ANTICANCER RESEARCH *41*: 4077-4082 (2021)
doi:10.21873/anticanres.15209

Knockdown of D-dopachrome Tautomerase Inhibits Cell Proliferation in Human HepG2 Cell Line

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Abstract. *Background/Aim: D-dopachrome tautomerase (DDT) is a macrophage migration inhibitory factor (MIF) homolog that promotes cell growth via CD74, a MIF cell surface receptor, in some types of tumors. We previously found that DDT acts as an anti-obesity adipokine independent of MIF. To understand the intrinsic properties of these two cytokines, a comparison of their actions in various tissues is necessary. In this study, we investigated the involvement of DDT in HepG2 cell (a human hepatoma cell line) proliferation, which is known to be promoted by MIF. Materials and Methods: Cell proliferation and gene expression were evaluated in HepG2 cells expressing short hairpin RNA against the DDT gene. Results: Inhibition of cell proliferation and reduced expression levels of cyclin D1 were observed in DDT-knockdown HepG2 cells. The inhibited proliferation was restored by administration of recombinant DDT. Conclusion: DDT promotes cell proliferation in HepG2 cells; therefore, its action may be similar to that of MIF.*

D-dopachrome tautomerase (DDT) was originally reported to catalyze the conversion of D-dopachrome to 5,6-dihydroxyindole (1); however, its physiological role is unclear as the substrate is inactive in mammals. DDT is composed of 118 amino acids and expressed in various organs, such as the liver, heart, lungs, and pancreas (2). Its tertiary structure is similar to that of macrophage migration inhibitory factor (MIF), a proinflammatory cytokine that activates macrophages and other immune response cells (3). Additionally, DDT and MIF have similar functions. For example, DDT is reported to induce inflammation by activating the extracellular signal-regulated kinase (ERK) pathway *via* CD74, a protein composed of the MIF receptor complex, which promotes tumor growth conjointly with MIF in various tumors (4, 5). Furthermore, MIF catalyzes tautomerization of the D-dopachrome (a substrate of DDT) into 5,6-dihydroxyindole-2-carboxylic acid (6). Thus, DDT is thought to be a MIF homolog.

In our previous studies, using proteomics approaches, we identified DDT as an adipokine that is secreted into the conditioned medium from human differentiated adipocytes (7). The expression patterns of DDT in adipose tissue are different from those of MIF. DDT is expressed more in mature adipocytes than in stromal vascular cells of adipose tissue. Further, its mRNA level in adipocytes, fractionated from visceral adipose tissue, showed a negative correlation with obesity-related clinical parameters, such as body mass index and fat area (7, 8). Conversely, MIF mRNA is expressed at almost the same level in both adipocytes and preadipocytes (9, 10), and its expression in subcutaneous adipose tissue is positively correlated with adipocyte size and insulin resistance (11). Administration of recombinant DDT (rDDT) in obese mice showed improvement in glucose intolerance (7) and inhibited adipogenesis of Simpson-Golabi-Behmal (SGBS) cells, a human preadipocyte cell line (12), suggesting that DDT acts as an anti-obesity adipokine. In contrast, MIF knockout mice exhibited improvement in insulin sensitivity and suppression of macrophage infiltration into adipose tissue (13), and MIF knockdown in 3T3-L1 cells (a mouse preadipocyte cell line) inhibited adipogenesis (10). Thus, DDT and MIF have opposite effects on the development of obesity, indicating that DDT has functions other than being a MIF homolog.

To develop anti-cancer or anti-obesity drugs targeting DDT or its downstream signaling molecules, it is necessary to compare the action and signaling pathways of DDT and MIF in various tissues. As few studies have compared the effects of both DDT and MIF, here, we investigated the involvement of DDT in the proliferation of HepG2 cells, a human hepatoma cell line, in which MIF has been reported to increase cell proliferation (14).

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Key Words: Adipokine, liver cancer, cell proliferation, apoptosis.

Table I. *Nucleotide sequences of primers used for RT-qPCR.*

Gene		Primer sequence
GAPDH	F	5'-GAAGGTGAAGGTCGGAGTC-3'
	R	5'-GAAGATGGTGATGGGATTTC-3'
CCND1	F	5'-GCTGCTCCTGGTGAACAAGC-3'
	R	5'-CACAGAGGGCAACGAAGGTC-3'
BAX	F	5'-TTTGCTTCAGGGTTTCATCC-3'
	R	5'-CAGTTGAAGTTGCCGTCAGA-3'
BIM	F	5'-GAGATATGGATCGCCCAAGA-3'
	R	5'-GTGCTGGGTCTTGTTGGTTT-3'
BCL-2	F	5'-GGATGCCTTTGTGGAACTGT-3'
	R	5'-AGCCTGCAGCTTTGTTTCAT-3'
DDT	F	5'-CTTGGAGTCCTGGCAGATTG-3'
	R	5'-AATGTTGCATGCGGGATAAT-3'

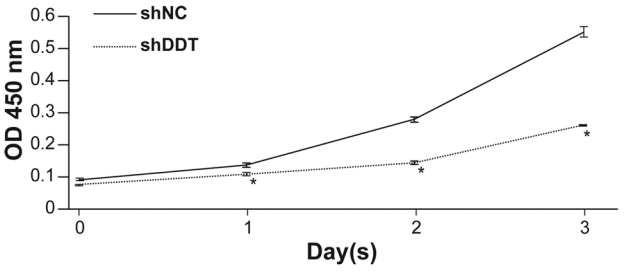

Figure 1. *Effect of D-dopachrome tautomerase (DDT) knockdown on the proliferation of HepG2 cells. After 24 h of transfection with non-targeting control shRNA (shNC) (solid line) or shRNA against the DDT gene (shDDT) (dashed line), HepG2 cells were seeded into 24-well plates and cell viability was measured every 24 h using Cell Counting Kit-8. *p<0.05, n=3.*

Materials and Methods

Cell culture. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Fuji Film, Tokyo, Japan) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO, USA) and Penicillin-Streptomycin-Amphotericin B suspension (Fuji Film) in an incubator with humidified air at 37˚C and 5% CO_2.

Knockdown of DDT. Knockdown of human DDT in HepG2 cells was achieved by transfection with plasmid pBAsi-hU6 Neo (Takara, Shiga, Japan) with DNA encoding a short hairpin RNA (shRNA) against the *DDT* gene (shDDT). The vector containing non-targeting control shRNA (shNC) sequences was used as a control. The sequences of shDDT and shNC were 5'-CCATTCGTTGAGTTGGAAATAGTGCT CCTGGTTGTTTCCAA-CTCAACGAA-3' and 5'-GGTAGATAGGAT GGTAAATTAGTGCTCCTGGTTGATT-TACCATCCTATCTACC-3', respectively. The HepG2 cells were transfected with these plasmids using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA), as per the manufacturer's instruction.

Cell proliferation assay. After 24 h of transfection with shNC or shDDT, the cells were plated at a density of 10,000 cells/well in a 24 well-plate. Cell viability was measured every day for up to 3 days using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), as per the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). After 24 h of transfection, total RNA from the cells was extracted using ISOGEN (Nippongene, Toyama, Japan). Complementary DNA (cDNA) was synthesized from total RNA using the ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan) as per the manufacturer's protocol. The cDNA was subjected to RT-qPCR on a Thermal Cycler Dice® Real Time System (Takara) using Thunderbird™ SYBR® qPCR Mix (Toyobo) *via* the following program: 10 min at 95˚C, followed by 40 cycles at 95˚C for 15 s and 60˚C for 1 min, and an additional cycle of dissociation curves to ensure unique amplification. Nucleotide sequences of the used primer sets are shown in Table I. The relative mRNA levels of each target gene were normalized using human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene.

Western blot analysis. After 48 h of transfection, the cells were lysed in radioimmunoprecipitation assay buffer [50 mM Tris HCL (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X, 0.1% SDS, 0.5% sodium deoxycholate] supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland). After incubation on ice for 30 min, the lysate was centrifuged at 21,500 × *g* and 4˚C for 30 min, and the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto polyvinylidene fluoride (PVDF) membranes. These membranes were blocked using Blocking One (Nacalai Tesque, Kyoto, Japan) for 30 min at 22˚C and then incubated with rabbit anti-cyclin D1 (Sigma; diluted 1:1,000), rabbit anti-caspase 3 antibody (Sigma; diluted 1:1,000), rabbit anti-DDT antibody produced previously (7) (diluted 1:2,000), and mouse anti-β actin (Sigma; diluted 1:2,000) at 4˚C overnight, followed by 1:80,000 or 1:40,000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA) at 22˚C for 40 min. The chemiluminescence signals were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) and detected using an imaging analyzer (Luminograph III; ATTO, Tokyo, Japan).

Preparation of rDDT. Human DDT recombinant protein was obtained as described by Iwata *et al.* (7). A pGEX 6p-3 vector (GE Healthcare, Buckinghamshire, UK) containing human DDT cDNA was introduced into competent BL21 (DE3) cells (Takara), and expression of the DDT-glutathione-S-transferase (GST) fusion protein was induced by 1 mM isopropyl β-D-thiogalactoside at 18˚C for 16 h. The bacterial lysate was applied to a glutathione-S-sepharose column (GE Healthcare), and GST was removed by digestion with PreScission Protease (GE Healthcare) at 4˚C. Thereafter, rDDT was purified by Mono Q column chromatography (GE Healthcare) and concentrated with a Centriplus YM-3K (Millipore). Using the ToxinSensor Endotoxin Detection System (Genscript, Piscataway, NJ, USA), the endotoxin concentration in the recombinant protein was confirmed to be less than 1 EU/lg.

Statistical analysis. Results are expressed as the mean±SE. Statistical analyses were performed using Student's *t*-test. Differences were considered significant when the *p*-value was less than 0.05.

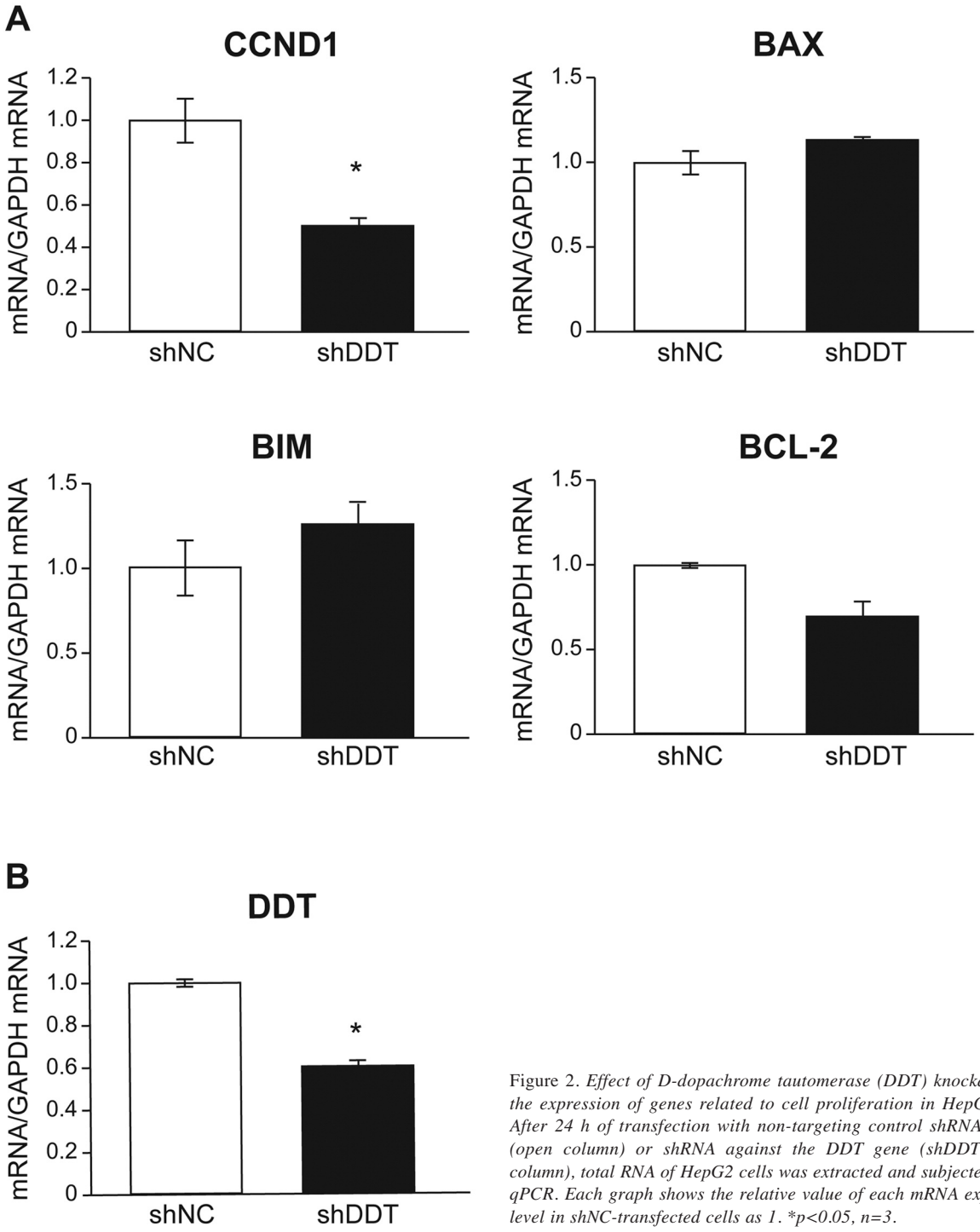

Figure 2. *Effect of D-dopachrome tautomerase (DDT) knockdown on the expression of genes related to cell proliferation in HepG2 cells. After 24 h of transfection with non-targeting control shRNA (shNC) (open column) or shRNA against the DDT gene (shDDT) (filled column), total RNA of HepG2 cells was extracted and subjected to RT-qPCR. Each graph shows the relative value of each mRNA expression level in shNC-transfected cells as 1. *p<0.05, n=3.*

Results

DDT knockdown inhibits cell proliferation in HepG2 cells. To investigate the involvement of DDT in cell proliferation, cell viability was compared between HepG2 cells transfected with shDDT and shNC. The cell viability of HepG2 cells transfected with shDDT was inhibited from day 1 after passage (2 days after transfection) compared with the cells transfected with shNC, and the inhibition was effective for up to 3 days (Figure 1). This result indicated

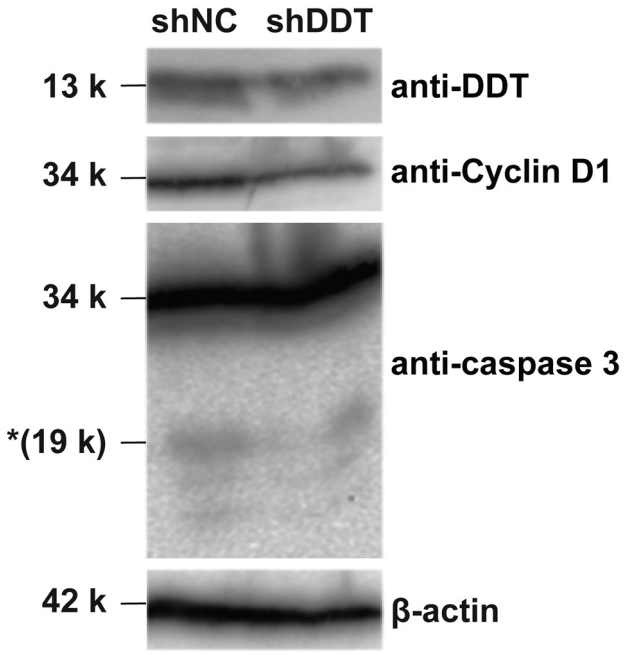

Figure 3. *Effects of D-dopachrome tautomerase (DDT) knockdown on caspase-3 activation and cyclin D1 protein expression. Lysates of HepG2 cells transfected with non-targeting control shRNA (shNC) or shRNA against the DDT gene (shDDT) were subjected to western blotting using the indicated antibodies. β-Actin was used as an internal control. The numbers at the left of the image indicate molecular weight in Da, and the asterisk indicates the predicted electrophoretic position of active caspase-3.*

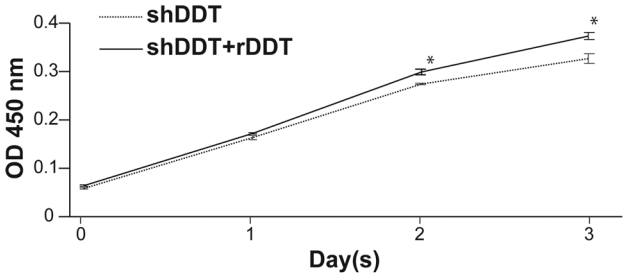

Figure 4. *Effect of recombinant D-dopachrome tautomerase (rDDT) on the viability of DDT-knockdown HepG2 cells. After 24 h of transfection with a short hairpin RNA (shRNA) against the DDT gene (shDDT), HepG2 cells were sub-cultured into 24-well plates in the presence (solid line) or absence (dashed line) of 10 nM rDDT and the cell viability was measured every 24 h using a Cell Counting Kit-8. *p<0.05, n=3.*

that DDT knockdown suppressed cell proliferation in HepG2 cells.

DDT knockdown inhibits cyclin D1 expression in HepG2 cells. To examine the mechanisms underlying the inhibitory effect of DDT knockdown on cell proliferation, the mRNA expression levels of cyclin D1 and apoptosis-related genes in HepG2 cells transfected with shNC and shDDT were examined using RT-qPCR. Transfection with shDDT decreased the mRNA expression level of cyclin D1, but not of Bcl-2 associated X-protein (BAX), Bcl-2-like protein 11 (BIM), and B-cell leukemia/lymphoma 2 (BCL-2) (Figure 2A). Furthermore, knockdown of DDT mRNA in HepG2 cells transfected with shDDT was confirmed (Figure 2B).

The correlation between DDT and cyclin D1 expression was also observed at the protein level (Figure 3). To evaluate the involvement of apoptosis in the inhibition of cell proliferation in HepG2 cells transfected with shDDT, the degradation of procaspase-3 by western blotting was examined. There was no difference in procaspase-3 degradation between cells transfected with shNC and shDDT (Figure 3).

Recombinant DDT restores the cell viability of DDT-knockdown HepG2 cells. To investigate whether the inhibitory effect of DDT knockdown on cell proliferation is mediated by the reduction in the extracellular signals of DDT, cell proliferation was evaluated by culturing HepG2 cells transfected with shDDT in either the presence or absence of 10 nM rDDT. Administration of rDDT in the culture medium slightly, but significantly, promoted the proliferation of DDT-knockdown HepG2 cells (Figure 4), suggesting that DDT, as a cytokine, was at least partially involved in the cell proliferation of HepG2 cells.

Discussion

In the present study, we demonstrated that knockdown of DDT suppressed the proliferation of the human hepatoma cell line HepG2 in a manner similar to that caused by the knockdown of MIF. MIF has been implicated in the tumorigenesis and metastasis of various cancer phenotypes (15). It is over-expressed in almost all cancer types and its serum level in patients with oral carcinomas and ovarian cancer correlates with the progression of these cancer types (16, 17). In comparison, there have not been many reports relating DDT to cancer. Similar to MIF, DDT has been reported to be expressed significantly in various types of malignant tumors. DDT cooperates with MIF for tumor survival and the formation of non-small cell lung carcinomas (18) and clear cell renal cell carcinomas (19). Double knockdown of DDT and MIF promotes proliferation of lung adenocarcinoma (20), pancreatic ductal adenocarcinoma (21), cervical cancer (22), and melanoma (23) cell lines. Considering these reports and the finding of this study, DDT may have functions similar to MIF in a wide range of cancer types.

In HepG2 cells, MIF knockdown inhibits cell proliferation by down-regulating cyclin D1 and up-regulating apoptosis-inducing factors, such as BAX and BIM (14). In the present

study, DDT knockdown suppressed cyclin D1 expression; however, DDT knockdown did not affect the mRNA expression of BAX, BIM, and BCL-2 or the activation of caspase-3. This suggests that DDT regulates cell proliferation *via* cell cycle progression rather than by inhibiting apoptosis induction. The subtle difference in the molecular mechanisms of HepG2 cells subjected to knockdown of DDT and MIF might be caused by the fact that DDT and MIF share some common signals but are involved in different signaling pathways.

We examined the effect of biologically active exogenous DDT on cell proliferation in DDT-knockdown HepG2 cells. Administration of rDDT enhanced the proliferation of DDT-knockdown HepG2 cells, suggesting that DDT is a cytokine involved in cell proliferation. However, this result cannot rule out that intracellular DDT contributes to the proliferation of HepG2 cells. The action mechanisms of MIF and DDT have not yet been elucidated. When acting as cytokines, MIF and DDT have signals allied in a CD74-dependent manner within various cancer cell lines. MIF acts as an intracellular functional molecule, at least in part. For example, MIF exhibits oxidoreductase and tautomerase activity (6), and reduces prooxidative stress-induced apoptosis *via* its enzymatic activity (24). Although DDT does not have a Cys-Ala-Lys-Cys motif, which is critical for the oxidoreductase activity of MIF, it may affect cell proliferation *via* unidentified intracellular functions.

In adipose tissue, DDT act as an anti-obesity cytokine and this action is distinctly different from that of MIF (7, 12). Whether DDT acts as an MIF homolog or has a unique action may depend on the receptor. MIF has been reported to bind to either the extracellular domain of CD74, a potential MIF receptor, and initiate signaling by co-activating CD44 (25), or the chemokine receptors CXCR2, CXCR4, or CXCR7 (26, 27). The effects of DDT have been reported to be mediated by CD74, but the signaling or partner molecule of CD74 is largely unknown. DDT-induced IL-6 gene expression in preadipocytes was attenuated by knockdown of CD74 and not of CD44, suggesting that the DDT receptor complex may comprise CD74 and component(s) other than CD44 (12). Hence, a complete picture of the receptor mechanisms of DDT and MIF would aid in developing drugs that would enhance the effects of DDT unobserved with MIF or inhibit the effects of the MIF homolog: such drugs may be functional as anti-obesity and anti-cancer medications, respectively.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

T.I. conceived and designed the study. A.T, and T.I. performed the experiments and wrote the manuscript. K.Y. revised the manuscript. All the Authors approved the final version of the manuscript.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Priority Research Promotion Program, Niigata University of Pharmacy and Applied Life Sciences, Japan.

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Received May 22, 2021
Revised June 3, 2021
Accepted June 4, 2021