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ELUCIDATION OF THE MECHANISM UNDERLYING APOPTOSIS INDUCED BY VITAMIN K_3 ANALOGS IN A HUMAN LEUKEMIA-DERIVED CELL LINE

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ABSTRACT – *Objective:* Thioether analogs of vitamin K_3 (VK_3) promote the generation of reactive oxygen species (ROS) and enhance the expression of the apoptosis-inducing receptor Fas and its ligand, thereby inducing apoptosis through activation of mitogen-activated protein kinase (MAPK) and caspase-3. They can also induce apoptosis without generating ROS by maintaining phosphorylation *via* inhibition of mitochondrial or tyrosine and serine/threonine phosphatases involved in intracellular signal transduction. Moreover, VK_3 analogs have been shown to upregulate death receptor 5 (DR5) and induce apoptosis in human leukemia-derived cells through the intracellular DR signaling pathway. In this study, we aimed to investigate the effect of the VK_3 analog 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone (CPD5) on the expression of factors acting further upstream in this signaling pathway.

Materials and Methods: Apoptosis induction in human leukemia-derived cells following CPD5 treatment was examined by assessing intracellular protein expression levels using Western blotting. The analyzed proteins were factors involved in apoptosis signaling upstream of DR5.

Results: Treatment with CPD5 (10 μM) activated caspase-3 and caspase-8 and increased the expression of DR5. Further investigation of upstream signaling revealed elevated levels of tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL), which acts on death receptors. Expression of the TRAIL-cleaving enzyme was also increased.

Conclusions: These findings indicate that CPD5 acts on the TRAIL-cleaving enzyme in leukemia-derived cells, leading to the excessive release of soluble TRAIL (sTRAIL) and subsequent apoptosis *via* DR5. By elucidating its upstream effects and site of action, CPD5 can be proposed as a potential anti-tumor agent.

KEYWORDS: Apoptosis, Caspase, Death receptor 5, Lymphocytic leukemia, Matrix metalloproteinase-8, TNFα-related apopto-sis-inducing ligand, Vitamin K_3 analog.

ABBREVIATIONS: APO-2L: Apoptosis antigen 2 ligand; APO-3L: Apoptosis antigen 3 ligand; ART: Antiretroviral therapy; CPD5: 2-(2-Mercaptoethanol)-3-methyl-1,4-naphthoquinone; DMSO: Dimethyl sulfoxide; DR: Death receptor; DR3: Death receptor 3; DR4: Death receptor 4; DR5: Death receptor 5; FADD: Fas-associated death domain protein; Fas/CD95: Fas cell surface death receptor (CD95); FLIP: FLICE inhibitory protein; MAPK: Mitogen-activated protein kinase; MMP-8: Matrix metalloproteinase-8; mTRAIL: Membrane-bound TNF-related apoptosis-inducing ligand; sTRAIL: Soluble TNF-related apoptosis-inducing ligand; TNF-R1: Tumor necrosis factor receptor 1; TNF-R2: Tumor necrosis factor receptor 2; TNF-α: Tumor necrosis factor alpha; TRAIL: TNF-related apoptosis-inducing ligand; TRANCE: TNF-related activation-induced cytokine; TWEAK: TNF-related weak inducer of apoptosis; VK_3: Vitamin K_3.

INTRODUCTION

Vitamin K exists in the naturally occurring forms, vitamin K_1 and K_2, as well as the synthetic analog, vitamin K_3 (VK_3). Phylloquinone (vitamin K_1) and menaquinone (vitamin K_2) exhibit procoagulant activity by acting as cofactors for γ-glutamyl carboxylase, facilitating the γ-carboxylation of glutamate residues in prothrombin and other vitamin K–dependent coagulation factors[1]. These vitamins are clinically used to treat vitamin K deficiency and osteoporosis, and for detoxification in cases of warfarin poisoning.

Menadione (VK_3) exerts its biological effects primarily through interference with redox-sensitive processes, including the inhibition of enzyme activities by disrupting disulfide bond formation. This disruption affects the three-dimensional conformation of enzymes, potentially leading to their inactivation and altering various cellular processes[2,3]. Thioether-containing VK_3 analogs can form disulfide bonds by linking their thioether group to the sulfhydryl (SH) group of active-site cysteine residues in proteins such as protein tyrosine phosphatases (PTPases). This interaction can interfere with phosphate group binding between protein tyrosine kinases (PTKs) and PTPases[4], thereby preserving PTK phosphorylation and modulating downstream cellular signaling pathways. Among VK_3 analogs, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone (CPD5), which contains a hydroxyl group at the terminal position of its thioether moiety, has been shown to maintain epidermal growth factor receptor (EGFR) phosphorylation, an event involved in various signaling cascades. In addition, CPD5 suppresses cell proliferation by directly inhibiting the expression of the cell-cycle regulatory protein Cdc25A[1].

The apoptosis-inducing effects of menadione are reportedly mediated by the generation of reactive oxygen species (ROS) and the upregulation of the apoptosis-inducing receptor Fas and its ligand[5-7]. Apoptosis requires activation of mitogen-activated protein kinases (MAPKs) and caspase-3[8-10]. CPD5 inhibits PTPase activity, leading to MAPK activation, which in turn suppresses cell proliferation[4,11] and induces apoptosis[12-14]. Furthermore, CPD5 promotes apoptosis by activating extracellular signal-regulated kinase (ERK), a key event in MAPK signaling[12,13]. Collectively, these findings indicate that CPD5 induces apoptosis by activating MAPK signaling through inhibition of PTPase activity.

The present study aimed to elucidate the intracellular signal transduction pathways in human leukemia-derived cells treated with CPD5 and to investigate the involvement of death receptors (DRs) and their associated signaling mechanisms.

MATERIALS AND METHODS

All cell culture and biochemical experiments in this study were conducted as previously described by Asami et al[14].

Cell Culture

Jurkat cells were obtained from the RIKEN BioResource Research Center (RCB3052, Ibaraki, Japan) and cultured at a density of 2 × 10^6 cells/5 mL per 60-mm cell culture dish in RPMI-1640 medium (FUJIFILM Wako Pure Chemical, Osaka, Japan, 189-02025). The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FUJIFILM Wako Pure Chemical). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Subsequently, the cells were treated with reagents dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO; D8418-100ML). Control cells received an equivalent amount of DMSO.

Mycoplasma contamination was not detected in the cells used in this study. Testing was performed using the Myco-Visible Mycoplasma LAMP Detection Kit (MP Biomedical, Chiba, Japan; 093050601).

As this study utilized *in vitro* cultured cells, ethical approval was not required.

Reagents

VK_3 was purchased from FUJIFILM Wako Pure Chemical (132-08132), and the VK_3 analog (CPD5) was obtained by synthesis. VK_3 and CPD5 were dissolved in DMSO at concentrations ranging from 1×10^{-4} to 1.5×10^{-3} M and stored at 20°C in the dark. Reagents were added at 1% of the total medium volume for each experiment, resulting in final concentrations of 1×10^{-6} to 1.5×10^{-5} M.

Caspase Activity Measurements

Caspase activity in CPD5-treated Jurkat cells was measured using fluorometric assay kits: APOPCYTO Caspase-3 Fluorometric (#4815), APOPCYTO Caspase-8 Fluorometric (#4820), and APOPCYTO Caspase-9 Fluorometric (#4825) (Medical and Biological Laboratories Co., Ltd., Tokyo, Japan).

Caspase-3 activity was assessed in cells treated with 1 or 10 µM CPD5 for 0, 4, 8, 12, and 24 h. Similarly, caspase-8 and caspase-9 activities were measured in cells treated with 10 µM CPD5 for the same time points.

Western Blotting

Jurkat cells were cultured for 24 h before treatment with VK_3 or CPD5 at the desired final concentrations. After treatment, the cells were harvested, washed with Tris-Buffered Saline (Sigma-Aldrich, T5912), and lysed in an extraction buffer containing 20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, protease inhibitor cocktail I (1:200; Sigma-Aldrich, 20-201), phosphatase inhibitor cocktail II (1:100; Sigma-Aldrich, 524625), 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The cells were disrupted by sonication (30 s, twice), and the supernatants were collected by centrifugation at 20,000 × *g* for 10 min at 4°C. Protein concentrations were determined using a Rapid Protein Assay Kit (FUJIFILM Wako Pure Chemical Co., Ltd., 293-56101), with bovine serum albumin (BSA) as the standard. Aliquots of the supernatants were mixed with sample buffer containing 240 mM Tris–HCl (pH 6.8), 312 mM sodium dodecyl sulfate (SDS), 30% glycerol, 15% 2-mercaptoethanol, and 1% bromophenol blue. Samples were boiled for 3 min at 100°C and then centrifuged at 20,000 × *g* for 1 min at 4°C to prepare the final gel-loading samples. Equal amounts of protein (7.5 µg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, IL, USA). Membranes were blocked with 5% skim milk and incubated with primary antibodies overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at 25°C. The blots were visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare) and imaged with a Lumino Image Analyzer LAS-1000 (FUJIFILM Wako Pure Chemical Co., Ltd.). Band intensities were quantified using NIH ImageJ software (version 1.53; https://imagej.net/ij/).

Expression of DR-Related Factors

Jurkat cells were treated with VK_3 (10 µM) or CPD5 (10 µM) for 0 or 24 h (0 h representing untreated controls). The following primary antibodies were used: tumor necrosis factor receptor 1 (TNF-R1; C25C1, Cell Signaling Technology, Danvers, MA, USA; #3736), death receptor 5 (DR5; D4E9 XP®, Cell Signaling Technology; #8074), death receptor 3 (DR3; D5Q2R, Cell Signaling Technology; #45901), FLICE-inhibitory protein (FLIP; D5J1E, Cell Signaling Technology; #56343), and Fas ligand (FasL; D1N5E, Cell Signaling Technology; #68405). Additional antibodies included anti–Fas-associated death domain (FADD) antibody (EPR5030, Abcam, Cambridge, MA; ab124812), anti–β-tubulin (Sigma-Aldrich, T4026), IgG (Sigma-Aldrich, A9044), and IgG (Sigma-Aldrich, A9169). In this Western blot analysis, β-tubulin was used as the loading control during sample preparation.

Expression of Apoptosis Ligands

Jurkat cells were treated with CPD5 (1, 5, 10, or 15 µM) for 24 h (0 µM representing untreated controls). The primary antibodies used were tumor necrosis factor α–related apoptosis-inducing ligand (TRAIL; C92B9, Cell Signaling Technology; #3219) and TNF-like weak inducer of apoptosis (TWEAK, Cell Signaling Technology; #4437), both used at a dilution of 1:5,000. The amount of protein loaded in each lane was adjusted using β-tubulin as a reference to ensure equal loading.

Expression of Enzymes Involved in TRAIL Shedding

Jurkat cells were treated with CPD5 (1, 5, 10, or 15 µM) for 24 h (0 µM representing untreated controls). The primary antibody used was anti–matrix metalloproteinase-8 (MMP-8) antibody (EP1252Y [ab81286], Abcam) at a dilution of 1:5,000. β-Tubulin was used to normalize sample loading and confirm equal protein input across lanes.

Statistical Analyses

All data are expressed as mean ± standard error of mean (SEM; n = 3). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test. Analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Caspase Activity

Caspase-3 activity in cells treated with VK_3 and CPD5 at final concentrations of 1 and 10 µM is shown in Figure 1A and 1B, respectively. No caspase-3 activity was observed in cells treated with either reagent at 1 µM (Figure 1A). In contrast, cells treated with 10 µM CPD5 for 4 and 8 h exhibited caspase-3 activity significantly higher than that of DMSO-treated controls at the same time points ($p < 0.001$; Figure 1B). Similarly, CPD5-treated cells at 12 and 24 h showed significantly increased caspase-3 activity compared with DMSO-treated controls ($p < 0.01$ and $p < 0.05$, respectively). However, no significant differences in caspase-3 activity were observed between VK_3-treated and DMSO-treated groups at any time point.

To investigate the upstream regulation of caspase-3, caspase-8 (Figure 1C), and caspase-9 (Figure 1D) activities were measured. Caspase-8 activity in VK_3-treated cells did not differ significantly from that in DMSO-treated controls at any time point. In contrast, CPD5-treated cells exhibited significantly higher caspase-8 activity than DMSO-treated cells at 4 and 8 h ($p < 0.05$ and $p < 0.01$, respectively). No significant differences in caspase-9 activity were observed among the VK_3-, CPD5-, and DMSO-treated groups at any time point.

Expression Levels of DR-Related Factors

Given the observed involvement of caspase-8 in CPD5-mediated apoptosis, we next examined the expression of DRs and associated adaptor proteins (Figure 2). The analyzed proteins included Fas (40-50 kDa, Figure 2A), TNF-R1 (55 kDa, Figure 2B), DR3 (55-60 kDa, Figure 2C), DR5 (40 kDa, Figure 2D), and DR5 (48 kDa, Figure 2E). In addition, caspase-8–associated factors were evaluated, including FADD (28 kDa, Figure 2F), FLIP-L (55 kDa, Figure 2G), and FLIP-S (25 kDa, Figure 2H). β-Tubulin (55 kDa, Figure 2I) was used as the loading control.

After treatment with VK_3, CPD5, or DMSO for 0 or 24 h, the expression levels of each factor were analyzed, with DMSO-treated cells serving as the control. Fas receptor expression significantly decreased following treatment with either VK_3 or CPD5 ($p < 0.001$). In contrast, 40-kDa DR5 expression was significantly upregulated after CPD5 treatment ($p < 0.01$).

Expression Levels of Apoptosis Ligands

To determine whether CPD5 treatment increases the expression of apoptotic ligands upstream of death receptors, Jurkat cells were treated with 0, 1, 5, 10, or 15 µM CPD5 for 24 h, after which total cellular proteins were extracted. Protein amounts were normalized using β-tubulin as the loading control, and the expression levels of TRAIL (Figure 3A) and TWEAK (Figure 3B), both of which bind to DR5, were analyzed. The expression of TRAIL, a ligand with high affinity for DR5, significantly increased in cells treated with 10 or 15 µM CPD5 compared with untreated controls (0 µM) ($p < 0.001$). In contrast, TWEAK expression did not change significantly across the 0-15 µM CPD5 treatment range.

Expression Levels of Enzymes Involved in TRAIL Shedding

Membrane-associated TRAIL is cleaved by matrix metalloproteinase-8 (MMP-8), resulting in the extracellular release of soluble TRAIL (sTRAIL). To assess the effect of CPD5 on this process, we measured MMP-8 expression in cells treated with 0, 1, 5, 10, or 15 µM CPD5 for 24 h (Figure 4). To further clarify whether CPD5 modulates TRAIL shedding, we examined the expression of enzymes involved in this process using proteins extracted from the same samples analyzed for apoptosis ligands. MMP-8 expression

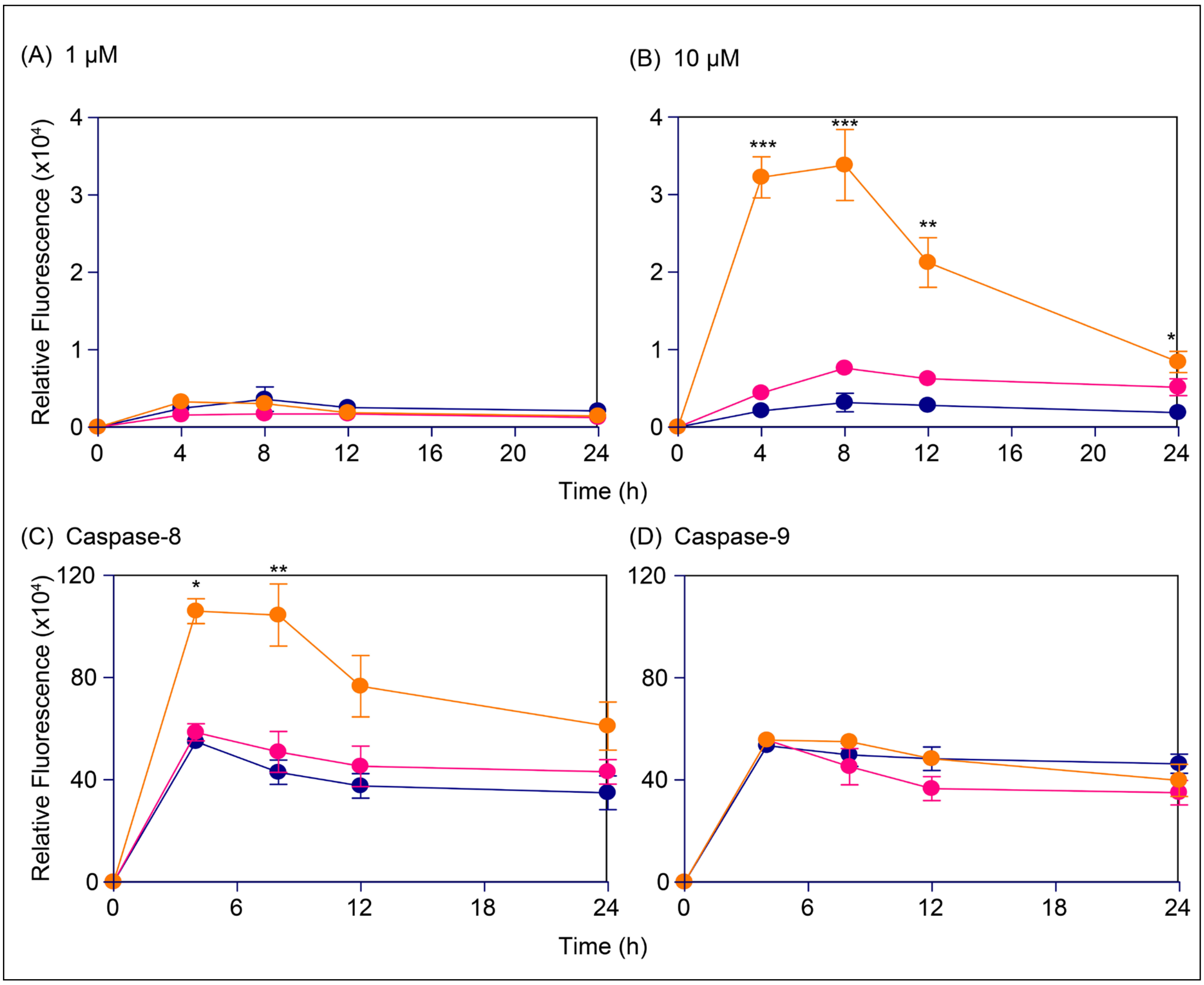

Figure 1. Caspase activity following CPD5 treatment. Caspase activity was measured in Jurkat cells treated with the VK_3 analog 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone (CPD5) for 24 h. The vertical axis indicates fluorescence intensity, and the horizontal axis indicates treatment duration. The graphs show caspase activity in cells treated with CPD5 (-●-, orange), VK_3(-●-, pink), or control (-●-, blue) at 1 μM (A) or 10 μM (B, C, D). Caspase-3 (A, B), caspase-8 (C), and caspase-9 (D) activities were analyzed. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001; n = 3).
Abbreviations: CPD5, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone; VK_3, vitamin K_3; ANOVA, analysis of variance.

showed a tendency to increase in cells treated with CPD5 at concentrations ≥10 μM, with a significant upregulation observed in cells treated with 15 μM CPD5 ($p < 0.05$).

DISCUSSION

Treatment with CPD5 is known to induce apoptosis in Jurkat cells[14]. To determine whether CPD5, a thioether analog of VK_3, induces apoptosis *via* caspases, we measured the activity of caspase-3, a key downstream effector in the caspase cascade. A previous study reported that CPD5 induces apoptosis in human leukemic cells through MAPK activation within the mitochondrial system[14]. However, the present findings suggest that CPD5-mediated apoptosis may be primarily driven by DR-mediated signaling rather than by mitochondria mechanisms. Apoptosis was induced at CPD5 concentrations ≥10 μM, with peak caspase-3 activity observed between 4 and 12 h of treatment. To further delineate the apoptotic pathway, we examined the activities of caspase-8 (associated with DR signaling[15]) and caspase-9 (associated with the mitochondrial pathway[16]). Notably, only caspase-8 activity was observed.

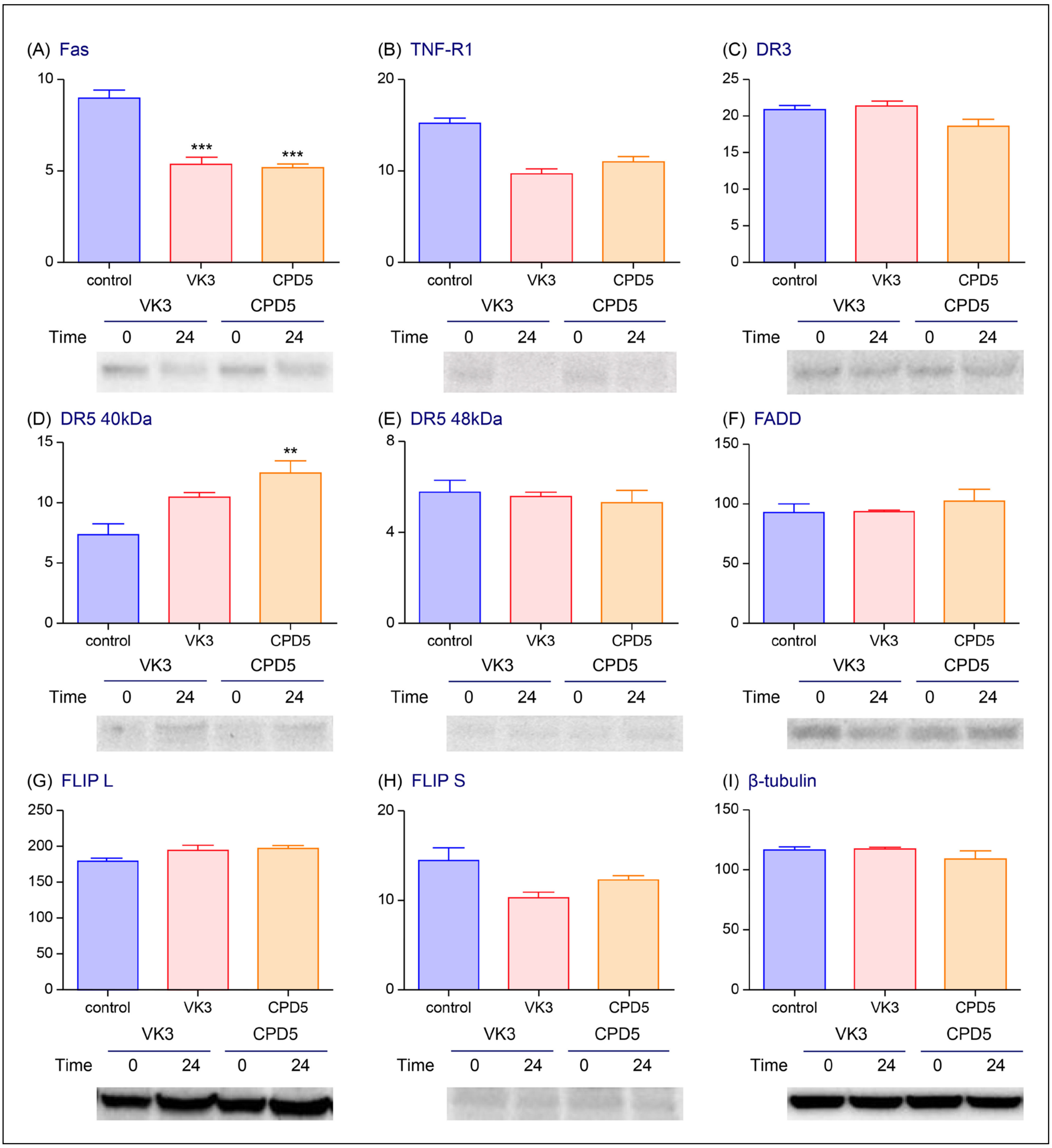

Figure 2. Expression levels of death receptor-related factors were determined by Western blotting. Jurkat cells were treated with menadione (10 μM) or CPD5 (10 μM) for 24 h. β-Tubulin was used as the loading control. The molecular weights of the detected proteins were as follows: (A) Fas (CD95/APO-1), 40 kDa; (B) tumor necrosis factor receptor 1 (TNF-R1), 55 kDa; (C) death receptor 3 (DR3), 58 kDa; (D) DR5, 40 kDa; (E) DR5, 48 kDa; (F) Fas-associated death domain protein (FADD), 28 kDa; (G) FLICE-inhibitory protein–long form (FLIP-L), 55 kDa; (H) FLIP–short form (FLIP-S), 25 kDa; and (I) β-tubulin (loading control), 55 kDa. Statistical significance was determined using one-way ANOVA, followed by Bonferroni's test (**$p < 0.01$, ***$p < 0.001$; n = 3).

Abbreviations: CPD5, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone; DR, death receptor; FADD, Fas-associated death domain; FLIP, FLICE-inhibitory protein; TNF-R1, tumor necrosis factor receptor 1; VK$_3$, vitamin K$_3$; ANOVA, analysis of variance.

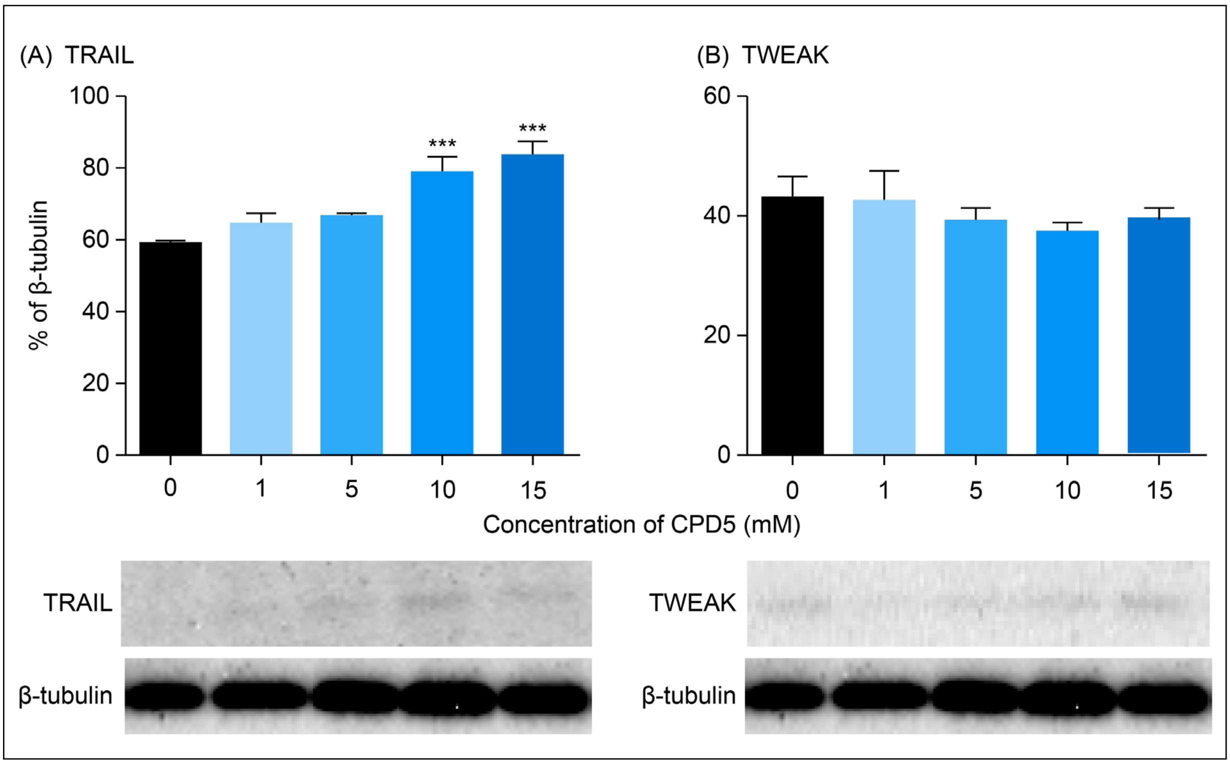

Figure 3. Expression levels of apoptosis ligands were determined by Western blotting. Jurkat cells were treated with CPD5 (0, 1, 5, 10, or 15 µM) for 24 h. β-Tubulin was used as the loading control. The detected proteins were: (A) tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), 28 kDa and (B) TNF-like weak inducer of apoptosis (TWEAK), 28 kDa. Statistical significance was determined using one-way ANOVA, followed by Bonferroni's test (***$p < 0.001$, n = 3).

Abbreviations: CPD5, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-like weak inducer of apoptosis; ANOVA, analysis of variance.

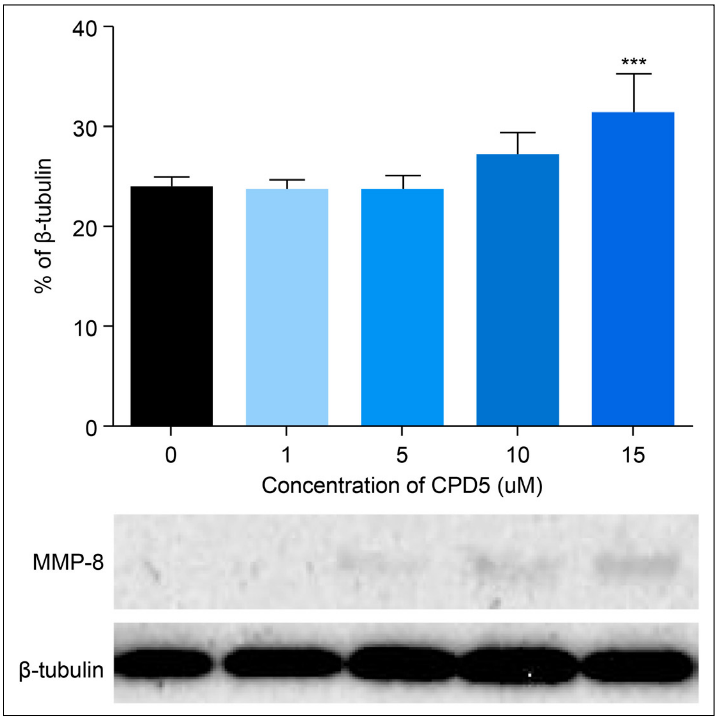

Figure 4. Expression of the enzyme involved in TRAIL shedding determined by western blotting. Jurkat cells were treated with CPD5 (0, 1, 5, 10, or 15 µM) for 24 h. β-Tubulin was used as the loading control. The predicted molecular weight of matrix metalloproteinase-8 (MMP-8) was 53 kDa, while the observed band size was 72 kDa. Statistical significance was determined using one-way ANOVA, followed by Bonferroni's test (*$p < 0.05$, n = 3).

Abbreviations: CPD5, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone; MMP-8, matrix metalloproteinase-8; TRAIL, TNF-related apoptosis-inducing ligand; ANOVA, analysis of variance.

To better understand the involvement of the DR pathway, we analyzed the expression of various DRs, including Fas[17], TNF-R1, TNF-R2, DR3, DR4, and DR5[18,19], as well as their associated ligands TRAIL[20,21], TNF-related activation-induced cytokine (TRANCE), and TWEAK. Additionally, we investigated the expression of intracellular apoptotic regulators, including FADD[22,23] and FLIP[24].

Based on the increased caspase-8 activity observed in this study, TNF-R2 was excluded because it does not participate in caspase-8–dependent intracellular signaling. Therefore, the expression levels of Fas, TNF-R1, DR3, and DR5 (including both DR4/DR5 variants) were measured. Cells treated with VK_3 or CPD5 for 24 h exhibited decreased Fas expression compared with DMSO-treated controls. Among the DR5 isoforms, the 48-kDa DR5 showed minimal changes across all treatment conditions (DMSO, VK_3, and CPD5), whereas the 40-kDa DR5 was significantly upregulated following CPD5 treatment compared with DMSO. As the Fas/CD95 receptor pathway activates caspase-3 *via* the mitochondrial system, an increase in caspase-9 activity was expected to accompany Fas receptor expression. However, caspase-9 activity in VK_3- or CPD5-treated cells remained comparable to that of controls, suggesting that caspase-9 is unlikely to be the primary downstream effector of CPD5. FADD, a key adaptor protein in the intracellular signal transduction of all DRs, also showed no significant change in expression in VK_3- or CPD5-treated cells, indicating that CPD5 does not directly affect FADD-mediated signaling. Furthermore, the expression of FLIP, a known caspase-8 inhibitor, remained unchanged upon VK_3 or CPD5 treatment. Collectively, these findings suggest that CPD5 is unlikely to exert its proapoptotic effects through direct intracellular signaling mechanisms. Therefore, we considered the possibility that CPD5 may act *via* extracellular ligands. FasL, a ligand for the Fas/CD95 receptor, participates in caspase-9–mediated signaling, while TNF-α, a ligand for TNF-R2, is not involved in the caspase-8 pathway; hence, both were excluded from further analysis. Instead, we hypothesized that CPD5 acts through apoptosis antigen (APO)-3L (a ligand for DR3, TWEAK, and DR4) and APO-2L (a ligand for DR5 or TRAIL)[25,26]. To test this hypothesis, we examined the expression of these ligands.

Treatment with ≥10 μM CPD5 resulted in a significant increase in TRAIL expression compared with baseline (0 h). Although APO-3L and TWEAK are known ligands for DR3, TWEAK expression remained unchanged even after 15 μM CPD5 treatment. These results suggest that CPD5 primarily modulates TRAIL expression. TRAIL expression has been documented in various tissues and cell types, including T cells, natural killer cells, monocytes, dendritic cells, and neutrophils, as well as in tumor sites and cancer cells[27,28]. TRAIL exists in two forms: membrane-bound TRAIL (mTRAIL), which is localized on the cell surface, and soluble TRAIL (sTRAIL), which is released extracellularly following cleavage by the TRAIL-shedding enzyme MMP-8[29]. To further explore the role of TRAIL in CPD5-mediated apoptosis, we examined MMP-8 expression.

MMP-8 expression in cells treated with <10 μM CPD5 was comparable to that in control cells. In contrast, MMP-8 expression in cells treated with >10 μM CPD5 increased in a dose-dependent manner, with significant upregulation observed following 15 μM CPD5 treatment compared with controls (0 h). These results suggest that 10 μM CPD5 induces apoptosis in Jurkat cells *via* the extrinsic pathway, which is activated through DRs and caspase-8 signaling. Notably, intracellular signaling analysis revealed a decrease in Fas expression and an increase in DR5 expression. However, no changes were observed in FADD (a key component of intracellular caspase-8 activation complexes) or FLIP (a known caspase-8 inhibitor) expression. Furthermore, significant increases in TRAIL and MMP-8 expression were detected upstream. mTRAIL present on T cells and natural killer cells is cleaved by a shedding enzyme to produce sTRAIL, which serves as a ligand for DR5. MMP-8 has been identified as this shedding enzyme[29] (Figure 5). These findings suggest that CPD5 may influence the TRAIL-cleaving activity of MMP-8 in leukemia-derived cells, leading to excessive release of sTRAIL and subsequent activation of DR5-mediated apoptosis.

TRAIL induces apoptosis in cancer cells by binding to DR4 and DR5, while exerting minimal effects on normal cells[27,28,30]. Preclinical trials have demonstrated that recombinant TRAIL (rTRAIL) exhibits strong tumor-regression effects[31,32]. Consequently, rTRAIL and DR4/DR5-targeting antibodies are currently being explored as next-generation anticancer therapies with fewer adverse effects[33]. Furthermore, studies using *TRAIL receptor (R)*–knockout mice have shown increased rates of carcinogenesis and metastasis, suggesting that TRAIL plays a critical role in suppressing cancer growth[34,35]. However, tumor tissues and cancer cells typically express very low levels of TRAIL compared with normal cells, with TRAIL expression often reduced during tumor progression[36]. Based on these findings, CPD5 may enhance TRAIL expression, positioning it as a promising candidate for cancer therapy. Understanding its mechanism of action could be pivotal in developing strategies for suppressing carcinogenesis and preventing cancer progression. In future work, we aim to elucidate the intracellular signal mecha-

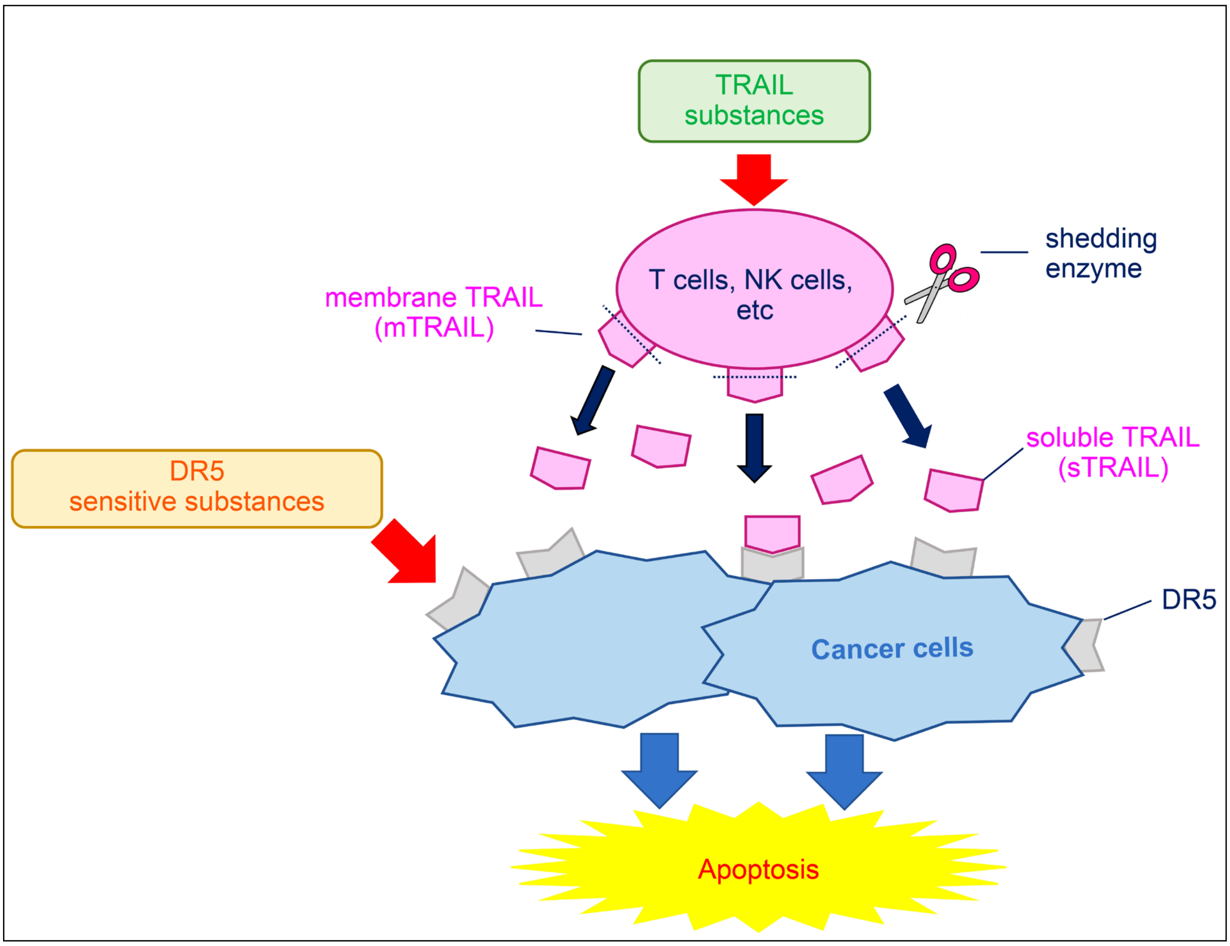

Figure 5. TRAIL-induced apoptosis pathway. TRAIL is primarily produced by immune cells such as neutrophils, monocytes, T cells, NK cells, and dendritic cells. It exists in two forms: membrane-bound TRAIL (mTRAIL) and soluble TRAIL (sTRAIL). sTRAIL is generated by enzymatic cleavage (shedding) of mTRAIL. Both mTRAIL and sTRAIL bind to death receptor 5 (DR5) on cancer cells, triggering apoptosis. DR5-sensitive compounds, such as CPD5, may enhance this pathway.
Abbreviations: CPD5, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone; DR5, death receptor 5; mTRAIL, membrane-bound TRAIL; sTRAIL, soluble TRAIL; NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand.

nisms regulating TRAIL expression and to further explore the potential of CPD5 as an antitumor agent for drug development.

A limitation of this study is that the expression of DR5, other receptors, intracellular factors, and the activity of the VK_3 thioether analog were quantified simultaneously, which limited the number of samples used for each measurement. Future studies with larger sample sizes are warranted to validate the observed changes in expression. Additionally, further investigations are needed to differentiate between mTRAIL and sTRAIL and to determine the precise site of CPD5 action.

CONCLUSIONS

This study confirmed that CPD5, a thioether analog of VK_3, influences MMP-8 expression, an upstream regulator of TRAIL shedding. Our findings suggest that CPD5 induces apoptosis through the extrinsic pathway by promoting TRAIL release and DR5 activation. Given its targeted mechanism of action, CPD5 holds potential as a novel antitumor agent, warranting further investigation for clinical application. Future studies should focus on elucidating its precise molecular mechanisms and optimizing its therapeutic potential.

Conflict of Interest:

The authors declare that they have no conflicts of interest to report for the present study.

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Authors' Contributions:

The authors confirm contributions to this work as follows: study design, Mikana Suzuki, Kohei Watanabe, and Satoru Asami; administrative support, Koichi Kato and Hisao Kano; experiment conduction, data collection, and analysis, Mikana Suzuki, Kohei Watanabe, and Satoru Asami; provision of critical reagents, Kohei Watanabe and Satoru Asami; manuscript writing, Mikana Suzuki; and critical revision of the article, Satoru Asami. All authors reviewed the results and approved the final version of the manuscript.

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Data Availability:

All data supporting the findings of this study are included within the article. Additional data are available from the corresponding author upon reasonable request.

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