTA, 1 M DTT, protease inhibitor cocktail, and 10% NP40) and followed by centrifugation at 12,000 × *g* for 15 min.

2.6. Immunoblot analysis

Nuclear proteins separated on 6% or 12% SDS-polyacrylamide gels were transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Membranes were blocked with 5% skimmed milk for 1 h in TPBS buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L KH_2PO_4, 0.24 g/NaH_2PO_4 and Tween-20 2 mL/L). Following incubation with the primary and secondary antibodies, the detection was done using Enhanced Chemiluminescence Reagent (Amersham Biosciences, Buckinghamshire, UK).

2.7. DNA transfection and luciferase activity

Cells were plated in 24-well plates at a density of 6×10^4 cells/well. The transfection complex containing 0.5 μg Nqo1 ARE-luciferase plasmid and 0.8 μL Lipofectamine™ 2000 (Invitrogen) was prepared in Opti-MEM® I medium (Invitrogen). At 60% of cell confluence, the transfection complex was added in to each well and incubated with cells for 24 h. After the recovery for 6 h in the complete medium, cells were treated with vehicle (DMSO) or D3T (5 and 10 μM) for 24 h. Obtained cell lysates were used for the measurement of the luciferase activity (Promega) by using a 20/20^n Luminometer (Turner BioSystems, Sunnyvale, CA).

2.8. Measurement of total GSH

Cells were grown in 96-well plates for 24 h and treated with D3T or cisplatin for a further 24 h. Cells were then washed with PBS and 30 μL of 5% metaphosphoric acid (MPA) solution was added to each well. For measuring GSH content, optical densities were monitored for 4 min following addition of 30 μL DTNB, GR and β-NADPH [25]. Protein concentration was determined by BCA™ protein assay kit (Pierce, Rockford, IL, USA). Total GSH content in mouse kidney was measured in tissue homogenates prepared in a 5% MPA solution.

2.9. MTT assay

Cells were plated at a density of 5×10^3 cells/well in 96-well plates. The next day, cells were treated with vehicle (DMSO) or D3T (5, 10, and 20 μM) for 24 h and then cisplatin treatment was followed for a further 9 h. MTT solution (2 mg/mL) was added into each well and cells were incubated for 4 h. After removing the MTT solution, 100 μL DMSO was added and absorbance was measured at 540 nm using a Versamax Microplate Reader (Sunnyvale, CA, USA).

2.10. Measurement of renal function

Renal injury was monitored by measuring levels of blood urea nitrogen (BUN) and creatinine. Blood was collected from D3T or cisplatin-treated mice from the retro orbital sinus or from the vena cava. Obtained blood was kept at room temperature for coagulation and centrifuged at 6000 × *g* for 20 min for serum preparation. Then, BUN-E (ASAN, South Korea) and DICT-500 (BioAssay Systems, Hayward, CA, USA) were added into serum samples for measuring levels of BUN and serum creatinine, respectively. Optical density values of BUN were measured at 570 nm using a UV–vis Spectrophotometer (Shimadzu, JAPAN). Levels of serum creatinine were measured at 510 nm using a Versamax Microplate Reader.

2.11. Histology and histomorphometry evaluation

Kidney was fixed in 10% neutral buffered formalin and a paraffin embedding was carried out. Tissue sections with 3–4 μm thickness were stained with Hematoxylin and Eosin for microscopic evaluation. Percentage of degenerative regions of kidney parenchyma (mm^2 of kidney parenchyma) and abnormal desquamated tubule numbers among 1000 observed tubules were measured using digital image analyzer (DMI-300, DMI, South Korea).

2.12. Statistical analysis

Statistical significance was determined by paired *t*-test or one-way ANOVA followed by Newman–Keuls multiple comparison test (GraphPad Prism4, San Diego, CA, USA).

3. Results

3.1. The expression of Nrf2-target genes was increased following treatment with D3T in murine kidney epithelial cells

We investigated whether D3T activates the Nrf2 pathway and induces the expression of Nrf2-target genes in cultured murine renal cells. Tubular epithelial TCMK-1 cells were isolated from the kidney of mouse and were immortalized by transformation with SV40 [26]. Concentrations of 2.5–20 μM D3T did not significantly affect cell survival of TCMK-1 cells (data not shown) and were used for the subsequent *in vitro* studies. First, in an attempt to evaluate the effect of D3T on the

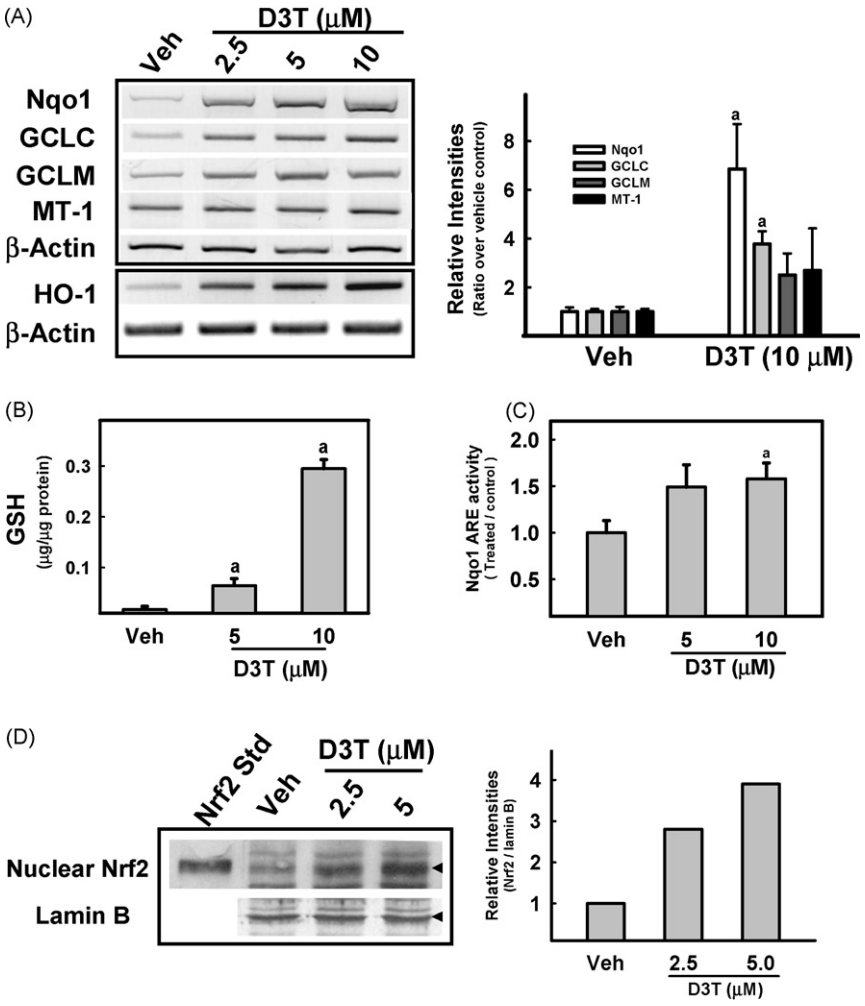

Fig. 1 – D3T induced the expression of Nrf2-target genes and level of total GSH in murine kidney tubular epithelial cells (TCMK-1). (A) Effect of D3T on transcript levels for Nrf2-target genes. Transcript levels of Nqo1, GCLC, GCLM, MT-1 and β-actin were measured using RT-PCR analyses following treatment with vehicle (Veh, DMSO) or D3T (2.5, 5, 10 μM) for 24 h. Levels of HO-1 were measured following incubation of cells with D3T (2.5, 5, 10 μM) for 4 h. Relative intensities were obtained following normalization by levels of β-actin. Values are mean ± S.E. from three experiments. [a]P < 0.05 compared with vehicle control. (B) Total GSH levels following treatment with D3T. TCMK-1 cells were treated with vehicle (Veh, DMSO) or D3T (5 and 10 μM) for 24 h, and total GSH content was measured using the kinetic enzymatic recycling assay based on the oxidation of GSH by DTNB. Measured total GSH contents were normalized by protein amounts. Values are mean ± S.E. from three experiments. [a]P < 0.05 compared with vehicle control. (C) Effect of D3T on ARE-driven luciferase activities. TCMK-1 cells were transfected with the ARE containing reporter plasmid and were incubated with vehicle (Veh, DMSO) or D3T (5 and 10 μM) for 24 h. *Firefly* luciferase activities were measured in cell lysates. Values are mean ± S.E. from three experiments. (D) Effect of D3T on nuclear Nrf2 levels. Cells were incubated with vehicle (Veh, DMSO) or D3T (2.5 and 5 μM) for 6 h, and cellular crude nuclear extracts were used for immunoblot analysis of nuclear Nrf2 or lamin B. Relative band intensities were obtained following normalization by levels of lamin B.

Nrf2 system, transcript levels for Nrf2-target genes were examined following treatment with D3T by using RT-PCR analysis. Incubation of cells with D3T for 24 h caused a concentration-dependent increase in Nqo1, which is a prototypic Nrf2-target gene in many cell types (Fig. 1A). Transcript levels for GSH synthesizing enzyme GCLC were also elevated by D3T treatment compared to vehicle control. By contrast, transcript levels for MT-1 were not affected by D3T treatment in these cells. Elevation of heme oxygenase-1 (HO-1) which is an early response antioxidant and anti-inflammatory enzyme in animal was also observed following incubation of cells with D3T for 4 h. In accordance with the increase in GCLC following D3T treatment, total content of cellular GSH was significantly enhanced: about 17-fold following 10 μM D3T incubation for 24 h (Fig. 1B). These results indicate that enhanced expression of GSH synthesizing enzymes by D3T treatment increased the total pool of GSH in murine kidney epithelial cells. Next, in order to determine whether D3T-induced expression of antioxidant genes is mediated by Nrf2, effects of D3T on ARE activation and nuclear Nrf2 accumulation have been

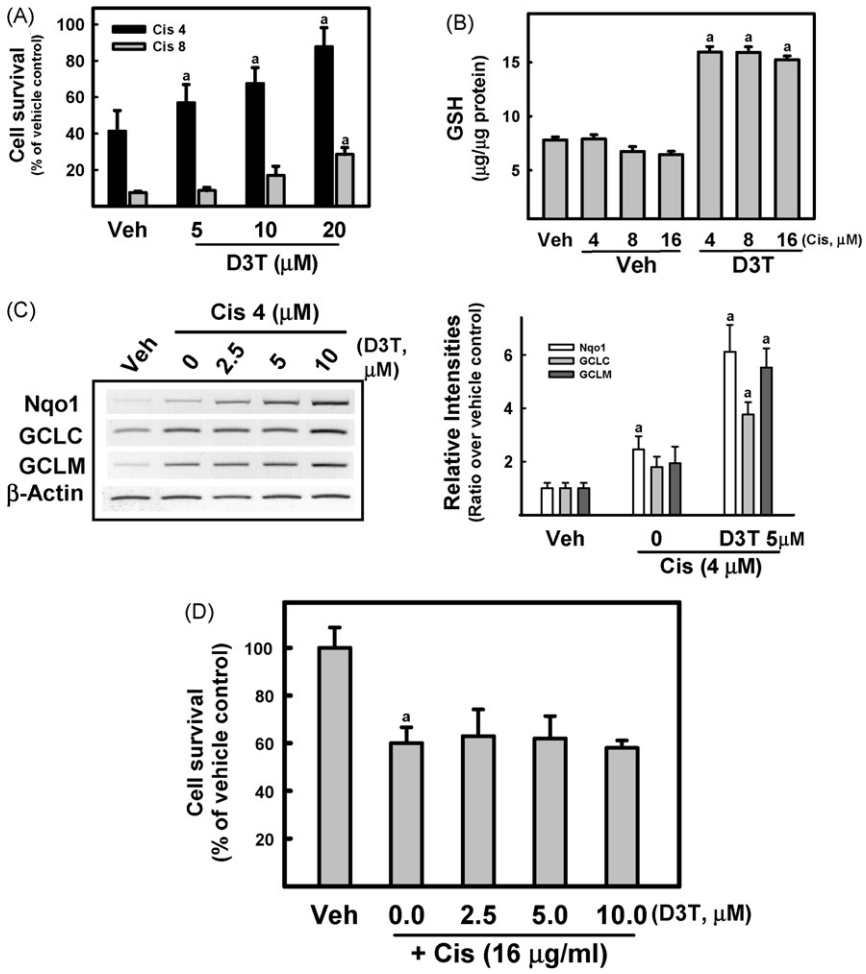

Fig. 2 – D3T protected murine renal epithelial cells from cisplatin-mediated cytotoxicity. (A) Effect of D3T on cell survival following treatment with cisplatin. Cells were treated with vehicle (Veh, DMSO) or D3T (5, 10 and 20 μM) for 24 h, and cisplatin treatment (4 and 8 μM) was followed for the next 24 h. Cell viability was monitored by MTT analysis. Values are mean ± S.E. from eight measurements. $^{a}P < 0.05$ compared with cisplatin alone group. (B) Effect of D3T pre-treatment on total GSH level in cisplatin-treated TCMK-1 cells. Cells were treated with vehicle (Veh, DMSO) or D3T (10 μM) for 24 h. Then, cisplatin (4, 8, and 16 μM) was added and incubated for a further 24 h. Contents of total GSH were measured by using DTNB. Values are mean ± S.E. from three experiments. $^{a}P < 0.05$ compared with vehicle control. (C) Effect of D3T and cisplatin on transcript levels of Nrf2-target genes. Cells were pre-incubated with vehicle (Veh, DMSO) or D3T (2.5, 5 and 10 μM) for 24 h and cisplatin incubation (4 μM) was followed for 9 h. Relative intensities were obtained following normalization by levels of β-actin. Values are mean ± S.E. from three experiments. $^{a}P < 0.05$ compared with vehicle control. (D) Cell viability measured in human ovarian cancer SK-OV3 cells. Cells were pre-incubated with D3T (2.5, 5, and 10 μM) for 24 h, and cisplatin (53 μM)-incubation was followed for a further 24 h. Cell viability was assessed by MTT analysis.

examined in these cells. The luciferase reporter plasmid containing the ARE from the rat *Nqo1* was transfected into TCMK-1 cells, and luciferase activities were measured in cell lysates following incubation with D3T for 24 h. D3T elevated the Nqo1 ARE reporter expression with 1.5-fold increase at 10 μM D3T (Fig. 1C). This low inducible level of the ARE reporter gene expression might be due to the SV-40 antigen in TCMK-1 cells. Increased replication of transfected plasmid in these cells increased the basal expression of the ARE-luciferase gene with a great extent, which might result in the interference with the inducible expression of this plasmid following D3T treatment. Activation of Nrf2 by D3T in TCMK-1 cells could be confirmed in immunoblot analysis of nuclear levels of Nrf2. D3T incubation (2.5 and 5 μM) for 6 h increased levels of nuclear Nrf2 compared to vehicle control by up to 4-fold (Fig. 1D). Taken together, these results indicate that D3T can activate the transcription factor Nrf2 in murine kidney epithelial cells and as a consequence, elevate the expression of Nrf2-target genes and levels of total GSH.

3.2. *D3T protected murine kidney epithelial cells from cisplatin-mediated cytotoxicity*

Next, we examined the potential protective effect of D3T on cisplatin-mediated cytotoxicity in TCMK-1 cells. First, the IC_{50} value of cisplatin was determined to be 3 μM in these

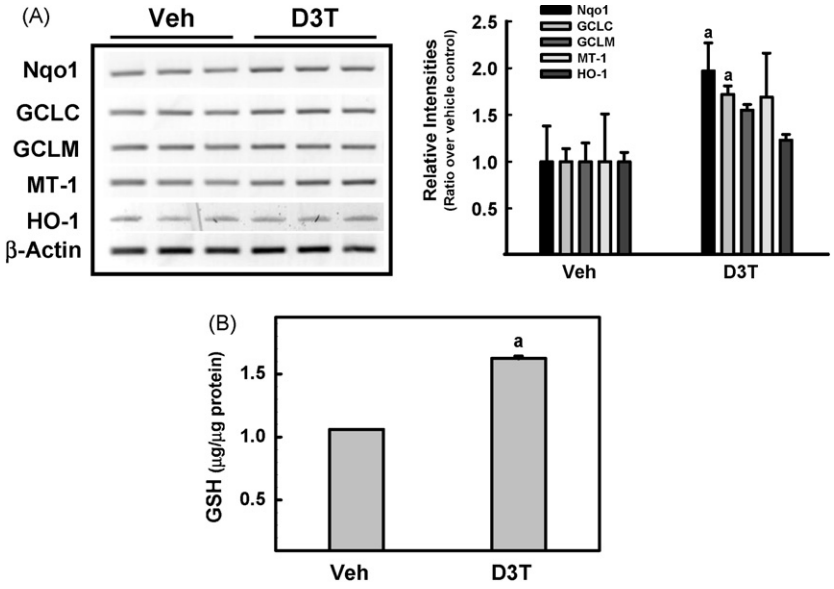

Fig. 3 – D3T induced the expressions of Nrf2-target genes and increased total GSH levels in the kidney of mice. (A) Effect of D3T on renal transcript levels of Nrf2-target genes in mice. Mice were treated with vehicle (Veh) or D3T (0.25 mmol/kg) by *gavage* in a suspension consisting of olive oil (10% of total volume), glycerol (5 mL/kg, 25% of total volume) and β-cyclodextrin (1:1 molar ratio). Mouse kidneys were isolated and total RNAs were obtained. Transcript levels of Nqo1, GCLC, GCLM, MT-1, HO-1 and β-actin were measured using RT-PCR analyses. $^{a}P < 0.05$ compared with vehicle control. (B) Renal GSH levels following treatment with D3T in mice. Levels of total GSH were measured using the kinetic enzymatic recycling assay based on the oxidation of GSH by DTNB in tissue homogenates from kidney. Measured total GSH contents were normalized by protein amounts and expressed as amounts of GSH per μg protein. Values are mean ± S.E. from three experiments. $^{a}P < 0.05$ compared with vehicle control.

cells, and subsequent studies used 4 and 8 μM cisplatin to assess the protective effect of D3T on cisplatin cytotoxicity. Cells were pre-incubated with D3T (5, 10, and 20 μM) for 24 h and cisplatin treatment followed for a further 24 h. Pre-incubation with D3T protected cells from cisplatin (4 μM)-mediated cell death: complete protection was observed with 20 μM D3T, while lower concentrations produced partial protections (Fig. 2A). Significant cytoprotection was also shown against a higher concentration of cisplatin (8 μM). D3T-pre-treated cells maintained elevated levels of GSH pool in the presence of cisplatin, indicating that the cytoprotective effect of D3T might be associated with elevation of GSH pool (Fig. 2B). It is notable that cisplatin treatment increases the expression of some of Nrf2-target genes (Fig. 2C). Transcript levels for Nqo1 were elevated by incubation with 4 μM cisplatin for 24 h; further increases were observed in the D3T-pre-treated group. In case of GCLM and GCLC, elevation of transcripts was only observed in D3T-pre-treated cells (Fig. 2C). This protective effect of D3T in renal cells can be argued by the potential of Nrf2 activators to reduce the anticancer efficacy of cisplatin in cancer cells. In an effort to determine this possibility, human ovarian cancer SK-OV3 cells were pre-incubated with D3T (2.5, 5, and 10 μM) and cisplatin (16 μg/mL)-mediated cytotoxicity was evaluated by using MTT analysis. SK-OV3 cells were known to have high levels of the basal expression of Nrf2-target genes with enormous increases in total GSH pool [27]. As a result, pre-incubation with D3T did not show any protective effect on cisplatin-mediated cytotoxicity in these cancer cells. Collectively, these results suggest that the D3T-mediated increase in total GSH may contribute to cytoprotection against cisplatin.

3.3. *The expression of Nrf2-target genes was increased in D3T-treated mice*

Next, we investigated whether D3T treatment can induce the expression of Nrf2-target genes in the kidney of mice. ICR mice were treated with vehicle or D3T (0.25 mmol/kg) by *gavage* and sacrificed 24 h after treatment. The transcript levels for Nrf2-target genes were determined in the kidney by using RT-PCR analysis. Levels of renal Nqo1 were increased 2-fold in D3T-treated mice (Fig. 3A). Levels for GCLC were also elevated by 1.7-fold in D3T-treated mice. The expression of cisplatin-detoxifying MT-1 appears to be altered by D3T; however there was no statistically significant difference between vehicle-treated and D3T-treated group. In accordance with increases in GCLC mRNA, renal levels of total GSH were increased 1.6-fold following treatment of mice with D3T (Fig. 3B). A higher dose of D3T (0.5 mmol/kg) did not show further increases in the expression of Nrf2-target genes, as well as total GSH content (data not shown). Taken together, these results indicate that oral administration of D3T into mice can elevate the expression of Nrf2-target genes, and can lead to enhancement of total GSH in the kidney.

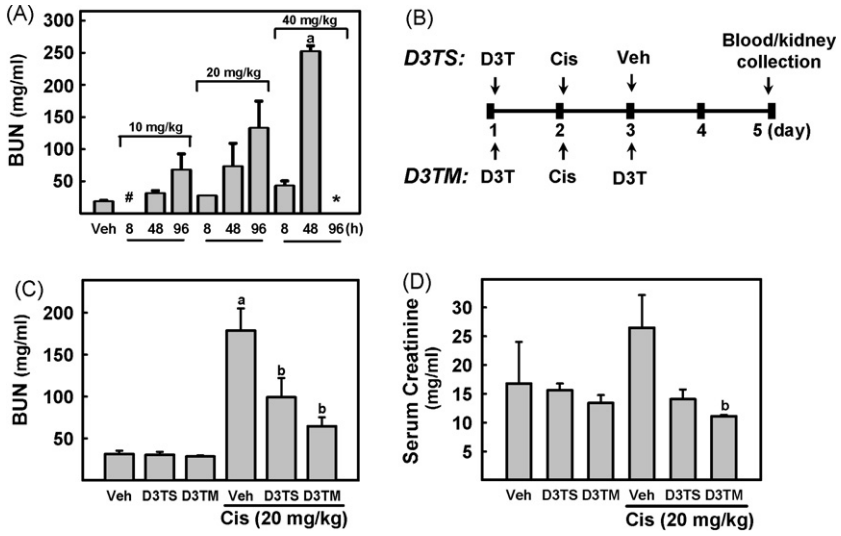

Fig. 4 – D3T protected the kidney from cisplatin-mediated toxicity in mice. (A) Cisplatin-mediated renal toxicity in mice. Mice were treated with vehicle (Veh, saline) or cisplatin (10, 20 and 40 mg/kg) by intraperitoneal injection and the blood was collected from the retro-orbital sinus 8 h, 48 h and 96 h after cisplatin. The degree of cisplatin-mediated renal toxicity was monitored by measuring the levels of BUN (blood urea nitrogen). Values are mean ± S.E. from three animals. $^{a}P < 0.05$ compared with vehicle group. $^{\#}$Not determined. *Mice died 48–96 h after cisplatin in 40 mg/kg group. (B) Dosing schedule of D3T and cisplatin. At day 1, mice were treated with vehicle or D3T (0.25 mmol/kg) by *gavage* in a suspension consisting of 10% olive oil, 25% glycerol (5 mL/kg) and β-cyclodextrin (1:1 molar ratio). Cisplatin (20 mg/kg, *i.p.*) was injected into mice at day 2. Then at day 3, mice in the single administration group (D3TS) were treated with vehicle, and mice in the multiple administration group (D3TM) were treated with D3T. Mice were sacrificed at day 5 (72 h after cisplatin) and the kidneys and blood were collected. (C) Levels of BUN in the serums obtained from D3T- and cisplatin-treated mice. Values are mean ± SE from 3 to 4 individual animals. $^{a}P < 0.05$ compared with vehicle group. $^{b}P < 0.05$ compared with cisplatin alone group. (D) Levels of serum creatinine in the serum obtained from D3T- and cisplatin-treated mice. Values are mean ± S.E. from 3 to 4 individual animals. $^{b}P < 0.05$ compared with cisplatin alone group.

3.4. *D3T treatment protected mice from cisplatin-induced renal toxicity*

Next, the protective effect of D3T against cisplatin-induced renal toxicity was evaluated in an animal model. In order to characterize cisplatin-induced nephrotoxicity in ICR mice, animals were injected with cisplatin (10, 20, and 40 mg/kg, *i.p.*) and renal injury was monitored 8, 48, and 96 h after cisplatin by measuring levels of blood urea nitrogen (BUN), which is a marker of renal dysfunction. The level of BUN was gradually elevated in cisplatin-treated mice, with a maximal increase observed 96 h after cisplatin administration (Fig. 4A). The increase of BUN was proportional to dose of cisplatin: 1.7-, 4.1-, and 14.1-fold increases were observed in mice treated with 10, 20, and 40 mg/kg cisplatin, respectively. All mice treated with 40 mg/kg cisplatin died 48–96 h after cisplatin due to a severe toxicity (Fig. 4A). Therefore, to evaluate the protective effect of D3T, mice were injected with 20 mg/kg cisplatin and renal toxicity was monitored 72 h later cisplatin. Our previous result indicated that 0.25 mmol/kg D3T effectively increased Nrf2-target gene expression, as well as total GSH in the kidney. Therefore, for the single dosing group, 0.25 mmol/kg D3T was administered to mice 24 h prior to cisplatin challenge (D3TS, Fig. 4B). For the multiple dosing group, additional administration of D3T was followed 24 h after cisplatin (D3TM). First, D3T alone treatment did not alter levels of BUN and serum creatinine, indicating that D3T itself does not affect the renal function of mice (Fig. 4C and D). Second, the single or multiple dosing of D3T could suppress cisplatin-induced increases in BUN and serum creatinine. Cisplatin-mediated increase of BUN was inhibited by 45% and 64% following the single and multiple dosing of D3T, respectively. A similar pattern was observed in serum creatinine levels, and repeated dosing of D3T could suppress the increase of serum creatinine by up to 58%. Next, the histological profiles of kidney were also examined in mice. Cisplatin treatment induced acute renal failure (ARF)-related histopathological changes such as epithelial necrosis and desquamation affecting cortex, glomeruli, and proximal/distal tubules (Fig. 5e and f). In addition, the percentage of degenerative regions and abnormal tubule numbers were significantly increased in cisplatin-treated mice: 12-fold and 78-fold increases in degenerative region percentages and abnormal tubule numbers were obtained, respectively (Table 1). These ARF-related changes were remarkably decreased by multiple dosing of D3T (Fig. 5i and j). Cisplatin-mediated changes in degenerative region percentages and abnormal tubule numbers were also repressed in multiple dosing group (Table 1). The single dose of D3T only showed a slight reduction in the ARF-related histopathological changes (Fig. 5g and h and Table 1). Collectively, these results suggest that oral administration of D3T can protect the kidney from cisplatin-mediated toxicity and preserves renal function

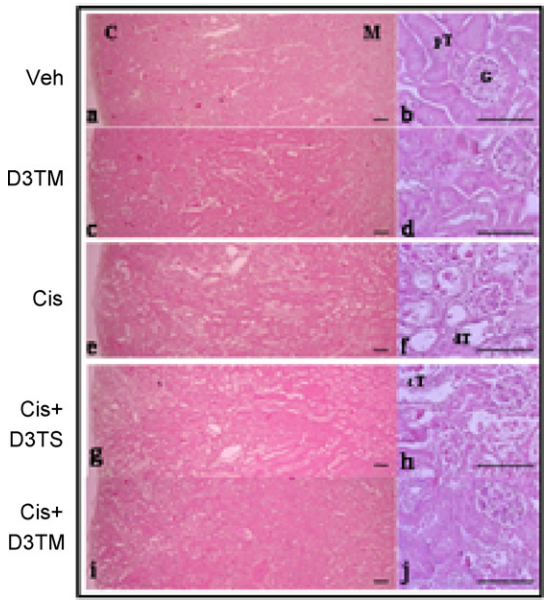

Fig. 5 – Histopathological changes of the kidney from D3T- and cisplatin-treated mice. Histological profiles were detected by H&E staining in mice treated with vehicle (a and b), multiple dosing of D3T (D3TM; c and d), cisplatin alone (e and f), cisplatin + D3TS (g and h), and cisplatin + D3TM (i and j). The glomeruli and tubules of cortex were severely and locally disrupted in cisplatin-treated mice. While, cisplatin-induced ARF-related histopathological changes were remarkably decreased by multiple dosing of D3T (D3TM). Different magnifications of histology were shown in each treatment group. C, cortex; M, medulla; G, glomeruli; pT, proximal convoluted tubule; dT, distal convoluted tubule; cT, collecting duct; Scale bar, 80 μm.

of mice. In particular, repeated administration of a low dose D3T might be more effective for this protection.

4. Discussion

Cisplatin produces a wide-range of cellular perturbations: DNA adduct formation, lipid peroxidation, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, cytoskeletal rearrangement, and oxidative stress [4,28–30]. The formation of inter- and intra-strand cross-links of DNA by the binding of cisplatin has been suggested to be largely responsible for cytotoxicity and mediates transcription inhibition, cell-cycle arrest, and activation of the apoptosis signal pathways [1]. Cell death by either necrosis or apoptosis occurs depending on the concentration of cisplatin. High concentrations of cisplatin (800 μM) led mouse proximal tubular cells to necrotic cell death within a few hours, while lower concentration of cisplatin (8 μM) caused apoptotic cell death over several days [31,32].

Nephrotoxicity occurs in approximately 20–41% of patients receiving cisplatin and is the major limitation in cisplatin-based chemotherapy. Accumulated cisplatin in the kidney affects the proximal tubules and distal nephron, and causes cell death. The production of reactive oxygen species (ROS) is associated with cisplatin-induced apoptotic cell death. Increased levels of ROS following cisplatin treatment can be explained by several observations. First, cisplatin reduced the activities of antioxidant enzymes such as manganese superoxide dismutase and glutathione (GSH) peroxidase [33]. Second, it has been demonstrated that GSH, which is the major cellular ROS-removing antioxidant within cells, is depleted in cisplatin-treated cells [9]. Third, cisplatin inhibits the respiratory chain in mitochondria, which can result in enhanced superoxide formation [28]. It has been reported that NADPH oxidase can be activated by cisplatin in some types of cells [34]. Based on these observations, oxidative stress is thought to be one of components participating in cisplatin-induced nephrotoxicity. Indeed, treatment with antioxidants delayed cisplatin-induced apoptosis in many studies. Pre-treatment with N-acetylcysteine and post-treatment with sodium thiosulfate were effective in protection of cisplatin-mediated renal toxicity *in vitro* and *in vivo* [35].

Now, it is widely accepted that the transcription factor Nrf2 governs the expression of various antioxidant proteins and plays a crucial role in the adaptive response of mammalian cells to oxidative and other stresses. Therefore, small molecule activators of Nrf2, which can be obtained from natural and synthetic sources, show striking protective efficacy against oxidative stress-associated damage [13,14]. Dithiolethiones including D3T and oltipraz are known to be potent chemopreventive agents that inhibit the carcinogenicity of various chemicals by modulating Nrf2-target gene expression. Due to their modulatory effects on Nrf2-target

Table 1 – Histomorphometric evaluation of cisplatin renal toxicity

Groups	Histomorphometry	
	Degenerative region percentages (%)	Abnormal tubule numbers (%)
Vehicle	7.3 ± 2.3	1 ± 0.7
D3TM	7.6 ± 2.0	1 ± 0.4
Cisplatin	85.9 ± 8.0[a]	78 ± 13.6[a]
Cisplatin + D3TS	79.7 ± 4.4	74 ± 7.0
Cisplatin + D3TM	68.2 ± 4.9[b]	57 ± 11.2

Values are mean ± S.D. $N = 5$ (5 fields of microscopy).
[a] $P < 0.01$ compared with vehicle control.
[b] $P < 0.01$ compared with cisplatin control.

gene expression and the subsequent antioxidative effect, these Nrf2 activators are often called indirect antioxidants. In our present study, D3T-treatment effectively activated the Nrf2 pathway in murine renal epithelial cells, and led to a protection against cisplatin (4–8 μM). In accordance with results obtained *in vitro* study, oral administration of D3T (0.25 mmol/kg) increased transcript levels for Nrf2-target genes and as a consequence, cisplatin-mediated renal dysfunction was largely ameliorated by D3T. These results indicate that pharmacological application of indirect antioxidants can be beneficial to protect normal tissues, in particular the kidney, from toxicities of cisplatin-based chemotherapy.

However, chemoresistance is often associated with increased levels of GSH and antioxidant proteins in cancer cells. It can be hypothesized that D3T-mediated increase in Nrf2-target gene expression might lead to a facilitated detoxification of cisplatin in cancer cells. Therefore, selective activation of the Nrf2 pathway in normal cells, not in cancer cells, would be a critical point to avoid chemoresistance. Several recent studies have proposed that some types of cancer cells accumulate Nrf2 within the nucleus, which result in constitutive activation of the target gene expression. Genetic mutations in *Keap1* have been identified in lung cancer cell lines and tumors, and these abnormal Keap1 proteins were found to be incapable of anchoring Nrf2 in the cytoplasm [36,37]. These observations provide a notion that indirect antioxidants may activate the Nrf2 pathway primarily in normal cells but not in tumor cells. In fact, our result obtained from human ovarian cancer cells, which are resistant to cisplatin treatment, were not rescued from cisplatin-mediated cytotoxicity by pre-incubating with D3T (Fig. 2D).

There have been several strategies to protect the renal toxicity of cisplatin-based chemotherapy. Exogenous administration of thiol compounds including GSH and sodium thiosulfate can protect cisplatin cytotoxicity in animal and human studies [35,38]. It has been shown that procainamide, which is a thiol-containing antiarrhythmic drug, reduces the nephrotoxicity of cisplatin [39]. Significant attenuation of cisplatin-induced renal dysfunction has been observed in several studies with the targeted delivery of superoxide dismutase into the proximal tubular and treatment with hydroxyl radical scavenger DMTU [40,41]. The beneficial effects of direct antioxidants in cisplatin-induced renal failure imply the involvement of oxidative stress by cisplatin. Our present study demonstrated that Nrf2 activator D3T can enhance the cellular antioxidative capacity in the kidney and significantly attenuated cisplatin-mediated ARF in mice. Increased renal levels of GSH in D3T-treated mice can account for the protective effects of this Nrf2 activator. GSH is the major source that detoxifies cisplatin through adduct formation and the subsequent efflux from the cell. MTs, which are cysteine-rich low-molecular-weight proteins, are also thought to be involved in the detoxication process of cisplatin [42]. Pre-administration of MT-inducing metals can suppress the renal toxicity of cisplatin. Furthermore, the sensitivity to cisplatin-mediated renal toxicity is increased in *MT*-deficient mice [43]. D3T administration to mice did not show significant increases in renal MT-1 levels, indicating that MT-1-mediated effect may not participate in the protective effect of D3T against cisplatin.

It has been demonstrated that single administration of cisplatin caused increased expression of cytokines such as TNFα, and chemokine receptors, and endothelial adhesion molecules ICAM-1/VCAM-1 in mice, indicating that inflammation is one of components participating in cisplatin-mediated cytotoxicity [44]. Indeed, administration of anti-inflammatory fibrate remarkably ameliorated cisplatin-induced ARF in the animal model [44,45]. Accumulating lines of evidence suggests that Nrf2-target antioxidant genes are strongly associated with the inhibition of inflammation. Disruption of *nrf2* in mice induces marked inflammatory injuries following exposure to LPS, carageenan, and cigarette smoke [46–48]. Furthermore, LPS-induced inflammatory response and mortality in mice were effectively suppressed by triterpenoid analog that activate Nrf2 signaling [49]. These results suggest that D3T treatment might modulate the inflammatory response by activating the Nrf2 pathway, which can result in an effective protection against cisplatin toxicity.

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