

Cantharidin alters the expression of genes associated with the NKG2D-associated immune response in TSGH-8301 human bladder carcinoma cells

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Abstract. Cantharidin (CTD) is a natural toxin in beetles of the *Mytilabris* genus (blister beetle), which has been revealed to induce cell death in various types of human cancer cells. However, to the best of our knowledge, no previous studies have investigated the effect of CTD on the expression of genes and their associated signaling pathways in human bladder carcinoma cells. In the present study, CTD-induced cell morphological changes and apoptosis were observed using phase-contrast microscopy and the terminal deoxynucleotidyl transferase dUTP nick end labeling assay, respectively, in TSGH-8301 human bladder carcinoma cells. In addition, a complementary DNA microarray analysis demonstrated that CTD treatment led to a >2-fold upregulation of 269 genes. For example, the DNA damage-associated gene DNA-damage-inducible transcript 3 had a 4.75-fold upregulation. Furthermore, another 286 genes

were >2-fold downregulated in response to CTD treatment. Matrix-remodeling associated 5, which is associated with cell migration and invasion, was downregulated 7.98-fold.

Introduction

Cancer has long been recognized as a major causes of mortality in humans. Bladder cancer, a heterogeneous neoplasm, is either a low-grade tumor located in the superficial mucosa (80% of cases) or a muscle-invasive carcinoma (20% of cases) (1). Urothelial carcinoma is the most common type of bladder cancer and is a major cause of morbidity and mortality (2). In Western populations, bladder cancer represents the fourth most common malignancy affecting males (3,4) and ranks thirteenth in terms of cancer-associated mortality worldwide (5,6). The majority (>90%) of bladder tumors are of epithelial origin and arise from the bladder urothelium (7). The 2014 report from the Ministry of Health and Welfare of Taiwan indicated that bladder cancer was one of the most common types of cancer in Taiwan in 2006, with 1,985 new cases and 681 mortalities (8). Currently, the majority of treatments for superficial bladder cancer are derived from natural products. The aim of this treatment is to reduce tumor recurrence and prevent tumor progression.

Cantharidin (CTD), a natural toxin, is secreted by beetles of the *Mytilabris* genus (blister beetle) and has been used as a traditional drug to treat *Molluscum contagiosum* viral infections and cancer in China and Vietnam (9,10). For treating superficial bladder cancer, the correct dosage of CTD is important; an overdose of CTD may lead to mortality (11,12). A number of studies have demonstrated that CTD induces cytotoxic effects

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on numerous human cancer cell lines. CTD was demonstrated to repress cancer cell growth through cell cycle arrest and the induction of apoptosis in cancer cells, including pancreatic cancer (13-15), leukemia U937 (16), human HepG2 (17), human colon cancer colo205 (18), human lung cancer A549 (19) and human bladder cancer (20) cells, in addition hepatocellular carcinoma *in vivo* (21). It has been reported that tamoxifen represses the phosphorylation of protein kinase C (PKC) and amplifies the anticancer effect induced by CTD (22). Previous studies have revealed a CTD-induced cytotoxic effect in melanoma A375.S (23), lung cancer NCI-H460 (24) and bladder cancer TSGH-8301 (25) cells.

Cancer arises from alterations in the structure, expression and function of tumor suppressor genes (26). A number of genetic mutations have been identified as biomarkers for the diagnosis and treatment of certain types of human cancer, including glioblastoma multiforme, but intratumoral heterogeneity presents challenges for personalized treatment strategies (27). Furthermore, genetic mutations in oncogenes and tumor suppressors have been identified in numerous types of cancer cells (28,29). It has been determined, through constructing a protein-protein interaction network of differentially expressed genes and co-expressed genes, that G₂/M phase-specific cyclin B1 and H2A histone family member Z are significantly associated with bladder cancer (30). A previous study suggested that fibroblast growth factor receptor 2 gene mutations in bladder cancer may only serve a minor role in bladder carcinogenesis (31). Another previous study demonstrated that CTD alters gene expression in human lung cancer NCI-H460 cells *in vitro* (32). It has also been hypothesized that CTD alters the gene expression of bladder cancer cells; however, to the best of our knowledge this has not yet been tested. In the present study, the effects of CTD treatment on NKG2D-associated immune response in TSGH-8301 human bladder carcinoma cells were investigated, including its effects on gene expression.

Materials and methods

Chemicals and reagents. CTD, propidium iodide, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). All organic solvents used were of high performance liquid chromatography grade. RPMI-1640 medium, fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Tissue culture plastic wares were obtained from TPP Techno Plastic Products AG (Trasadingen, Switzerland). The ApoBrdU DNA Fragmentation Assay kit [terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)] was obtained from BioVision, Inc. (Milpitas, CA, USA). CTD was dissolved in DMSO and stored at -20°C prior to use.

Human bladder cancer cell culture. TSGH-8301 human bladder carcinoma cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in T75 tissue culture flasks with RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml

Table I. Number of genes that were upregulated or downregulated in TSGH-8301 cells following treatment with 7.5 µM CTD compared with untreated control cells.

A, Upregulated genes		
Fold change	Number of genes	Total
≥5 and <10	7	269
≥4 and <5	12	
≥3 and <4	19	
≥2 and <3	231	
B, Downregulated genes		
Fold change	Number of genes	Total
>-3 and ≤-2	233	286
>-4 and ≤-3	34	
>-5 and ≤-4	11	
>-10 and ≤-5	8	

streptomycin). Cell cultures were maintained in a humidified incubator at 37°C with 5% CO₂.

Cell morphology assay. Cell morphology was examined as described previously (25). Briefly, TSGH-8301 cells (1x10⁵ cells/well) were trypsinized, seeded into a 12-well plate with RPMI-1640 medium and cultured for 24 h. The culture medium was then replaced with fresh medium containing CTD (7.5 µM) and the plates were incubated for 12, 24 and 48 h at 37°C. TSGH-8301 cell morphology was subsequently examined using an Olympus phase-contrast microscope (Olympus Corporation, Tokyo, Japan).

TUNEL assay for cell apoptosis. The TUNEL assay was performed with the ApoBrdU DNA Fragmentation Assay kit according to the manufacturer's protocol (33). TSGH-8301 cells were seeded at a density of 1x10⁵ cells/well onto coverslips, cultured with 7.5 µM CTD for 48 h and then fixed with 4% formaldehyde in PBS for 15 min, permeabilized with 0.1% (v/v) Triton X-100 for 1 h and washed with PBS. TUNEL staining solution was added to the coverslips to bind the fluorescein-dUTP to the DNA break terminals. TUNEL-positive cells were examined and images captured using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany), using an excitation wavelength of 488-623 nm and a detection wavelength of 488-520 nm.

Complementary (c)DNA microarray assay. TSGH-8301 cells were seeded into a 10 cm dish at a density of 1.5x10⁶ cells/dish with RPMI-1640 medium and cultured for 24 h. Cells were treated with 7.5 µM CTD for 48 h and then collected and washed twice with PBS. Total RNA from the untreated control and CTD-treated groups were extracted using a Qiagen RNeasy Mini kit according to the manufacturer's protocol (Qiagen, Inc., Valencia, CA, USA). RNA concentrations were

Table II. Representative genes that were upregulated or downregulated in response to CTD treatment in TSGH-8301 cells.

Probe set ID	Gene symbol	Fold change	Gene description
7998927	TRNAP24P	6.7	Transfer RNA proline 24 (anticodon AGG) pseudogene
7903530	FNDC7	6.59	Fibronectin type III domain containing 7
8114572	HBEGF	6.15	Heparin-binding EGF-like growth factor
7973433	DHRS2	6.08	Dehydrogenase/reductase (SDR family) member 2
7909610	ATF3	5.41	Activating transcription factor 3
8092578	ETV5	5.26	Ets variant 5
8122724	ULBP1	5.11	UL16 binding protein 1
7982868	CHAC1	4.89	ChaC, cation transport regulator homolog 1 (<i>E. coli</i>)
8160912	C9orf131	4.82	Chromosome 9 open reading frame 131
7964460	DDIT3	4.75	DNA-damage-inducible transcript 3
7963534	KRT4	-4.76	Keratin 4
7960919	MFAP5	-4.79	Microfibrillar associated protein 5
8040430	VSNL1	-5.25	Visinin-like 1
8015337	KRT15	-5.28	Keratin 15
8115623	ATP10B	-5.36	ATPase, class V, type 10B
8058857	IGFBP5	-5.41	insulin-like growth factor binding protein 5
8136336	AKR1B10	-5.48	Aldo-keto reductase family 1, member B10 (aldose reductase)
8104758	NPR3	-5.78	Natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)
8113709	LOX	-6.35	Lysyl oxidase
8171172	MXRA5	-7.98	Matrix-remodelling associated 5

A positive fold-change number indicates upregulation and a negative fold-change number indicates downregulation.

determined using a Qubit™ Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.) as described previously (32). For cDNA synthesis, the oligo (dT) Maxime RT premix kit according to the manufacturer's protocol (Intron Biotechnology, Inc., Seongnam, Korea) was used. The samples were hybridized to the Affymetrix GeneChip® Human Gene 1.0 ST array as described previously (Affymetrix, Inc., Santa Clara, CA, USA) (34), then the sample fluorescence was quantified by Asia BioInnovations Corporation (Taipei, Taiwan). The data was analyzed using Affymetrix® Expression Console™ software (Soft version 1.1.2; Affymetrix, Inc.) with default robust multiarray parameters. Following comparison of the control group with the CTD treated group, a 2-fold change in gene expression was used as the threshold to indicate a significant effect on expression (7-10).

Statistical analysis. Data are representative of three assays. Differences between control and CTD-treated groups were presented when they were >2-fold.

Results

CTD induces morphological changes in TSGH-8301 cells in vitro. Following treatment with 7.5 μ M CTD for 12, 24 and 48 h, cells were examined and imaged using phase-contrast microscopy at a magnification of x200 (Fig. 1). Control cells without CTD treatment were incubated under identical conditions. The results indicated that CTD induced

cell morphological changes, including cell size reduction, and decreased cell number in a dose-dependent manner.

CTD induces TSGH-8301 cell death in vitro. Following treatment with 7.5 μ M CTD for 48 h, cells were examined using a TUNEL assay (Fig. 2). TUNEL-positive cells were observed in the CTD-treated group, but not in the control group. The number of apoptotic nuclei (green color) was higher in the CTD treated group compared with that of the control group (data not shown). These observations indicate that CTD induces apoptotic cell death in TSGH-8301 cells.

CTD alters the expression of genes associated with the NKG2D-associated immune response in TSGH-8301 human bladder carcinoma cells. Following treatment with 7.5 μ M CTD for 48 h, total RNA was extracted from TSGH-8301 cells for cDNA microarray analysis in order to examine changes in gene expression. The genes that were upregulated or down-regulated, calculated from the microarray data, are displayed in in Tables I and II. A total of 269 genes were >2-fold upregulated and 286 genes were >2-fold downregulated (Table I). DNA-damage-inducible transcript 3 (DDIT3) was 4.75-fold upregulated, activating transcription factor 3 was 5.41-fold upregulated and dehydrogenase/reductase (SDR family) member 2 was 6.08-fold upregulated compared with the untreated control cells. Microfibrillar associated protein 5 was 4.79-fold downregulated, insulin-like growth factor binding protein 5 was 5.41-fold downregulated and matrix-remodeling

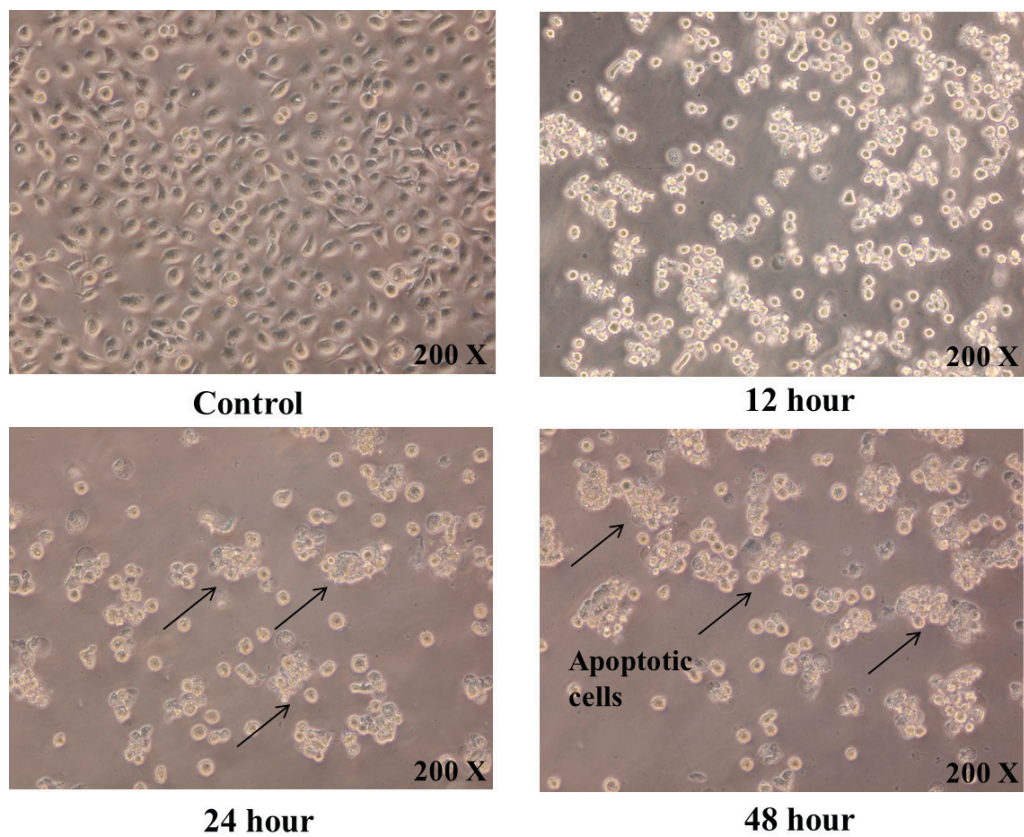


Figure 1. Morphology of TSGH-8301 human bladder carcinoma cells following treatment with 7.5 μ M cantharidin for 12, 24 and 48 h. Arrows indicate apoptotic cells. Cell morphology was examined using a phase-contrast microscope (magnification, x200).

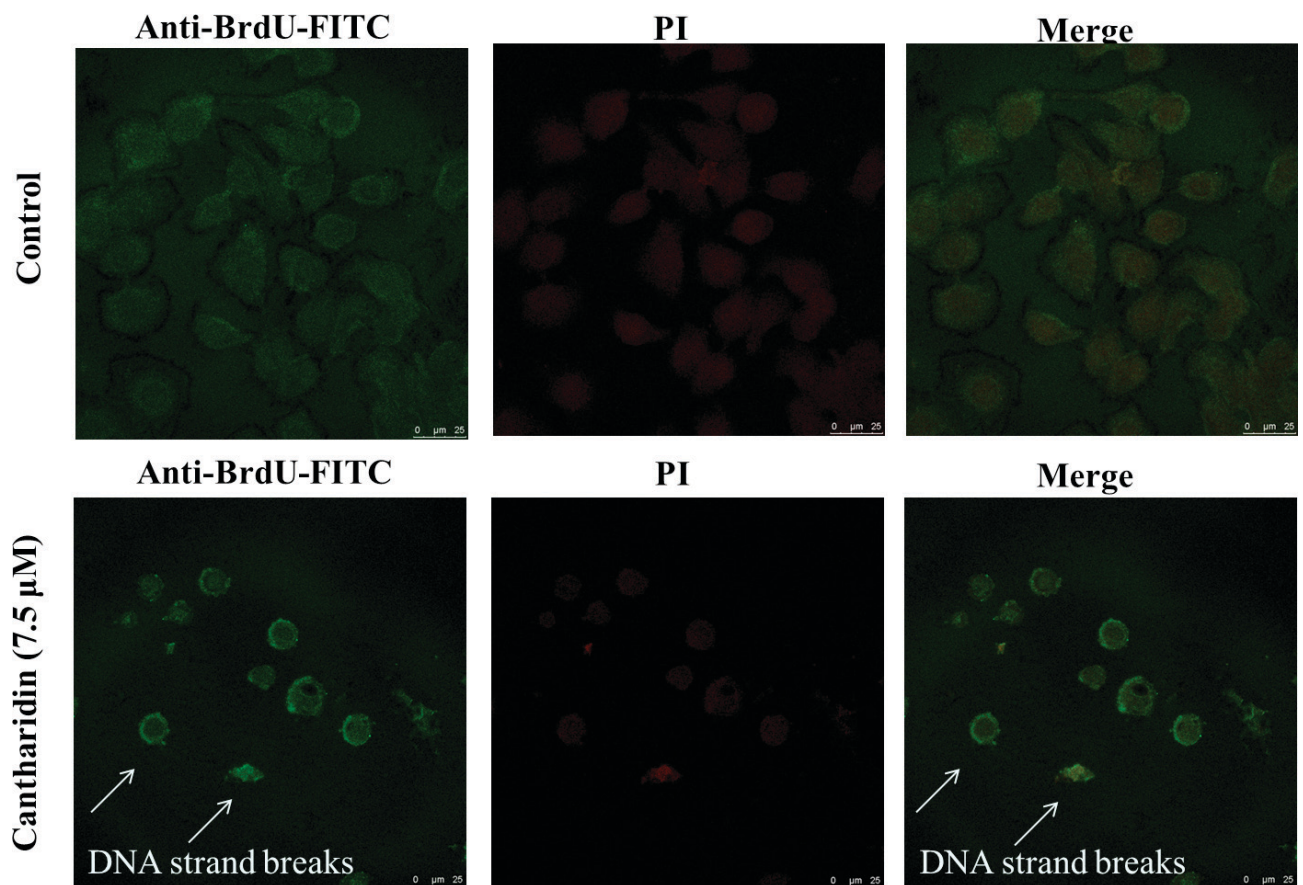


Figure 2. Cantharidin increases DNA fragmentation-associated BrdU-FITC signaling in TSGH-8301 cells compared with untreated control cells. BrdU, bromodeoxyuridine; FITC, fluorescein isothiocyanate; PI, propidium iodide.

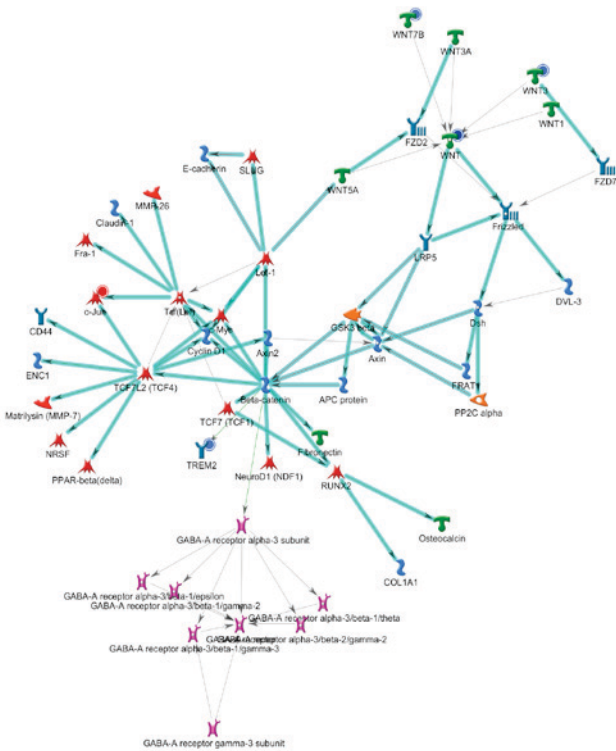


Figure 3. Top scored (by the number of pathways) Analyze Network network. Thick cyan lines indicate the fragments of canonical pathways. Upregulated genes are marked with red circles and downregulated genes with blue circles.



Figure 4. Second highest scored (by the number of pathways) Analyze Network network. Thick cyan lines indicate the fragments of canonical pathways. Upregulated genes are marked with red circles and downregulated genes with blue circles.

associated 5 (MXRA5) was 7.98-fold downregulated compared with the untreated control cells (Table II).

CTD-treatment alters signaling pathways in TSGH-8301 cells. In order to investigate the molecular signaling pathways associated with the genes whose expression was altered by CTD, a GeneGo analysis was performed. The analysis from GeneGo for immune responses for human NKG2D type II integral membrane protein signaling were the top, second and third highest scored Analyze Network (AN) networks (data not shown), by the number of pathways (Figs. 3-5). Therefore, NKG2D was selected as there were significant effects following CTD treatment compared with that of control.

Discussion

The results from the present study demonstrated that CTD induces cell morphological changes and apoptosis, as revealed using phase-contrast microscopy and a TUNEL assay, respectively, in TSGH-8301 human bladder carcinoma cells. Tumor cell lines are invaluable research tools that are able to be experimentally manipulated (35). The TSGH-8301 cells are derived from a well-differentiated human transitional cell carcinoma of the urinary bladder (36). In addition, CTD treatment resulted in the upregulation and downregulation of a number of immune response-associated genes. Numerous studies have demonstrated that CTD induces a cytotoxic effect in a number of human cancer cell lines. For example, CTD affects gene expression in human lung cancer NCI-H460 cells (32). Additionally, CTD induces cell apoptosis through mitochondrial-dependent signaling pathways (25), and inhibits

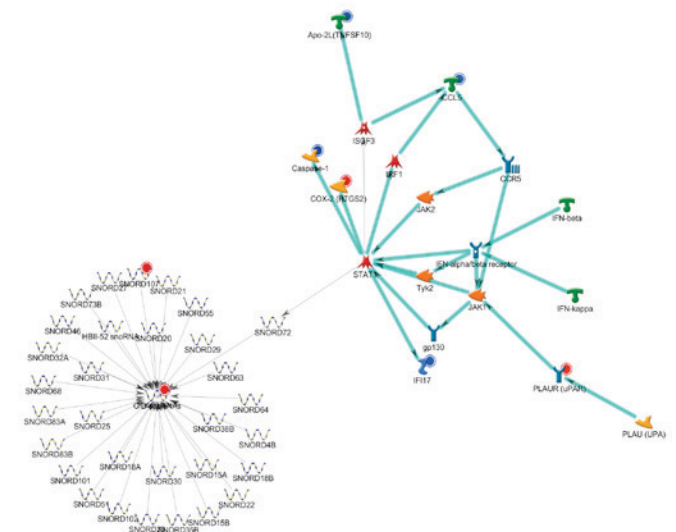


Figure 5. Third highest scored (by the number of pathways) Analyze Network network. Thick cyan lines indicate the fragments of canonical pathways. Upregulated genes are marked with red circles and downregulated genes with blue circles.

cell migration and invasion through the inhibition of the matrix metalloproteinase-2/-9 signaling pathway in human bladder cancer TSGH-8301 cells (37). Other studies in human bladder cancer T24 cells have also demonstrated that CTD induces apoptosis through the calcium/PKC-regulated endoplasmic reticulum stress signaling pathway (38). However, to the best of our knowledge no studies have demonstrated the effect of

CTD on gene expression in human bladder cancer cells. Thus, in the present study, the effect of CTD on gene expression in human bladder cancer TSGH-8301 cells was investigated *in vitro*.

In the present study, human bladder cancer TSGH-8301 cells were treated with 7.5 μ M CTD, followed by analysis of cell morphology and apoptotic cell death using phase-contrast microscopy and the TUNEL assay. The results demonstrated that CTD induces apoptotic cell death and morphological changes in human bladder cancer TSGH-8301 cells. Compared with the control group, cells treated with CTD exhibited increased DNA strand breaks, as visualized using an ApoBrdU DNA Fragmentation Assay kit. This result indicates that CTD induces apoptotic cell death via DNA fragmentation in TSGH-8301 cells.

The changes in cell morphology observed in the present study as a result of CTD treatment were dose-dependent. Additionally, CTD induced apoptotic cell death in a dose-dependent manner, as indicated by TUNEL staining.

A previous study demonstrated that CTD affects gene expression in the human lung cancer NCI-H460 cell line (32). Additionally, a recent study identified, using next generation sequencing, that human bladder cancer has numerous gene alterations compared with normal tissue, with most predicted to be loss-of-function mutations; however, evaluating the functional impact of each genetic alteration is impractical (39). The present study, to the best of our knowledge, is the first to demonstrate the effects of CTD on gene expression in human bladder TSGH-8301 cancer cells. Thus, the results from the present study offer an insight into CTD-induced gene expression changes in bladder cancer cells, which will aid in the identification of potential biomarkers for patients with bladder cancer that require targeted therapy.

A number of studies have shown that the matrix proteins, stromal cells and associated secreted molecules that comprise the tumor microenvironment serve a role in mediating responses to cancer drugs (40-42). Therefore, in the present study, changes in gene expression in TSGH-8301 cells following exposure to CTD for 24 h were analyzed using a cDNA microarray. CTD treatment resulted in an >2-fold upregulation of 269 genes and an >2-fold downregulation of 286 genes in TSGH-8301 cells. DDIT3, which is associated with DNA damage, was upregulated 4.75-fold and MXRA5, which is associated with cell migration and invasion, was downregulated 7.98-fold. In order to further investigate the genes whose expression was altered in response to CTD, and their associated molecular signaling pathways, a GeneGo process network analysis was performed. This suggested that CTD treatment affects a number of associated signaling pathways, including the NKG2D signaling pathway, in TSGH-8301 cells.

In conclusion, the data from the present study indicates that CTD induces cytotoxic effects in TSGH-8301 cells, based on the observed decrease in the number of cells and increase in apoptotic cell death in CTD-treated cells compared with control untreated cells. Additionally, CTD treatment resulted in the upregulation or downregulation of numerous genes that are associated with a number of immune response-associated molecular signaling pathways. These findings may aid in future research into the molecular targets of CTD.

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