Accelerated degradation of cFLIP<sub>L</sub> and sensitization of the TRAIL DISC-mediated apoptotic cascade by pinoresinol, a lignan isolated from *Rubia philippinensis*

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Plant-derived lignans have numerous biological effects including anti-tumor and anti-inflammatory activities. Screening of purified constituents of *Rubia philippinensis* from human glioblastoma cells resistant to TNF-related apoptosis-inducing ligand (TRAIL) has suggested that the lignan pinoresinol was a highly active TRAIL sensitizer. Here we show that treatment with nontoxic doses of pinoresinol in combination with TRAIL induced rapid apoptosis and caspase activation in many types of glioblastoma cells, but not in normal astrocytes. Analyses of apoptotic signaling events revealed that pinoresinol enhanced the formation of TRAIL-mediated death-inducing signaling complex (DISC) and complete processing of procaspase-8 within the DISC in glioblastoma cells, in which caspase-8 was inactivated. Mechanistically, pinoresinol downregulated the expression of cellular FLICE-inhibitory protein (cFLIP<sub>L</sub>) and survivin through proteasome-mediated degradation, without affecting death receptors or downstream intracellular apoptosis-related proteins. Furthermore, the sensitization of TRAIL-mediated apoptosis by pinoresinol strictly depended on the expression level of cFLIP<sub>L</sub>, which was regulated through de novo protein synthesis, rather than by NF-κB or p53 signaling. Taken together, our results indicate that pinoresinol facilitates DISC-mediated caspase-8 activation by targeting cFLIP<sub>L</sub> in an early event in apoptotic signaling, which provides a potential therapeutic module for TRAIL-based chemotherapy.
appearance of cancer cells that escape the cytotoxicity induced by TRAIL-targeted therapy. Thus, the discovery of a therapeutic strategy module that can eradicate cancer cells without restoring resistance has been pending in the field of TRAIL-based chemotherapy.

The genus *Rubia* (family Rubiaceae), a perennial herb, is widely distributed worldwide. It is one of the most attractive plant resources because of its potent and wide spectrum of *in vivo* and *in vitro* biological activities, which include anti-cancer, anti-inflammatory, and anti-angiogenic effects. In a recent phytochemical study of *Rubia philippinensis* Elmer, we isolated several compounds, including derivatives of anthraquinones, pentacyclic triterpenoids, cyclopeptides, and lignans. Although several studies have reported the anti-cancer effects of *Rubia* species, the effects of the principle constituents of *R. philippinensis* on DR-mediated cell death, particularly during TRAIL sensitization, have not yet been determined. As part of our ongoing search to identify potential therapeutic approaches for sensitizing TRAIL-mediated cell death, we tested 33 compounds isolated from *R. philippinensis* and found that nontoxic doses of pinoresinol, a lignan, drastically sensitized cancer cells against TRAIL-induced apoptosis. Pinoresinol facilitated DISC formation to trigger a caspase-8-dependent apoptotic cascade activation in TRAIL-resistant glioblastoma cells. Moreover, our findings revealed novel evidence that the prominent sensitizing effects of pinoresinol against TRAIL-mediated apoptosis involved the downregulation of levels of cellular FLICE-inhibitory protein (cFLIP) by a mechanism involving de novo protein synthesis.

**Results**

**Identification of pinoresinol from *R. philippinensis* as a TRAIL sensitizer in TRAIL resistant glioma cells.** We characterized a set of major compounds obtained from *R. philippinensis* to identify active constituents that synergistically sensitized the cytotoxic effects of TRAIL in TRAIL-resistant glioblastoma cells (Supplementary Table S1, Supplementary Figs 1–33). Treatment of LN428 cells with 50–200 ng/ml TRAIL alone induced a limited number of cell deaths (<5%) over 24 h (data not shown). In the screening assay, LN428 cells were sequentially treated with the purified compounds and 50 ng/ml TRAIL, followed by an ATP-based cell viability assay. In parallel, we tested the cytotoxicity of each compound on LN428 cells as single agents. Of the compounds screened, the lignin pinoresinol was a potent sensitizer of TRAIL-mediated cell death, we tested 33 compounds isolated from *R. philippinensis* and found that nontoxic doses of pinoresinol, a lignan, drastically sensitized cancer cells against TRAIL-induced apoptosis. Pinoresinol facilitated DISC formation to trigger a caspase-8-dependent apoptotic cascade activation in TRAIL-resistant glioblastoma cells. Moreover, our findings revealed novel evidence that the prominent sensitizing effects of pinoresinol against TRAIL-mediated apoptosis involved the downregulation of levels of cellular FLICE-inhibitory protein (cFLIP) by a mechanism involving de novo protein synthesis.
Sensitization of TRAIL-induced killing by pinoresinol is associated with a caspase-8-dependent apoptotic cascade in glioma cells. Next, to validate the above screening results, we performed kinetic experiments to evaluate the synergistic induction of cell death using a nontoxic concentration (0.5 μM) of pinoresinol. TRAIL-mediated cytotoxicity began to appear from 9 h after pinoresinol co-treatment, and rapidly increased up to 24 h (Fig. 2A). In addition, pinoresinol had a similar synergistic efficacy against TRAIL-mediated cytotoxicity in three other glioblastoma cell lines (U87MG, LNZ308, LN428, and U251) and normal primary astrocytes were treated with pinoresinol, TRAIL and TRAIL plus pinoresinol for 24 h. Cell death was quantified as in Fig. 1A. Data were normalized to the rate of spontaneous cell death occurring in untreated cells. Data represents the mean ± SE of three independent experiments. *p < 0.05, compared with the TRAIL only-treated group. (C,D) LN428 cells were treated with PINO, TRAIL and TRAIL plus PINO for 24 h in the absence or presence of caspase or necroptosis inhibitor z-VAD-fmk (20 μM)/z-IETD-fmk (50 μM) or Nec-1 (30 μM). (C) Cell death was quantified as in A. Data represents the mean ± SE of three independent experiments. *p < 0.05, compared with the PINO/TRAIL-treated group. (D) Cells were visualized using an inverted microscope. (E) LN428 cells were treated with PINO, TRAIL and TRAIL plus PINO for 24 h in the absence or presence of z-VAD-fmk. Cells were subjected to Annexin V/PI staining, and then analyzed by flow cytometry. (F) LN428 cells were treated with PINO (0.5 μM), TRAIL (50 ng/ml) and TRAIL plus PINO for indicated times. Whole cell lysates were subjected to immunoblotting with the indicated antibodies (left) and densitometry analysis of the bands from the relevant proteins was performed (right). Data represents the mean ± SE of three independent experiments. *p < 0.05, compared with the TRAIL only-treated group.
be limited to cancer cells. Although TRAIL-induced cancer cell death was mainly apoptotic, it might also induce non-apoptotic cell death via non-canonical TRAIL signaling depending on the cellular context. Thus, next, we examined whether cell death caused by pinoresinol plus TRAIL was associated with caspase-dependent apoptosis. As expected, pretreatment of LN428 cells with pancaspase and the irreversible caspase 8-inhibitors z-VAD-FMK and z-IETD-fmk completely abrogated the cytotoxicity induced by pinoresinol plus TRAIL (Fig. 2C,D). However, necrostatin-1, an inhibitor of programmed necrosis, failed to protect against cell death, indicating that pinoresinol predominantly triggered apoptotic, rather than necrotic, cell death. To confirm the mode of TRAIL-mediated cell death sensitized by pinoresinol, cell death was analyzed by Annexin V and propidium iodide (PI) staining followed by flow cytometry. Consistently, treatment of pinoresinol plus TRAIL drastically increased the population of an early phase of apoptosis (Annexin V+), whereas very few cells were stained exclusively with PI, and such increased apoptotic population was prevented by co-treatment with z-VAD-FMK (Fig. 2E). To get more insights into the mechanisms underlying TRAIL-sensitized apoptosis, we sequentially analyzed the activation of processes of caspase signaling cascade, including those of initiator caspase (caspase-8) and as executor caspases (caspase-3 or ~9) and the resultant PARP cleavage. In the kinetic analysis, we found that the treatment of pinoresinol plus TRAIL caused an activation of caspase-8 and ~3, and PARP cleavage from 6 h onwards (Fig. 2F). Furthermore, pretreatment with z-IETD-fmk completely inhibited the activation of caspase cascade induced by pinoresinol plus TRAIL treatment. These results clearly indicate that caspase-8 activation is essential in the sensitization of pinoresinol-induced apoptosis in TRAIL-resistant glioblastoma cells.

**Sensitizing efficacy of pinoresinol on TRAIL-mediated apoptosis is not associated with either NF-κB or p53.** Pinoresinol exhibits anti-inflammatory properties via blockade of the NF-κB pathway in several immune and cancer cells. Given the well-established ability of NF-κB to regulate TRAIL resistance through induction of its anti-apoptotic genes, it was hypothesized that the anti-NF-κB effects of pinoresinol might contribute to sensitization against TRAIL-induced apoptosis. Consistent with previous studies, pretreatment of LN428 cells with pinoresinol significantly decreased the transcriptional activity of NF-κB induced by either TNF or TRAIL, while pinoresinol alone did not affect the basal level of NF-κB activity (Fig. 3A). However, unexpectedly, LN428 cells with prevention of NF-κB activation by overexpression of the IkBα super-repressor (SR-IκBα), which could not be phosphorylated due to substitutions of serine 32 and serine 36 by alanine, were found to have a similar extent of cell death after TRAIL or pinoresinol plus TRAIL treatment, although TNF-induced cell death was drastically enhanced (Fig. 3B). In addition, pretreatment with the NF-κB inhibitor TPCA failed to affect cell death upon TRAIL or pinoresinol plus TRAIL treatment (Fig. 3C). These results suggest that the TRAIL-sensitizing efficacy conferred by pinoresinol was unlikely to be a result of NF-κB inhibition. Although p53 activation plays an important role in TRAIL sensitization, the involvement of p53 in pinoresinol-induced TRAIL sensitization was excluded because LN428 cells retained mutant-p53. Consistent with this possibility, we found that pinoresinol treatment also sensitized TRAIL-induced cell death in p53 null HCT116 cells to a similar extent as it did with wild type (WT) cells, despite the remarkable differences in cytotoxicity after camptothecin treatment between these two cell types (Fig. 3D). Moreover, no detectable induction of p53 and its target p21 upon pinoresinol alone or pinoresinol plus TRAIL treatment in WT-HCT116 cells, despite the fact that the substantial amounts of p53 and p21 were induced by a DNA damaging agent, camptothecin in WT-HCT116 cells but not in p53 null HCT116 cells (Fig. 3E). Such findings thus indicate that pinoresinol's effect on TRAIL-mediated cell death is independent of p53.

**Pinoresinol accelerates DISC formation by down-regulating cFLIP_L expression.** To characterize the underlying mechanism involved in pinoresinol-induced sensitization of glioma cells against TRAIL-mediated apoptosis, we determined the expression levels of several apoptosis-related proteins in the death receptor signaling pathway after exposure to pinoresinol for different times in LN428 cells. Notably, the protein expression levels of the long isoform of cellular FLIP (cFLIP_L) and survivin were drastically decreased in a time-dependent manner in LN428 cells with pinoresinol (Fig. 4A, panels 6,7). Reductions of cFLIP_L and survivin were accompanied by increased levels of cleaved-RIP1 and truncated-Bid (t-Bid) in cells upon pinoresinol plus TRAIL treatment (Fig. 4A, panels 4,11). Furthermore, we observed that pinoresinol was also able to down-regulate cFLIPS expression by increased levels of cleaved-RIP1 and truncated-Bid (t-Bid) in cells upon pinoresinol plus TRAIL treatment (Fig. 4A, panels 4,11). These results suggest that expression of cFLIP isoforms is highly cell type-specific and pinoresinol-induced cFLIP downregulation, especially in cFLIP_L, may play a predominant role in sensitizing TRAIL-mediated apoptosis in glioblastoma cells. By contrast, the protein levels of signaling components of TRAIL including death receptors, adaptor proteins, other inhibitor of apoptosis proteins, and Bcl-2 family proteins were not affected or only modestly affected in cells after pinoresinol treatment. The expression of cFLIP_L and survivin proteins are regulated by either transcriptional or post-translational modifications such as ubiquitin-mediated proteasomal degradation. In contrast to the observed down-regulation of cFLIP_L and survivin protein levels, treatment with pinoresinol did not change their mRNA levels at any of the time points examined (Fig. 4C). However, pretreatment with the proteasome inhibitor MG132 sufficiently prevented the down-regulation of cFLIP_L and survivin expression by pinoresinol (Fig. 4D), suggesting that pinoresinol might reduce the protein levels of cFLIP_L and survivin via proteasome-mediated degradation rather than through transcriptional control.

Next, we examined whether downregulation of cFLIP_L and survivin by pinoresinol affected the facilitated TRAIL-mediated cytotoxicity by overexpressing these genes in LN428 cells. Consistent with its critical function to antagonize caspase-8, overexpression of WT cFLIP_L resulted in a significant decrease in cell death and caspase cascade activation induced by pinoresinol plus TRAIL treatment (Fig. 5A,B). We also found that a cFLIP_L mutant (cFLIP_L-K167/195R containing modified major ubiquitin acceptor sites) more profoundly abrogated pinoresinol plus TRAIL-induced caspase-dependent apoptosis, compared to that of WT cFLIP_L. However, the cell death
induced by pinoresinol plus TRAIL was not affected by the overexpression of survivin, suggesting that downregulation of upstream anti-apoptotic protein anti-cFLIP L, rather than survivin, contributed to an important mechanism involving pinoresinol sensitization of TRAIL-induced cell death.

The cFLIP isoforms compete directly with procaspase-8 for binding to FADD in a TRAIL-dependent fashion, thus inhibiting procaspase-8/-10 recruitment to form the DISC31,32. It is therefore possible that downregulation of cFLIP L by pinoresinol might directly affect the formation of the DISC, an early signaling event in TRAIL-induced apoptosis. An immunoprecipitation assay using an anti-caspase-8 antibody revealed that treatment of LN428 cells with TRAIL in the absence of pinoresinol led to efficient recruitment of cleaved cFLIP L to the isolated DISC, whereas DISC-bound FADD and caspase-8/-10 were only weakly detected (Fig. 5C, left panel, rows 1–3). These results suggest that in LN428 cells, the TRAIL-induced recruitment of cFLIP L into the DISC was an important step before caspase-8 activation to exhibit resistance against TRAIL cytotoxicity. However, pretreatment with pinoresinol promoted an increase in TRAIL-mediated DISC formation and procaspase-8/10 processing, concomitant with decreasing amounts of DISC-bound cFLIP L (Fig. 5C, left panel, rows 4–6). More importantly, in pinoresinol-pretreated cells, activation of procaspase-8 processing within the DISC following TRAIL treatment proceeded to completion, as shown by the appearance of the active p18 subunit of mature caspase-8. Taken together, these results strongly suggest that a reduced amount of DISC-bound cFLIP L played a major role in TRAIL sensitization by pinoresinol.

Pinoresinol-mediated down-regulation of cFLIP L is mediated via de novo protein synthesis inhibition. Next, we identified the underlying mechanism by which pinoresinol directly controls ubiquitin-mediated degradation of cFLIP L. As expected, co-immunoprecipitation analyses showed that treatment of cells with MG132 led to an increase in polyubiquitinated cFLIP L, with concomitant enhanced protein levels (Fig. 6A). However, we unexpectedly detected lower levels of ubiquitinated cFLIP L in cells treated with pinoresinol plus MG132, compared to cells exposed to MG132 cells, indicating that the accelerated proteasomal degradation of cFLIP L by pinoresinol was not achieved through direct activation of the ubiquitination process.

Figure 3. Sensitizing effect of pinoresinol against TRAIL-mediated apoptosis is not associated with either NF-κB or p53 signaling pathway. (A) LN428 cells were transfected with an NF-κB-responsive reporter plasmid (p2xNF-B-Luc) and pRSV-β-gal. After 24 h, cells were treated with PINO (0.5 μM) alone for 6 h, or pretreated with PINO (0.5 μM) for 30 min, followed by TNF (30 ng/ml) or TRAIL (50 ng/ml) for additional 6 h. The luciferase assays were performed as describe in Methods, and the activity of each sample was normalized according to β-galactosidase activity. Each column shows the mean ± SE of three independent experiments. *p < 0.05, compared with the TNF or TRAIL only-treated group. (B) LN428 cells were transfected with mock or IkBα super-repressor (SR-IkBα) plasmid. After 24 h, cells were treated with PINO, TNF, TRAIL or TRAIL plus PINO for additional 24 h. (C) LN428 cells were pretreated with IKKα/β inhibitor TPCA-1 (0.5 μM) for 30 min, followed by PINO, TRAIL or TRAIL plus PINO for additional 24 h. (D) Wild-type and p53 null HCT116 cells were treated with PINO, TRAIL plus PINO and campothecin (Cpt, 100 μM) for 24 h. (B–D) Cell death was quantified as in Fig. 1A. Data were normalized to the rate of spontaneous cell death occurring in untreated cells. Data represents the mean ± SE of three independent experiments. *p < 0.05, compared with the mock-transfected group. *p < 0.05, compared with the wild-type HCT116 cells. (E) Wild-type and p53 null HCT116 cells were treated with PINO and campothecin for 24 h. Whole cell lysates were subjected to immunoblotting with the indicated antibodies (left) and densitometry analysis of the bands from the relevant proteins was performed (right). *p < 0.05, compared with the none-treated group.
Given that cFLIPL and survivin are unstable proteins with a rapid turnover, we addressed whether the reduced protein levels by pinoresinol were associated with de novo protein synthesis of cFLIPL and survivin. Treatment with either pinoresinol or cycloheximide (CHX) did not influence the cellular amounts of DRs and adaptor proteins, including DR4/5, FADD, RIP1, and TRAF2 (Fig. 6B). By contrast, pinoresinol was able to down-regulate the expression levels of cFLIPL and survivin with similar kinetics to that of CHX. Furthermore, the down-regulating effect by either pinoresinol or CHX was not accelerated by the combined treatment of pinoresinol and CHX. These results suggest that in a similar manner to CHX, pinoresinol inhibited de novo synthesis of proteins with a rapid turnover cFLIP and survivin.
To directly assess whether the down-regulation of protein expression by pinoresinol is due to the impairment of the general translational machinery, we conducted a cell-free in vitro transcription and translation assay. As shown in Fig. 6C, pinoresinol suppressed the production of green fluorescent protein (GFP), similar to a well-known protein translation inhibitor CHX, in a dosage dependent manner. To rule out the possibility that pinoresinol-dependent suppression of protein synthesis is caused by hampering transcriptional processes, subsequent in vitro translation response was assessed by using in vitro synthesized EGFP mRNA. Consistently, incubation with 1 μM pinoresinol completely interfered the EGFP protein production with a similar efficacy of 10 μM CHX (Fig. 6D). Taken together, these data indicate that pinoresinol directly interferes a de novo protein synthesis without affecting transcriptional machinery.

Discussion
Glioblastoma is a heterogenous group of invasive malignant primary brain tumors with high mortality. Although all populations of cancer cells contribute in their own way to drive tumor growth, the molecular changes disrupting the apoptotic pathway are considered a pathological hallmark of glioblastoma. Emerging evidence suggests that cells within the glioblastoma exhibit abnormalities of the cell death pathway such as over-expression of antiapoptotic proteins or silencing of key death effectors. Importantly, genomic analyses of human glioblastomas have shown that caspase-8, an essential component of the DISC, is frequently inactivated by either gene mutations or promoter methylation. Thus, resistance to DR-mediated cytotoxicity in glioblastoma cells might occur as a step of DISC assembly. Consistent with this possibility, we found that in a series of glioblastoma cells including LN428 cells treated with TRAIL, complete activation of caspase-8 and functional DISC formation were blocked. However, pinoresinol treatment resulted in cells resistant to apoptosis with an
increased recruitment of both procaspase-8 and FADD to the TRAIL DISC, and complete activation of caspase-8. It is therefore possible that pinoresinol-induced TRAIL sensitization was conducted at the level of the DISC/caspase-8 axis.

Accumulating evidence presently suggests that cFLIP is a key player in the DR-mediated apoptotic pathway that retains the sublethal activation of caspase-8 at the DISC 31,32,40. Consequently, elevated levels of cFLIP in tumor tissues from patients with a variety of cancers including lung cancer, Burkitt’s lymphoma, cervical carcinoma and colorectal carcinoma are correlated with poor clinical outcomes 41–44, implicating the existence of a strong association between suppression of DISC-mediated apoptosis by cFLIP and tumorigenesis. An important finding from the present study is that protein levels of cFLIPL were significantly reduced by pinoresinol treatment, and the ectopic overexpression of cFLIPL, significantly suppressed caspase-8 activation and reduced the susceptibility to cell death caused by pinoresinol/TRAIL treatment in LN428 cells. Changes in the expression levels of cFLIPL by pinoresinol therefore appear to be responsible for TRAIL sensitization in glioma cells. In this regard, it is important to determine the potential mechanism involved in the pinoresinol-induced downregulation of cFLIPL.

**Figure 6.** Pinoresinol-induced down-regulation of cFLIPL is mediated via de novo protein synthesis inhibition. (A) LN428 cells were co-transfected with plasmids expressing flag-tagged cFLIPL and HA-tagged ubiquitin plasmids for 24 h. The cells were then treated with PINO (0.5 μM) for 4 h in the absence or presence of MG-132 (10 μM). Cell extracts from each sample were subjected to immunoprecipitation with anti-flag antibody followed by immunoblotting with anti-HA antibody for detection of ubiquitinated cFLIPL. A total of 1% of the cell extract volume from each sample was used as input control (left). Densitometry analysis of the bands from the relevant proteins was performed (right). Data represents the mean ± SE of three independent experiments. *p < 0.05, compared with none-treated group. (B) LN428 cells were treated with PINO (0.5 μM), cycloheximide (CHX, 10 μM) and PINO (0.5 μM) plus CHX (10 μM) for indicated times. Whole cell extracts were subjected to immunoblotting with the indicated antibodies (left). Densitometry analysis of the bands from the relevant proteins was performed (right). Data represents the mean ± SE of three independent experiments. *p < 0.05, compared with none-treated group. (C) The in vitro coupled transcription and translation assay was performed using 1-Step Human Coupled IVT Kit as described in Methods. pCFE-GFP plasmid as template in HeLa cell lysate supplemented with indicated concentrations of CHX and PINO. (D) In vitro translation inhibition assay with increased amount of PINO. In vitro transcribed mRNA encoding EGFP was purified as described in Methods. 3 μg of EGFP mRNA each in HeLa cell lysate was incubated for 6 h with indicated concentrations of CHX and PINO. The in vitro translation yield of EGFP protein was monitored by immunoblotting with anti-Turbo GFP or anti-GFP antibody, respectively and quantified using a densitometry from the relevant proteins (top). Percent inhibition of protein synthesis was calculated by dividing the band intensities from each concentration of PINO over control DMSO-treated samples (bottom).
cFLIP_L expression. It has previously been reported that cFLIP_L expression is tightly regulated at the transcriptional level by a number of stimuli, including NF-κB transcription factor and protein kinase B/Akt and protein kinase B/Akt and protein kinase B/Akt and protein kinase B/Akt and protein kinase B/Akt and protein kinase B/Akt and protein kinase B/Akt. Previous pharmacological and biochemical studies have reported that pinoresinol exhibits anti-inflammatory and anti-cancer effects, in part through the inhibition of NF-κB. It is therefore possible that pinoresinol suppresses cFLIP_L expression through NF-κB inhibition. In accordance with previous observations, we found that pinoresinol potently inhibited the NF-κB activity in LN428 cells in response to TNF and TRAIL. However, we did not observe a decrease in cFLIP_L mRNA expression levels in cells treated with pinoresinol concentrations of 0.5–1 μM, which caused cFLIP_L depletion and maximal TRAIL sensitization. Furthermore, the expressions of a subset of NF-κB-inducible genes, including TRAF2, cIAP1/2, XIAP, and Bcl-X_L, were unaffected by pinoresinol treatment. These findings raise the possibility that down-regulatory effects of pinoresinol on cFLIP_L expression are not associated with transcriptional regulation of NF-κB. These discrepancies of transcriptional regulation between cFLIP_L expression and NF-κB may have resulted from different concentrations of pinoresinol. Indeed, pinoresinol must be used at a relatively high concentration (≥10 μM) to exhibit anti-NF-κB activity in several types of cells, suggesting that other factors may be involved in the effects of cFLIP_L depletion by low concentrations of pinoresinol.

On the other hand, the expression levels of cFLIP_L were regulated by the ubiquitin–proteasomal pathway with a short half-life. In this respect, it is of particular interest that, in LN428 cells, pinoresinol induces proteasomal degradation of cFLIP_L or ectopic expression of the cFLIP_L mutant (cFLIP_L-K167/195 R), which significantly abolishes sensitization by pinoresinol to TRAIL-induced cytotoxicity. Furthermore, the results of an in vitro translational assay showed that pinoresinol directly inhibited de novo protein synthesis, which has similar efficiency with the well-known protein synthesis inhibitor CHX. These results raise the possibility that pinoresinol disrupts de novo protein synthesis, particularly for fastidious proteins such as cFLIP_L, leading to proteasomal degradation with decreased stability. Nevertheless, it is currently unclear how pinoresinol inhibits de novo protein synthesis. Further studies to identify the ribosomal proteins that interact with pinoresinol in the translational machinery will be critical for a complete understanding of the mechanism of action, and for the development of novel TRAIL-based chemotherapeutics. Earlier, it has been reported that survivin plays an essential role in cell cycle progression. In this study, we found that pinoresinol induced a G2/M arrest with an increase in the G2 population (Supplementary Fig. 34), suggesting that down-regulation of survivin by pinoresinol using preclinical models are required to further validate TRAIL and pinoresinol-based therapeutic development for glioblastoma.

Methods

Isolation of pinoresinol. Pinoresinol was isolated from our chemical study on *Rubia philippinensis*, as described previously. Briefly, a methylene chloride (CH2Cl2)-soluble fraction (50 g) was prepared from solvent extraction of *R. philippinensis* extract (150 g) using CH2Cl2 and water. Vacuum liquid chromatography of CH2Cl2-soluble fraction on silica gel column (20 x 20 cm) eluting with n-hexanes-EToAc (20:1, 10:1, 5:1, 3:1, 2:1) and CHCl3-MeOH (8:1) resulted in the preparation of six column fractions (D-1 → D-6). Fraction D-6 (10 g) was separated by reversed-phase C18 column chromatography [column: SNAP cartridge KP-C18-HS (400 g), mobile phase: MeOH-H2O (10:90 → 100:0, 7:1)] and 11 subfractions (D-6-1 → D-6-11) were obtained. Pinoresinol (t_r 34.0 min, 40 mg) was purified from D-6-4 (360 mg) by preparative HPLC [column: Phenomenex C18 (250 x 21.0 mm), mobile phase: MeOH-H2O (50:50, 4 mL/min)]. Pinoresinol: brownish amorphous powder; ESIMS m/z 359.2 [M + H]^+^, 381.1 [M + Na]^+^, 357.1 [M−H]^−^, 393.1 [M + Cl]^−^; 1H NMR (300 MHz, methanol-d_4): 3.13 (2H, m, H-8, H-8′), 3.80 (2H, Ha-9, Ha-9′), 3.81 (6H, s, OCH3-3, OCH3-3′), 4.22 (2H, Hb-9, Hb-9′), 4.70 (2H, d, J = 2.7, H-7, H-7′), 6.77 (2H, overlapped, H-5, H-5′), 6.79 (2H, 1H), 6.95 (2H, H-2), 2H, 2H, 2H); 13C NMR (75 MHz, methanol-d_4): 133.8 (C-1, C-1′), 111.0 (C-2, C-2′), 149.1 (C-3, C-3′), 147.3 (C-4, C-4′), 116.1 (C-5, C-5′), 120.0 (C-6, C-6′), 87.5 (C-7, C-7′), 55.3 (C-8, C-8′), 72.6 (C-9, C-9′), 56.4 (OCH3-3, OCH3-3′).

Structure determination of pinoresinol. The molecular formula of purified compound was deduced as C_{39}H_{59}O_{6}, based on the ESIMS protonated ion at m/z 359.2, the sodium-adduct ion at m/z 381.1, the deprotonated ion at m/z 357.1, and the chloride-adduct ion at m/z 393.1 (calcd. for C_{39}H_{59}O_{6}, m/z 358.1). The 1H NMR data displayed a pair of symmetric benzene ring system at δ_1 6.77 (2H, overlapped, H-5, H-5′), 6.79 (2H, overlapped, H-6, H-6′), 6.95 (2H, H-2, H-2′). The proton signals at δ_3 3.13 (2H, m, H-8, H-8′), 3.80 (2H, Ha-9, Ha-9′), 4.22 (2H, Hb-9, Hb-9′), 4.70 (2H, d, J = 2.7, H-7, H-7′) indicated two symmetrical tetrahydrofuran substructure of lignan. In the 13C NMR spectroscopic data, six benzene signals at δ_1 111.0–149.1, two oxygenated carbons at δ_1 55.3, and a methoxy group at δ_1 56.4 supported the symmetric structure of tetrahydrofuran-type lignan. The chemical structure was identified as pinoresinol by NMR spectroscopic and LC-MS data analyses.

Antibodies and chemicals. The antibodies and chemicals were obtained from the following resources; anti-PARP (#556362), anti-XIAP (#610716), anti-FADD (#610399), anti-RIP1 (#610459), anti-p53 (#545147) and anti-p21 (#556430) antibodies (BD Biosciences, San Diego, CA, USA); anti-caspase-3 (#9662), anti-caspase-8 (#9746), anti-caspase-9 (#9508) and anti-Bid (#2002) antibodies (Cell signalling Technology, Beverly, MA, USA); anti-caspase-10 (M059-3) antibody (MBL, WOBURN, MA, USA); anti-Bcl-X_L (sc-271121), anti-survivin
Human malignant glioblastoma cells (LN428, LN2308, U87MG, and U251MG) were kindly provided by Dr. Yongwan Kim (Dongsung Cancer Center, Daegu, Korea). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) including 10% fetal bovine serum, 2 mmol/L glutamine and 100 U/mL penicillin/streptomycin. The normal primary astrocytes were prepared from the neonatal rats, as described previously [58], and cultured in Dulbecco’s modified Eagle’s medium (DMEM) including 10% fetal bovine serum, 2 mmol/L glutamine and 100 U/mL penicillin/streptomycin. The normal primary astrocytes were prepared from the neonatal rats, as described previously [58], and cultured in Dulbecco’s modified Eagle’s medium (DMEM) including 10% fetal bovine serum, 2 mmol/L glutamine and 100 U/mL penicillin/streptomycin. The primary astrocytes were transfected with the luciferase assay kit (Promega, Madison, CA, USA) according to the manufacturer’s instructions. Luciferase activity obtained were normalized to β-galactosidase activity of each sample.

Cell death assessment. Cell death was determined using Cell Titer-glo Luminescent Cell Viability Assay kit (Promega Co., Fitchburg, WI, USA), according to the manufacturer’s instructions. Luminescent signals were measured by Tecan Infinite Plate reader (Tecan group Ltd., Männedorf, Switzerland), and Viability rates were calculated following the formula: viability rates = (1 − medicating/control) × 100%. Representative images were also taken by an inverted microscope.

Flow cytometry analysis. After LN428 cells were treated with pinoresinol, as described in the figure legend, the cells were harvested and examined for the mode of apoptotic and necrotic cell death by double staining with FITC-conjugated annexin V and propidium iodide (PI) in 10 mM HEPES buffer, pH 7.4, according to the manufacturer’s instructions (BD FITC Annexin V kit). For cell cycle analysis, cells were suspended with ice-cold phosphate buffered saline (PBS) and fixed in 70% ethanol. Cells were washed with PBS and added 100 μg/ml PI solution including 50 μg/ml RNase in PBS for 30 min at room temperature. The cells were analyzed with a FACScan flow cytometer (BD Biosciences).

Immunoblotting and immunoprecipitation. Upon treatment, cells were lysed in ice-cold M2 buffer (20 mM Tris, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 2 mM dithiothreitol, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin). For immunoblot analysis, cell lysates were resolved on 8–12% SDS-polyacrylamide gel (PAGE), transferred onto the PVDF membrane (GE Healthcare Life Sciences). Membranes were serially incubated with specific primary antibodies (1:1,000 dilution), followed by secondary antibody (1:2,000 dilution), and immunoblots were visualized by enhanced chemiluminescence reagent (Invitrogen). For immunoprecipitation assays, the cell lysates were incubated with anti-caspase-8 antibody and protein A-agarose beads at 4 °C overnight. The immunoprecipitants were washed three times with PBS containing 0.5% Triton X-100, boiled in Laemmli buffer for SDS-PAGE. The expression levels of EGFP proteins were examined by immunoblot analysis with anti-EGFP antibody. For in vitro translational analysis, EGFP mRNA was generated by using a mMESSAGE mMACHINE T7 Transcription kit (ThermoFisher Scientific) following manufacturer’s instructions. Each 3 μg of purified EGFP mRNA was incubated with HeLa cell lysates for 6 hours at 30 °C with indicated concentrations of PINO and CHX as described in figure legends. The expression level of EGFP was measured through immunoblotting with anti-EGFP antibody and then quantified by densitometry using an image J software.
Statistical analysis. Data are expressed as the mean ± S.E. from at least three separate experiments performed in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the Bonferroni t-test for multi-group comparison tests. Student's t-test was used to compare the mean values from the two groups. P < 0.05 was considered as statistically significant.

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Author Contributions

S-R.L. participated in the design of the study, carried out bench experiments and analyzes data. H.S.B., K.K., X.P., E.J. helped carrying out bench experiments related to this study. H.R. helped the experiments for the *in vitro* translation assay. K.Q., I.P., M.N. carried out the experiments for the purification of pinoresinol and LC-MS analysis. G.M.H. designed this study and wrote the manuscript with comments from the coauthors, and all authors collaborated on the work.

Additional Information

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