In vitro and in vivo anti-leukemic effects of cladoloside C2 are mediated by activation of Fas/ceramide synthase 6/p38 kinase/c-Jun NH2-terminal kinase/caspase-8

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ABSTRACT

We previously demonstrated that the quinovose-containing hexaoside stichoposide C (STC) is a more potent anti-leukemic agent than the glucose-containing stichoposide D (STD), and that these substances have different molecular mechanisms of action. In the present study, we investigated the novel marine triterpene glycoside cladoloside C2 from Cl adol abes schmelt zii, which has the same carbohydrate moiety as STC. We assessed whether cladoloside C2 could induce apoptosis in K562 and HL-60 cells. We also evaluated whether it showed antitumor action in mouse leukemia xenograft models, and its molecular mechanisms of action. We investigated the molecular mechanism behind cladoloside C2-induced apoptosis of human leukemia cells, and examined the antitumor effect of cladoloside C2 in a HL-60 and K562 leukemia xenograft model.

Cladoloside C2 dose- and time-dependently induced apoptosis in the analyzed cells, and led to the activation of Fas/ceramide synthase 6 (CerS6)/p38 kinase/JNK/caspase-8. This cladoloside C2-induced apoptosis was partially blocked by specific inhibition by Fas, CerS6, and p38 siRNA transfection, and by specific inhibition of JNK by SP600125 or dominant negative-JNK transfection. Cladoloside C2 exerted antitumor activity through the activation of Fas/CerS6/p38 kinase/JNK/caspase-8 without showing any toxicity in xenograft mouse models. The antitumor effect of cladoloside C2 was reversed in CerS6 shRNA-silenced xenograft models. Our results suggest that cladoloside C2 has in vitro and in vivo anti-leukemic effects due to the activation of Fas/CerS6/p38 kinase/JNK/caspase-8 in lipid rafts. These findings support the therapeutic relevance of cladoloside C2 in the treatment of human leukemia.

INTRODUCTION

Leukemia is a heterogeneous clonal disorder characterized by defects in cell differentiation and the death of hematopoietic progenitor cells. Despite major advances in drug development, leukemia treatment remains limited by resistance to chemotherapeutic agents in many patients [1, 2]. Thus, there remains a need for new therapeutic agents and strategies to improving the leukemia cure rate.
The tumor-suppressor lipid ceramide reportedly exhibits potent growth inhibition effects in a variety of cell types [3]. Ceramide can be generated by either ceramide synthases or sphingomyelinases [4, 5]. Sphingomyelinases (SMases) are categorized as acid, neutral, or alkaline [6, 7], according to the pH at which they show maximum activity [8]. Many anticancer agents increase ceramide levels to varying extents in different types of cancer cells [9]. Thus, the pharmacological modulation of sphingolipid metabolism to enhance ceramide in tumor cells represents a novel therapeutic approach.

Marine triterpene glycosides exhibit a wide range of biological activities, including antitumor activity [10, 11]. We previously demonstrated that stichoposide C (STC) (Figure 1A)—a hexaoside containing quinovose as the second monosaccharide unit—induces apoptosis of leukemia cells by generating ceramide. The mechanisms for ceramide generation include activation of acid SMase after activating caspase-8, and the activation of neutral SMase resulting from glutathione depletion and increased ROS production [12]. Another study reported that stichoposide D (STD) (Figure 1A)—a STC structural analog that contains glucose instead of quinovose in its carbohydrate chains—induces leukemia cell apoptosis through the activation of ceramide synthase 6 (CerS6) [13]. These studies suggest that marine triterpene glycosides, particularly hexaosides containing a quinovose as the second monosaccharide, are strong candidates for anti-leukemic agents.

Cladoloside C2, a novel marine triterpene glycoside with a quinovose as the second monosaccharide (Figure 1A), has been isolated from the holothurian Cladolabes schmeltzii (Subfamily Cladolabinae, Family Sclerodactylidae, Order Dendrochirotida). In our present study, we investigated the molecular mechanism underlying the anti-leukemic potential of cladoloside C2 in K562 and HL-60 cells, and mouse leukemia xenograft models. Our data provide the first evidence that cladoloside C2 induces apoptosis of human leukemia cells through an extrinsic, but not an intrinsic pathway. We further demonstrated that the in vitro and in vivo anti-leukemic effects of cladoloside C2 occur through a mechanism involving the activation of Fas/CerS6/p38 kinase/c-Jun-NH2-terminal kinase (JNK)/caspase-8 in lipid rafts.

RESULTS

**Cladoloside C2 induces apoptosis of leukemic cells through extrinsic pathway activation**

To examine whether cladoloside C2 can induce apoptosis of K562 and HL-60 cells, K562 and HL-60 cells were treated with various cladoloside C2 concentrations for different time periods, and co-stained with PI and FITC-conjugated annexin V. Cladoloside C2 treatment dose- and time-dependently increased the proportions of apoptotic cells (Figure 1B).

In contrast, the concentrations of cladoloside C2 that were used in this study (0.1–1.0 μM) did not increase apoptosis of normal human hematopoietic progenitor cells (CD34+ cells) compared to control, as further confirmed by annexin-V/PI staining (data not shown).

We further evaluated the cladoloside C2-induced apoptotic signaling in K562 and HL-60 cells, with particular focus on the caspase activation cascade. Cladoloside C2-induced caspase activation was suggested by cleavage of the caspase-3 substrate PARP, and was confirmed by the presence of cleaved caspase-3 and caspase-8 (Figure 1C). To investigate the functional involvement of caspases in cladoloside C2-induced apoptosis, we used the pan-caspase inhibitor (Z-VAD-FMK), and specific inhibitors of caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), and caspase-9 (Z-LEHD-FMK). Cladoloside C2-induced apoptosis was partially abolished by pretreatment with Z-VAD-FMK, Z-DEVD-FMK, or Z-IETD-FMK, but not Z-LEHD-FMK (Figure 1D). These data suggest that cladoloside C2-induced apoptosis in K562 and HL-60 cells is influenced by a caspase-dependent mechanism involving an extrinsic pathway.

To assess mitochondrial pathway activation by cladoloside C2 treatment, we measured the mitochondrial membrane potential (MMP) and examined mitochondrial protein expression in the cytosol using western blot analysis. Cladoloside C2-treated K562 and HL-60 cells showed no MMP loss (Figure 1E), as well as no cytoplasmic release of cytochrome c, Smac/DIABLO, or AIF (Figure 1F). These findings indicate that cladoloside C2 treatment of K562 and HL-60 cells activated extrinsic apoptotic pathways, but not intrinsic pathways. To explain this phenomenon, we further investigated how cladoloside C2 treatment affected the levels of the antiapoptotic proteins myeloid cell leukemia-1 (Mcl-1), B-cell lymphoma-2 (Bcl-2), and B-cell lymphoma extra large (Bcl-xL); and the proapoptotic protein Bcl-2-associated X protein (Bax). Interestingly, cladoloside C2 led to increased expressions of Mcl-1 and Bcl-xL, decreased expression of Bax, and no change in Bcl-2 expression (Figure 1C). The decreased Bax expression and increased expressions of Mcl-1 and Bcl-xL may support mitochondrial preservation.

**Cladoloside C2 generates ceramide through activation of ceramide synthase 6 following Fas activation in human leukemic cells**

We previously demonstrated that quinovose-containing STC induces apoptosis through ceramide generation via activation of acid and neutral SMase [12]. We expected that cladoloside C2 and STC would induce apoptosis through the same mechanism. Thus, we performed immunofluorescence staining, and found that cladoloside C2 increased ceramide generation (Figure 2A). To evaluate whether cladoloside C2-induced apoptosis was mediated by acid SMase, neutral SMase, or ceramide synthase, we incubated cells for 1 h with
Figure 1: Cladoloside C₂ induces apoptosis through extrinsic pathway activation in human leukemic cells. (A) Structures of cladoloside C₂ and stichoposides C and D. (B) Left panel: K562 and HL-60 cells were seeded, cultured for 4 h, and then treated for 6 h with various concentrations of cladoloside C₂ (0, 0.1, 0.3, or 0.5 µM). Right panel: K562 and HL-60 cells were seeded, cultured for 4 h, and then treated for the indicated times with 0.2 or 0.1 µM cladoloside C₂. The percentage of apoptotic cells was determined by annexin V-FITC/PI staining. Upper panels: Representative of three separate experiments. Lower panels: Mean ± SD of three independent experiments. **P < 0.01 vs. control cells. (C) K562 and HL-60 cells were treated with 0.2 or 0.1 µM cladoloside C₂ for the indicated times. Protein lysates were prepared and used for western blot analysis with the corresponding antibodies. β-actin was used as a loading control.
The blot is representative of three separate experiments. **(D)** Functional involvement of caspases in cladoloside C₂-induced apoptosis of K562 and HL-60 cells. Cells were pretreated for 1 h with the pan-caspase inhibitor Z-VAD-FMK (25 μM), the caspase-8 inhibitor Z-IETD-FMK (20 μM), the caspase-9 inhibitor Z-LEHD-FMK (20 μM), or the caspase-3 inhibitor Z-DEVD-FMK (50 μM), followed by treatment with 0.2 or 0.1 μM cladoloside C₂ for 6 h. The extent of apoptosis was measured by flow cytometry after annexin V staining. These data represent the mean ± SD of three independent experiments. "P < 0.01; ""P < 0.001 vs. cladoloside C₂-treated cells. **(E)** Left panel: K562 and HL-60 cells were treated with 0.2 or 0.1 μM cladoloside C₂ for 6 h. The extent of apoptosis was measured by flow cytometry after annexin V staining. These data represent the mean ± SD of three independent experiments. ""P < 0.001 vs. control cells. Right panel: K562 and HL-60 cells were treated with 0.2 or 0.1 mM cladoloside C₂ for 4 h or 2 h. The cells were stained with DiOC₆, and the reduction in Δψₘ was determined by monitoring the DiOC₆ uptake using flow cytometry. Low Δψₘ values are expressed as the percentage of cells exhibiting diminished mitochondrial potential. The values obtained from the DiOC₆ assays represent the mean ± SD of three independent experiments. **(F)** Western blot for the mitochondrial proteins AIF, Smac/DIABLO, cytochrome oxidase IV, and cytochrome c. Cytochrome oxidase IV (COX IV) was used as a mitochondrial marker. Protein lysates were prepared and subjected to western blot analysis using corresponding antibodies. β-actin was used as a loading control. The blot is representative of three separate experiments.

The acid SMase inhibitor desipramine, the neutral SMase inhibitor GW4869, the serine palmitoyl transferase inhibitor myriocin, or the ceramide synthase inhibitor fumonisin B₁, followed by treatment with cladoloside C₂. Cladoloside C₂-induced apoptosis was partially blocked by pretreatment with myriocin or fumonisin B₁, and was not blocked by pretreatment with desipramine or GW4869 (Figure 2B). Cladoloside C₂-induced ceramide generation in K562 and HL-60 cells was also blocked by pretreatment with myriocin or fumonisin B₁ (data not shown). We also examined the expressions of CerS4, CerS5, and CerS6 during cladoloside C₂-induced apoptosis. Cladoloside C₂ treatment markedly induced expression of CerS6, but not CerS4 or CerS5 (Supplementary Figure 1A, 1B). Thus, we focused on the role of CerS6 in cladoloside C₂-mediated cell death. Western blot analysis and immunofluorescence staining revealed that cladoloside C₂ treatment increased CerS6 expression in K562 and HL-60 cells (Figure 2C).

To verify the essential role of CerS6 activation in cladoloside C₂-mediated apoptosis, we transfected K562 and HL-60 cells with siRNA against CerS6 or nonspecific control siRNA. Western blot analysis and immunofluorescence staining confirmed CerS6 knockdown (Figure 2D, 2E). The extent of apoptosis was monitored in cladoloside C₂-treated transfected cells. CerS6 knockdown by siRNA partially protected cells from cladoloside C₂-induced apoptosis (Figure 2F). We observed similar results in CerS6 shRNA-silenced K562 and HL-60 cells (Supplementary Figure 2A–2C).

To establish the sequence of events accompanying cladoloside C₂-induced cell death, we monitored cladoloside C₂-induced activation of Fas, caspase-8, and caspase-3 in CerS6 siRNA-transfected K562 and HL-60 cells. CerS6 siRNA transfection reversed the activation of caspase-8 and caspase-3, but not Fas (Figure 2D). These data suggest that CerS6 activation occurred downstream of Fas activation and upstream of caspase-8 and caspase-3.

**Fas activation is involved in cladoloside C₂-induced CerS6 activation and apoptosis**

Since cladoloside C₂ treatment activated Fas (Figure 1C), we evaluated the functional significance of this activation by performing Fas knockdown with Fas siRNA in K562 and HL-60 cells. Western blot analysis and immunofluorescence staining confirmed Fas knockdown (Figure 3A, 3B), and the extent of apoptosis was monitored in cladoloside C₂-treated transfected cells. Fas knockdown partially protected cells from cladoloside C₂-induced apoptosis (Figure 3C), and reduced cladoloside C₂-induced CerS6 activation and ceramide generation (Figure 3A, 3B). Additionally, Fas siRNA transfection inhibited cladoloside C₂-induced activation of caspase-8 and caspase-3 (Figure 3A).

**Activation of p38 kinase and JNK occurs downstream of Fas and CerS6 activation, and may contribute to cladoloside C₂-induced apoptosis in human leukemic cells**

Ceramide activates multiple signaling pathways, including those involving mitogen-activated protein kinases (MAPKs) [14–18]. MAPKs—such as the extracellular signal regulating kinase (ERK), p38 kinase, and JNK—are centrally involved in stress-induced cell death, and in apoptotic signaling of ceramide. We explored the involvement of ERK, p38 kinase, and JNK in cladoloside C₂-induced apoptosis by treating K562 and HL-60 cells with cladoloside C₂ for various time periods, and then measuring MAPK protein levels by western blot analysis. Cladoloside C₂ time-dependently activated all MAPKs (Figure 4A). To ascertain the roles of activated ERK, p38 kinase, and JNK in cladoloside C₂-induced cell death, we used specific inhibitors of ERK (PD98059), p38 kinase, and JNK (SP600125) and measured the extent of apoptosis after 6 h of cladoloside C₂ treatment. Apoptosis was significantly reduced by inhibition of p38 kinase and JNK, but not ERK (Figure 4B). Inhibition of p38 kinase and JNK also significantly inhibited cladoloside C₂-induced caspase-8 activation, but not Fas activation, CerS6 activation, or ceramide generation (Figure 4C, 4D).

To further confirm the crucial role of p38 kinase in cladoloside C₂-induced apoptosis, we transfected K562 and HL-60 cells with p38 kinase siRNA. Western blot analysis confirmed p38 kinase knockdown (Figure 5A),...
### Table

| K562 | HL-60 |
|------|-------|
|      |       |

### Diagram

#### A

- K562
- HL-60
- Merge

#### B

- K562
- HL-60
- Merge

#### C

- K562
- HL-60

#### D

- K562
- HL-60

#### E

- K562
- HL-60

#### F

- K562
- HL-60
Cladoloside C₂ induces apoptosis of K562 and HL-60 cells through the activation of ceramide synthase 6 (CerS6). (A) K562 and HL-60 cells treated with cladoloside C₂ exhibited increased ceramide generation. (B) K562 and HL-60 cells (1 x 10⁶ cells/well) were incubated for 6 h with cladoloside C₂ in the presence or absence of myriocin, fumonisin B₁, desipramine, or GW4869. After treatment, the percentage of apoptotic cells was determined by annexin V-FITC/PI staining. Upper panel: Representative of three experiments in each cell line. Lower panel: Mean ± SD of three independent experiments. **P < 0.01; ***P < 0.001 vs. cladoloside C₂-treated cells. (C) K562 and HL-60 cells were incubated for 6 h with cladoloside C₂ in the presence or absence of myriocin or fumonisin B₁. Protein lysates were prepared and subjected to western blot analysis using corresponding antibodies. Western blots are each representative of three separate experiments. β-actin was used as a loading control. Densitometry results are expressed above the bands. (D–F) K562 and HL-60 cells were transiently transfected for 48 h by electroporation with CerS6 siRNA, nonspecific control (NC) siRNA, or no siRNA (shock). (D) Western blot analysis of protein lysates. (E) Transfected K562 cells were exposed to 0.2 μM cladoloside C₂ for 2 h, and then fixed and permeabilized. Samples were then stained with PE-conjugated antibodies against Fas, CerS6, ceramide, cleaved caspase-8, p-p38 kinase, p38 kinase, p-ERK, ERK, p-JNK, or JNK, and with Alexa 488-labeled cholera toxin B antibody. The pictures are representative of three separate experiments. (F) Upper panel: The culture medium was changed, and K562 and HL-60 cells were incubated for 6 h with or without 0.2 or 0.1 μM cladoloside C₂. The percentage of apoptotic cells was determined by annexin V-FITC/PI staining. Results are representative of three independent experiments in each cell line. Lower panel: Mean ± SD of three independent experiments. **P < 0.01, ***P < 0.001, cells treated with cladoloside C₂ alone versus cells transfected with CerS6 siRNA and treated with cladoloside C₂.

The above-described observations suggest that cladoloside C₂, activated Fas and CerS6, leading to ceramide generation, followed by the activation of caspase-8, caspase-3, and JNK. We next examined the clustering of these signaling molecules (Fas, CerS6, ceramide, p38 kinase, JNK, and active caspase-8) in lipid rafts during cladoloside C₂-induced apoptosis. K562 and HL-60 cells were pretreated with the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD) and nystatin for 1 h, followed by cladoloside C₂ treatment. Then we assessed the extent of apoptosis and the activation of Fas, caspase-8, caspase-3, and CerS6. Incubation of K562 and HL-60 cells with MβCD and nystatin inhibited cladoloside C₂-induced apoptosis, as well as the activation of Fas, caspase-8, caspase-3, and CerS6 (Figure 6A–6C).

The cholera toxin (CTx) B subunit predominantly localized in lipid rafts. We used the lipid raft marker Alexa 488-labeled CTx B, and found that cladoloside C₂ promoted the co-aggregation of Fas/CD95, CerS6, ceramide, p-p38 kinase, p-JNK, and active caspase-8, with lipid rafts in K562 and HL-60 cells (Figure 6C). Furthermore, incubation of K562 and HL-60 cells with MβCD inhibited cladoloside C₂-induced activation of caspase-8 and caspase-3 (Figure 6B).

Cladoloside C₂ induces antitumor activity through the activation of Fas, CerS6, p38 kinase, and JNK in K562 and HL-60 xenograft mouse tumor models

Finally, we observed that cladoloside C₂ significantly inhibited tumor growth in both HL-60 and K562 mouse xenograft models (Figure 7A). Tumors from control mice displayed the typical histologic appearance of leukemic cells. After 21 d, tumors from cladoloside C₂-treated mice showed mean volumes over 75% smaller than the volumes of tumors in vehicle-treated K562 and HL-60 xenograft mice (control group: 3174.18 ± 262.39 mm³, cladoloside C₂ group: 640.27 ± 159.74 mm³, cladoloside C₂ group: 685.51 ± 154.62 mm³). We used stable CerS6 shRNA-silenced K562 and HL-60 xenograft models to investigate the involvement of CerS6 in the in vivo antitumor activity of cladoloside C₂. In parallel, CerS6-silenced cells and nonspecific control (NC) cells were subcutaneously inoculated into 6-week-old nude mice. Next, cladoloside C₂ or vehicle was injected into each mouse. The anti-tumor effect of cladoloside C₂ was significantly inhibited in CerS6 shRNA-silenced K562 and HL-60 xenograft models (Figure 7B), with 75.6% and 84.1% inhibition of tumor growth in NC-shRNA-1 and NC-shRNA-3 xenograft models, respectively, vs. 15.6% and 25.6% inhibition of tumor growth by cladoloside C₂ in CerS6-shRNA-1 and CerS6-shRNA-5 xenograft models, respectively. As expected, western blot analysis of tumors from vehicle-treated NC-shRNA-1 and NC-shRNA-3 control mice revealed weak expressions of Fas, CerS6, p-p38, and p-JNK (Figure 7C), and we observed...
weak immunostaining for Fas, CerS6, ceramide, p-p38, and p-JNK (Figure 7D). In contrast, western blot and immunohistochemical analysis of tumors from cladoloside C₂-treated NC-shRNA-1 and NC-shRNA-3 shRNA mice revealed up-regulation of Fas, CerS6, ceramide, p-p38, and p-JNK (Figure 7C, 7D). The expression and staining of p38 kinase and JNK did not differ between control and cladoloside C₂-treated tumors (Figure 7C, 7D). These data were consistent with our in vitro findings.

DISCUSSION

Marine triterpene glycosides represent promising candidate anticancer agents. However, their detailed molecular mechanisms have not been clearly defined. We previously reported that triterpene glycosides from Thelenota anax induce leukemic cell apoptosis through ceramide generation. However, molecular mechanisms and potencies differ among structurally different triterpene glycosides, and a previous study suggests that STC has more potent anti-leukemia cell activity than STD. Here we investigated the novel nonsulfated triterpene glycoside cladoloside C₂, which contains the same carbohydrate chain as STC but with a different aglycone moiety. Our present results indicated that cladoloside C₂ is more potent than STC. Moreover, unlike STC, cladoloside C₂ apparently activates extrinsic apoptosis pathways, but not intrinsic pathways.

Interestingly, cladoloside C₂ did not seem to affect mitochondria, as we observed increased expressions of Mcl-1 and Bcl-xL and decreased Bax expression. Many cancer cells are apt to be resistant to chemotherapeutic agents due to overexpression of prosurvival factors, such as Mcl-1, Bcl-2, and Bcl-xL. Therefore, these proteins are important targets for the development of new anti-cancer agents [19–21]. Since cladoloside C₂ induces apoptosis despite enhancing the expressions of Mcl-1 and Bcl-xL, it may be useful as an anti-leukemic agent in leukemia cells that overexpress Mcl-1 and Bcl-xL. In particular, Mcl-1 overexpression reportedly leads to etoposide resistance [22], and we observed that cladoloside C₂ sensitized K562 cells to etoposide and Ara-C (Supplementary Figure 3). Further studies are required to confirm this sensitizing effect in various leukemic cells that are resistant to chemotherapeutic agents.

Since cladoloside C₂ and STC share the same carbohydrate chain structure, we expected that cladoloside C₂, like STC, would induce apoptosis through the activation of acid and neutral SMases. Unexpectedly, we found that cladoloside C₂-induced apoptosis through CerS6 activation, following to Fas activation, and the subsequent activation of p38 kinase/JNK/caspase-8. Similar to the mechanism of STD, Fas activation by cladoloside C₂ was not reversed by transfection with CerS6 siRNA. However, CerS6 activation and ceramide generation were reversed by Fas siRNA transfection.

In ceramide-induced apoptosis, MAPKs are important signaling molecules. Several previous studies have demonstrated that ceramide causes ERK dephosphorylation, and induces p38 kinase and JNK phosphorylation [14–18]. Ceramide-activated p38 kinase and JNK generally contribute to the induction of cell apoptosis through mitochondrial damage and caspase activation. Our present results demonstrated that cladoloside C₂-generated ceramide activated ERK, p38 kinase, and JNK, but did not induce mitochondrial damage. Since JNK and p38 kinase signaling targets the anti-apoptotic Mcl-1 and Bcl-2 proteins [23–25], we examined how p38 kinase and JNK inhibition (by chemical inhibitors or p38 kinase siRNA or DN-JNK) influenced Mcl-1 and Bcl-2 expressions, caspase-8 activation, and apoptosis. Interestingly, inhibition of p38 kinase by SB203580 and of JNK by SP600125 led to reduced Mcl-1 expression, and reversed caspase-8 activation and apoptosis, but did not change Bcl-2 expression. Similar results were obtained from silencing p38 kinase using p38 kinase siRNA, and inhibiting JNK via DN-JNK transfection. Thus, it appears that the activities of p38 kinase and JNK lead to enhanced Mcl-1 expression and are involved in cladoloside C₂-induced apoptosis.

Lipid rafts play crucial roles in the Fas receptor death pathway [26, 27]. Based on the recently demonstrated role of plasma membrane lipid rafts in STD-induced apoptosis [13], here we wed MβCD and nystatin to investigate the roles of the Fas death receptor pathway and ceramide-enriched membrane domains in cladoloside C₂-induced cell death. Cladoloside C₂-induced Fas clustering in lipid rafts. We also detected co-localization of the Fas downstream signaling molecules CerS6, ceramide, p38 kinase, JNK, and caspase-8 in lipid rafts upon cladoloside C₂ stimulation. MβCD blocked the cladoloside C₂-induced clustering of Fas and the downstream signaling molecules, we well as apoptosis. These results suggest that the clustering of Fas and downstream signaling molecules in lipid rafts was essential for cladoloside C₂-induced apoptosis.
Compared to controls, cladoloside C₂ treatment significantly inhibited tumor growth in mouse HL-60 and K562 leukemic xenograft models. Cladoloside C₂ treatment also led to up-regulation of Fas, CerS6, and ceramide and activation of p38 kinase, JNK, and caspase-8. The anti-tumor effect of cladoloside C₂ was significantly prevented in CerS6 shRNA-silenced xenograft models. These results are consistent with the in vitro data. Moreover, cladoloside C₂ showed a lack of toxicity towards normal hematopoietic progenitor cells and in mice, supporting it as a promising potential candidate for therapeutic use.

Future studies are needed to explore the antitumor activity of cladoloside C₂ in other types of leukemia, including chemotherapy-resistant leukemia cells, and in other types of cancer. There also remains a need to investigate other molecular mechanisms that may be involved in cladoloside C₂-induced apoptosis.

In summary, this study provides the first evidence that cladoloside C₂ induces apoptosis of human leukemic cells via activation of Fas/CerS6/p38 kinase/JNK/caspase-8 in lipid rafts (Figure 8). Cladoloside C₂ also exhibited in vivo antitumor activity through the activation of Fas/CerS6/p38 kinase/JNK/caspase-8. Our results suggest that cladoloside C₂ may be a useful candidate for the treatment of human leukemia overexpressing Mcl-1 and Bcl-xL.

MATERIALS AND METHODS

Cell preparations

The human leukemic cell lines K562 and HL-60 were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea), and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Human hematopoietic progenitor CD34⁺ cells were purchased from STEM CELL Technologies (Vancouver, BC), and cultured in Hematopoietic Progenitor Expansion Medium DXF with cytokine mix E (PromoCell, Heidelberg, Germany).

Reagents

Cladoloside C₂ was isolated and purified following the procedure published by Silchenko et al. [28], and was dissolved in sterilized distilled water. Annexin V was obtained from BD Biosciences Clontech (Palo Alto, CA, USA). Anti-Fas, anti-procaspase 8, anti-procaspase-3, anti-procaspase 9, and anti-cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against poly (ADP-ribose) polymerase (PARP), JNK, and p-JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-β-actin antibody was obtained from Sigma (St. Louis, MO, USA). Unless otherwise stated, all other chemicals were purchased from Sigma.

Aptosis analysis

The extent of apoptosis was evaluated using annexin V-FITC and flow cytometry as previously described [29].

Measurement of MMP

Variations in MMP (Δφ_m) were examined using DiOC₆₃ (Molecular Probes, Eugene, OR) as previously described [29].

Separation of the cytosolic and mitochondrial proteins

Cytosolic and mitochondrial proteins were extracted from cells treated with sterilized water or with cladoloside C₂ for the indicated times, and were separated as previously described [30, 31].

Western blot analysis

Cell lysis and western blot analysis were performed as described previously [29], using 30 μg protein for immunoblotting. β-actin was used as the loading control.

Immunofluorescence staining

Cells were fixed and permeabilized with 1% formaldehyde/methanol in PBS for 10 min at room temperature. Next, the cells were washed, and a series of antibodies was used as indicated, followed by staining with FITC- or PE-conjugated goat anti-mouse and anti-rabbit IgG (Calbiochem, San Diego, CA). The samples were then mounted using glycerol, and analyzed by
confocal microscope (Carl Zeiss LSM 510; Carl Zeiss, Thornwood, NY) with a 40× C-Apochromat objective. Negative control staining was performed using only secondary antibodies.

**siRNA transfection**

We purchased pre-designed siRNA targeted to human CerS6-1 mRNA (catalog number SI02758245; ID 253782), and AllStars negative control siRNA (catalog number 1027280) from Qiagen (Hilden, Germany). The siRNA sequence used for targeted silencing of CerS6 has been previously described [13]. Fas siRNA was obtained from Santa Cruz Biotechnology, and p38 kinase siRNA from Dharmacon (L-003512-00-0005; Thermo Scientific, Chicago, IL, USA), and their sequences have been previously published [13].

Cells were resuspended in PBS at 1.3 × 10⁷ cells/0.5 mL, and then mixed with 200 nM anti-CerS6 siRNA, anti-p38 kinase siRNA, anti-Fas siRNA, or non-silencing siRNA. This mixture was added to an electroporation cuvette with a 0.4-cm electrode gap, for transfection at 300 V and 950 μF in a Gene Pulser Xcell Electroporation System (Bio-Rad, Richmond, CA, USA). After electroporation, the cells were cultured for 48 h in RPMI1640 supplemented with 10% FBS, then treated with sterilized water or cladoloside C₂, Protein lysates were prepared and subjected to western blot analysis.

**Generation of CerS6-silenced K562 and HL-60 cell lines**

We obtained an shRNA construct containing CerS6 shRNA (MISSION® shRNA plasmid DNA; CerS6-pLKO.1-puro) and the non-targeting control construct NC-pLKO.1-puro from Sigma (St. Louis, MO, USA). The CerS6 shRNA and NC shRNA sequences have previously been published [13]. K562 and HL-60 cells (1 × 10⁶) were transfected with 2 μg of CerS6-pLKO.1-puro or NC-pLKO.1-puro using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s recommended protocol. Starting at 24 h post-transfection, the cells were selected with 2 μg/mL puromycin for 14 days to obtain stable clones, and positive clones were picked for identification. Stable cell lines were cultured in RPMI1640 supplemented with 10% FBS, and then treated for 6 h with or without cladoloside C₂. These cells were analyzed by annexin-V staining, immunofluorescence, and western blot.

**Establishment of HL-60 and K562 leukemia xenograft models**

All animal procedures and care were approved by the Institutional Animal Care and Usage Committee of Dong-A University. To determine the in vivo activity of cladoloside C₂, K562 cells (1.5 × 10⁶/100 μL PBS per mouse) and HL-60 cells (2 × 10⁶/100 μL PBS per mouse), which were confirmed to be viable by trypan blue staining, were injected into the right flanks of 6-week-old female Balb/c nude mice (n = 5 mice per group; Orient Bio Inc., Korea), as previously described [12, 29]. To confirm the essential role of CerS6 in the in vivo antitumor activity of cladoloside C₂, K562 and HL-60 cells expressing NC construct and K562 and HL-60 cells expressing CerS6 shRNA were injected, at the above-mentioned concentrations, into the right flanks of 6-week-old female Balb/c nude mice (n = 5 mice per group; Orient Bio Inc., Korea). When the average subcutaneous tumor volume reached 60–100 mm³, the mice were assigned
Figure 6: Clustering of Fas and its downstream molecules in lipid rafts during cladoloside C₂-induced apoptosis of K562 and HL-60 cells. (A) K562 and HL-60 cells were pretreated for 1 h with MβCD (20 μg/mL) and nystatin (20 μg/mL), and then cultured for 6 h in medium containing 0.2 or 0.1 μM cladoloside C₂. After treatment for the indicated times, the percentage of apoptotic cells was determined by annexin V-FITC/PI staining. Upper panel: Representative of three independent experiments. Lower panel: Mean ± SD of three independent experiments. **P < 0.01; ***P < 0.001 versus cladoloside C₂-treated cells. (B) Whole cell lysates were prepared from K562 and HL-60 cells incubated for 6 h with cladoloside C₂ in the presence or absence of MβCD, and were subjected to western blot analysis. (C) K562 cells were treated for 2 h with cladoloside C₂ in the presence or absence of MβCD or nystatin, and then fixed and permeabilized. These samples were then stained with PE-conjugated antibodies against Fas, CerS6, ceramide, cleaved caspase-8, p-p38 kinase, p38 kinase, p-ERK, ERK, p-JNK, or JNK, as well as Alexa 488-labeled cholera toxin B antibody. The pictures are representative of three separate experiments.
to either the cladoloside C₂ treatment or control group, receiving 7.19 μg/kg cladoloside C₂ or vehicle via the tail vein every 3 days. Tumor size was measured daily using a caliper: calculated volume = shortest diameter² × longest diameter/2. *P < 0.05, **P < 0.01, and ***P < 0.001, versus respective controls. (B) Left panel: NC-shRNA-transfected and CerS shRNA-transfected stable K562 (1.5 × 10⁷ cells/100 mL) were subcutaneously injected into Balb/c nude mice. Right panel: NC-shRNA-transfected and CerS shRNA-transfected stable HL-60 (2 × 10⁷ cells/100 mL) were subcutaneously injected into Balb/c nude mice. After the cells formed palpable tumors, the mice were randomized into two groups (n = 5 mice/group) and treated with vehicle control or cladoloside C₂ (7.19 μg/kg). Tumor size was measured daily using a caliper. *P < 0.05, **P < 0.01, and ***P < 0.001, versus respective controls. (C and D) Tumor tissues obtained from the above-described experiment on Day 14 were subjected to western blot analysis (C) and immunohistochemistry (D) using antibodies against Fas, CerS6, ceramide, p-p38, p38 kinase, p-JNK, or JNK. The sections were lightly counterstained with hematoxylin, and photographed with a ScanScope. K562 and HL-60 leukemia xenografts from cladoloside C₂-treated mice exhibited apoptosis and extensive necrosis (200×).

Histology and immunohistochemical analysis

Tumor sections were stained with hematoxylin/eosin, and immunohistochemistry was performed using the Discovery XT automated immunohistochemistry stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Tissue sections were deparaffinized using EZ Prep solution (Ventana Medical Systems). Antigen retrieval was performed by using CCI standard (pH 8.4 buffer containing Tris/Borate/EDTA; Ventana Medical Systems) for 24 min with anti-Fas antibody, 45 min with anti-ceramide antibody, 24 min with anti-CerS6 antibody, 45 min with anti-p-p38 antibody, 60 min with anti-p38 antibody, 15 min with anti-p-JNK antibody, and 24 min with anti-JNK antibody. The slides were then treated with Inhibitor D (3% H₂O₂, endogenous peroxidase; Ventana Medical Systems) for 4 min at 37°C. Next, the slides were incubated at 37°C for 32 min with anti-Fas

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Figure 7: Cladoloside C₂ inhibits the growth of K562 and HL-60 xenograft tumors and induces apoptosis through Fas/CerS6/p38 kinase/JNK activation in vivo. (A) K562 (1.5 × 10⁷ cells/100 μL) and HL-60 cells (2 × 10⁷ cells/100 μL) were subcutaneously injected into Balb/c nude mice. After the cells formed palpable tumors, the mice were randomized into two groups (n = 5 mice/group) and treated with vehicle control or cladoloside C₂ (7.19 μg/kg). Tumor size was measured daily using a caliper: calculated volume = shortest diameter² × longest diameter/2. *P < 0.05, **P < 0.01, and ***P < 0.001, versus respective controls. (B) Left panel: NC-shRNA-transfected and CerS shRNA-transfected stable K562 (1.5 × 10⁷ cells/100 mL) were subcutaneously injected into Balb/c nude mice. Right panel: NC-shRNA-transfected and CerS shRNA-transfected stable HL-60 (2 × 10⁷ cells/100 mL) were subcutaneously injected into Balb/c nude mice. After the cells formed palpable tumors, the mice were randomized into two groups (n = 5 mice/group), and treated with vehicle control or cladoloside C₂ (7.19 μg/kg). Tumor size was measured daily using a caliper. *P < 0.05, **P < 0.01, and ***P < 0.001, versus respective controls. (C and D) Tumor tissues obtained from the above-described experiment on Day 14 were subjected to western blot analysis (C) and immunohistochemistry (D) using antibodies against Fas, CerS6, ceramide, p-p38, p38 kinase, p-JNK, or JNK. The sections were lightly counterstained with hematoxylin, and photographed with a ScanScope. K562 and HL-60 leukemia xenografts from cladoloside C₂-treated mice exhibited apoptosis and extensive necrosis (200×).

Figure 8: Hypothetical molecular mechanisms of cladoloside C₂-induced apoptosis in human leukemia cells.
antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1 h with anti-ceramide antibody (1:10 dilution; Enzo Life Sciences, Inc., PA, USA), 30 min with anti-CerS6 antibody (1:200 dilution; Biorbyt Limited, Cambridge, UK), 1 h with anti-p-p38 antibody (1:200 dilution; Cell Signaling Technology, Beverly, MA, USA), 2 h with anti-p38 antibody (1:20 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), and 32 min with anti-JNK antibody (1:20 dilution; Cell Signaling Technology, Beverly, MA, USA). The slides were then incubated overnight at 4°C with anti-p-JNK antibody (1:20 dilution; Cell Signaling Technology, Beverly, MA, USA). Subsequently, the slides were treated at 37°C for 8 min with Dako REAL™ Envision™ anti-rabbit/mouse HRP (Dako) secondary antibody to anti-ceramide antibody, anti-CerS6 antibody, and anti-p-p38 antibody; for 16 min with the secondary antibody to anti-Fas antibody, anti-p38 antibody, and anti-JNK antibody; and for 30 min with the secondary antibody to anti-p-JNK antibody. Finally, the slides were incubated for 8 min in DAB/H₂O₂ substrate using the Ventana Chromo Map Kit (Ventana Medical Systems), followed by hematoxylin/eosin counterstaining. Sections were washed with PBS, mounted with VectaShield mounting medium (Vector Laboratories, Burlingame, CA, USA), coverslipped, and imaged using a ScanScope (Aperio Technologies, Inc., Vista, CA, USA).

Statistical analysis

Statistical analyses were performed using the SPSS 21.0 statistical package for Windows (SPSS, Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). We used one-way ANOVA to evaluate significant differences in cell viability between cladoloside C₂-treated and control cells. We assessed differences in tumor volume between treated and control groups using Student’s unpaired t-test. Statistical significance was defined as P < 0.05.

Abbreviations

STC: Sticholoside C; STD: Stichoposide D; CerS6: Ceramide synthase 6; SMases: Sphingomyelinases; PARP: Poly (ADP-ribose) polymerase; JNK: c-Jun-NH₂-terminal kinase; FBS: Fetal bovine serum; MMP: Mitochondrial membrane potential; DN-JNK: Dominant negative-JNK; Mcl-1: Myeloid cell leukemia-1 (Mcl-1); Bcl-2: B-cell lymphoma-2; Bcl-xL: B-cell lymphoma extra large; MAPKs: Mitogen-activated protein kinases; ERK: Extracellular signal regulating kinase; MβCD: Methyl-β-cyclodextrin; CTx: cholera toxin

Author contributions

SHY and JIP designed the research study and interpreted the data. SHY, EHS, SHH, TRK, MJH, JYH, JSJ, SHK and ASS performed the experiments. SHY, VAS and JIP wrote the manuscript. SHY performed the statistical analysis. JIP supervised the study. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors have no potential conflicts of interest to disclose.

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REFERENCES

1. Smith M, Barnett M, Bassan R, Gatta G, Tondini C, Kern W. Adult acute myeloid leukemia. Crit Rev Oncol Hematol. 2004; 50:197–222.  
2. Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ; American Cancer Society. Cancer statistics. CA Cancer J Clin. 2004; 54:8–29.  
3. Dbaibo GS, Pushkareva MY, Rachid RA, Alter N, Smyth MJ, Obeid LM, Hannun YA. P53-dependent ceramide response to genotoxic stress. J Clin Invest. 1998; 102:329–39.  
4. Brown DA, London E. Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol. 1998; 14:111–36.  
5. Kolesnick RN, Goni FM, Alonso A. Compartmentalization of ceramide signaling: physical foundations and biological effects. J Cell Physiol. 2000; 184:285–300.  
6. Ogretmen B, Hannun YA. Biologically active sphingolipids in cancer pathogenesis and treatment. Nat Rev Cancer. 2004; 4:604–16.  
7. Hannun YA, Obeid LM. Principles of bioactive lipid signaling: lessons from sphingolipids. Nat Rev Mol Cell Biol. 2008; 9:139–50.  
8. Strum JC, Ghosh S, Bell RM. Lipid second messengers. A role in cell growth regulation and cell cycle progression. Adv Exp Med Biol. 1997; 407:421–31.  
9. Taha TA, Mullen TD, Obeid LM. A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. Biochim Biophys Acta. 2006;1758: 2027–36.
10. Stonik VA, Kalinin VI, Avilov SA. Toxins from sea cucumbers (holothuroids): Chemical structures, properties, taxonomic distribution, biosynthesis and evolution. J Nat Toxins. 1999; 8:235–48.

11. Stonik VA. Some terpenoid and steroid derivatives from echinoderms and sponges. Pure Appl Chem. 1986; 58:423–36.

12. Yun SH, Park ES, Shin SW, Na YW, Han JY, Jeong JS, Shastina VV, Stonik VA, Park JI, Kwak JY. Stichoposide C induces apoptosis through the generation of ceramide in leukemia and colorectal cancer cells and shows in vivo antitumor activity. Clin Cancer Res. 2012; 18:5934–48.

13. Yun SH, Park ES, Shin SW, Ju MH, Han JY, Jeong JS, Kim SH, Stonik VA, Kwak JY, Park JI. By activating Fas/ceramide synthase 6/p38 kinase in lipid rafts, stichoposide D inhibits growth of leukemia xenografts. Oncotarget. 2015; 6:27596–612. https://doi.org/10.18632/oncotarget.4820.

14. Stoica BA, Movsesyan VA, Knoblach SM, Faden AI. Ceramide induces neuronal apoptosis through mitogen-activated protein kinases and causes release of multiple mitochondrial proteins. Mol Cell Neurosci. 2005; 29:355–71.

15. Willaime S, Vanhoucke P, Caboche J, Lemaigre-Dubreuil Y, Mariani J, Brugg B. Ceramide-induced apoptosis in cortical neurons is mediated by an increase in p38 phosphorylation and not by the decrease in ERK phosphorylation. Eur J Neurosci. 2001; 13:2037–46.

16. Kong JY, Klassen SS, Rabkin SW. Ceramide activates a mitochondrial p38 mitogen-activated protein kinase: a potential mechanism for loss of mitochondrial transmembrane potential and apoptosis. Mol Cell Biochem. 2005; 278:39–51.

17. Willaime-Moraweck S, Brami-Cherrier K, Mariani J, Caboche J, Brugg B, c-Jun N-terminal kinases/c-Jun and p38 pathways cooperate in ceramide induced neuronal apoptosis. Neuroscience. 2003; 119:387–97.

18. Kurinna SM, Tsao CC, Nica AF, Jiffar T, Ruvolo PP. Ceramide promotes apoptosis in lung cancer-derived A549 cells by a mechanism involving c-Jun NH2-terminal kinase. Cancer Res. 2004; 64:7852–6.

19. Azmi AS, Mohammad RM. Non-peptidic small molecule inhibitors against Bcl-2 for cancer therapy. J Cell Physiol. 2009; 218:13–21.

20. Azmi AS, Wang Z, Philip PA, Mohammad RM, Sarkar FH. Emerging Bcl-2 inhibitors for the treatment of cancer. Expert Opin Emerg Drugs. 2011; 16:59–70.

21. Quinn BA, Dash R, Azab B, Sarkar S, Das SK, Kumar S, Oyesanya RA, Dasgupta S, Dent P, Grant S, Rahman M, Curiel DT, Dmitriev I, et al. Targeting Mcl-1 for the therapy of cancer. Expert Opin Investig Drugs. 2011; 20:1397–411.

22. Zhou P, Qian L, Kozopas KM, Craig RW. Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood. 1997; 89:630–43.

23. Krishna M, Narang H. The complexity of mitogen-activated protein kinases (MAPKs) made simple. Cell Mol Life Sci. 2008; 65:3525–44.

24. Son JK, Varadarajan S, Bratton SB. TRAIL-activated stress kinases suppress apoptosis through transcriptional upregulation of Mcl-1. Cell Death Differ. 2010; 17:1288–301.

25. Trouillas M, Saucourt C, Duval D, Gauthereau X, Thibault C, Dembele D, Feraud O, Menager J, Rallu M, Pradier L, Boeuf H. Bel2, a transcriptional target of p38α, is critical for neuronal commitment of mouse embryonic stem cells. Cell Death Differ. 2008; 15:1450–9.

26. Verkleij AJ, Post JA. Membrane phospholipid asymmetry and signal transduction. J Membr Biol. 2000; 178:1–10.

27. Silchenko AS, Kalinovsky AI, Avilov SA, Andryjaschenko PV, Dmitrenok PS, Yurchenko EA, Dolmatov IY, Kalinin VI, Stonik VA. Structure and biological action of cladolosides B1, B2, C1, C2 and D, six new triterpene glycosides from the sea cucumber Cladolabes schmeltzii. Nat Prod Commun. 2013; 8:1527–34.

28. Shin SW, Seo CY, Han H, Han JY, Jeong JS, Kwak JY, Park JI. 15d-PGJ2 induces apoptosis by reactive oxygen species-mediated inactivation of Akt in leukemia and colorectal cancer cells and shows in vivo antitumor activity. Clin Cancer Res. 2009; 15:5414–25.

29. Lee CJ, Han JS, Seo CY, Park TH, Kwon HC, Jeong JS, Kim IH, Yun J, Bae YS, Kwak JY, Park JI. Pioglitazone, a synthetic ligand for PPARγ, induces apoptosis in RB-deficient human colorectal cancer cells. Apoptosis. 2006; 11:401–11.

30. Han H, Shin SW, Seo CY, Kwon HC, Han JY, Kim IH, Kwak JY, Park JI. 15d-Deoxy-D12,14-prostaglandin J2 (15d-PGJ2) sensitizes human leukemic HL-60 cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through Akt downregulation. Apoptosis. 2007; 12:2101–14.