Lambertianic Acid Sensitizes Non-Small Cell Lung Cancers to TRAIL-Induced Apoptosis Via Inhibition of XIAP/NF-κB and Activation of Caspases and Death Receptor 4

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Abstract: Lambertianic acid (LA) is a biologically active compound from the leaves of *Pinus koraiensis*. In the present study, apoptotic mechanisms of LA plus TNF-related apoptosis-inducing ligand (TRAIL) were elucidated in non-small cell lung cancer cells (NSCLCs). Cytotoxicity assay, flow cytometry, immunoprecipitation and Western blotting were performed. Here, combined treatment of LA and TRAIL increased cytotoxicity, sub-G1 population and cleaved poly (ADP-ribose) polymerase (PARP) and caspase3/8/9 in A549 and H1299 cells compared to LA or TRAIL alone. Furthermore, combined treatment of LA and TRAIL significantly decreased anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2), Fas-like inhibitor protein (FLIP) and X-linked inhibitor of apoptosis protein (XIAP) and enhanced the activation of pro-apoptotic proteins Bid compared to LA or TRAIL alone. In addition, combined treatment of LA and TRAIL upregulated the expression of Death receptor 4 (DR4) and downregulated phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells (p-NF-κB), inhibitory protein of kB family (p-IκB) and FLIP in A549 and H1299 cells along with disrupted binding of XIAP with caspase3 or NF-κB. Overall, these findings suggest that lambertianic acid enhances TRAIL-induced apoptosis via inhibition of XIAP/NF-κB in TRAIL resistant NSCLCs.

Keywords: Non-small cell lung cancer; Lambertianic acid; Apoptosis; TRAIL; XIAP; NF-κB

1. Introduction

The death ligand TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a member of the TNF superfamily, induces apoptosis in cancer cells with low toxicity and less resistance in normal cells [1]. TRAIL activation is mainly induced by binding to the DR4 and DR5 and subsequently leads to death-inducing signaling complexes (DISCs) via FAS-associated protein and caspase-8 protein leading to activation of apoptotic cell death [2, 3]. Though TRAIL is an important anticancer agent, TRAIL resistance is a major limitation to effective cancer therapy [4, 5]. Therefore, development of combination treatments to overcome resistance to TRAIL is requested for effective cancer therapy. Notably, non-small cell lung cancer cell lines such as A549, H1299 and H596 cells are known resistant to TRAIL-induced apoptosis [6]. Recently, several groups reported the synergistic or additive effect of phytochemicals, such as kaempferol [7], angelicin [8] and curcumin [9], on TRAIL-induced apoptosis.

LA (Figure 1a), a major active constituent of *Pinus koraiensis*, has been reported to have anti-obesity [10], anti-inflammatory [11], and anti-cancer effects [12]. Nevertheless, the underlying apoptotic mechanism of LA as a TRAIL sensitizer has never been demonstrated. In the present study,
the sensitizing mechanism of LA in TRAIL-induced apoptosis was investigated as a novel strategy to overcome the resistance of cancer cells to apoptosis in A549 and H1299 NSCLCs.

2. Results

2.1. Combined treatment of LA and TRAIL enhanced cytotoxicity in A549 and H1299 non-small cell lung cancer cells

The cytotoxicity of LA in A549 and H1299 non-small cell lung cancer cells was evaluated by MTT assay. As shown in Fig. 1b, combined treatment of LA (20 μM) and TRAIL (20 ng/ml) for 24 h showed significantly cytotoxicity in A549 and H1299 cells compared to treatment with LA or TRAIL alone (Figure 1b). Also, cell proliferation assay using crystal violet staining revealed that combined treatment of LA (20 μM) and TRAIL (20 ng/ml) significantly inhibited proliferation in A549 cells (Figure 1c).

![Figure 1](image.png)

**Figure 1.** Cytotoxicity of the combination of LA and TRAIL in A549 and H1299 non-small cell lung cancer cells. (a) Chemical structure of LA. Molecular weight = 316.43. (b) Cell viability was evaluated by MTT assay. Cells were seeded onto 96 well microplates and treated with various concentrations of LA (0, 5, 10, 20 μM) and TRAIL (0, 20, 40, 80 ng/ml) for 24 h. The cytotoxic effects of LA (20 μM) and TRAIL (20 ng/ml) on TRAIL in A549 and H1299 cells. Data represent means ± SD. * p<0.05, **p<0.01 versus LA-treated control (n=3). (c) A549 cells were treated with LA (20 μM) and/or TRAIL (20 ng/ml) for 24 h and then stained with crystal violet. Data represent means ± SD. **P<0.01 versus TRAIL alone (n=4).

2.2. Combined treatment of LA and TRAIL significantly increased the sub-G1 population and also increased the cleavage of PARP and caspase8/9/3 in A549 and H1299 non-small cell lung cancer cells

To confirm the apoptotic effect of combined treatment of LA and TRAIL, Western-blot assay and cell cycle analysis were performed in A549 and H1299 cells treated by combined treatment of LA and TRAIL. Combined treatment of LA and TRAIL increased sub-G1 population in A549 and H1299 cells (Figure 2a) and also increased the cleavage of PARP and caspase8/9/3 and decreased the expression of pro-PARP, pro-caspase8/9/3 in A549 and H1299 cells compared to LA or TRAIL alone (Figure 2b). To confirm the involvement of caspases, A549 and H1299 cells were pre-treated with caspase inhibitors for 1 h prior to the co-treatment. Here pan caspase inhibitor (z-VAD-fmk) and caspase-8 inhibitor (z-IETD-fmk) significantly blocked the increase of sub-G1 population by combined treatment of LA and TRAIL (Figure 2a). Consistently, Cell apoptosis assay using Annexin-V/PI double staining revealed that combined treatment of LA (20 μM) and TRAIL (20 ng/ml) for 24 h significantly increased the early and late apoptosis to 4.74 % and 10.21 % in A549 cells, 8.77 % and 8.71% in H1299 cells, respectively, compared to LA (20 μM) or TRAIL (20 ng/ml) alone by Annexin V and PI staining (Figure 2c).
Figure 2. Combined effect of LA and TRAIL on the sub-G1 population and apoptotic proteins in A549 and H1299 nonsmall cell lung cancer cells. (a) Cells were treated with LA (20 μM) and/or TRAIL (20 ng/ml) for 24 h. The treated cells were fixed with 70% ethanol, stained with propidium iodide (PI) and analyzed by flow cytometry with or without caspase inhibitors (pan caspase inhibitor; z-VAD-fmk (80 μM), caspase-8 inhibitor; z-IETD-fmk (50 μM)). Bar graphs show quantification of cell cycle population (%). Data represent means ± SD. ***P<0.001 versus TRAIL alone, #p<0.05, ###p<0.001 versus LA+TRAIL treated control. (n=3). (b) Cells were treated with LA (20 μM) and/or TRAIL (20 ng/ml) for 24 h. Cell lysates were prepared and subjected to Western blotting for procaspase-8,9,3,Pro-PARP,cleaved caspase-8,9,3 and cleaved-PARP. (c) Cells were treated with LA (20 μM) and/or TRAIL (20 ng/ml) for 24 h. The cells were stained using FITC-Annexin V/PI dye and early and late apoptotic portions were detected by flow cytometry.

2.3. Combined treatment of LA and TRAIL regulated anti-apoptotic and pro-apoptotic proteins in A549 and H1299 non-small cell lung cancer cells

To determine whether cotreatment of LA and/or TRAIL affects apoptosis, we assessed the expression levels of pro-apoptotic and anti-apoptotic proteins by Western blotting. Combined treatment of LA and TRAIL attenuated the expression of Bcl-2 and activated the expression Bid in A549 and H1299 cells (Figure 3a). Consistently, combined treatment of LA and TRAIL effectively blocked the expression of XIAP in A549 and H1299 cells compared to LA or TRAIL alone (Figure 3b).
2.4. Combined treatment of LA and TRAIL upregulated the expression of DR4 and inhibited the expression of p-NF-κB, p-IκB and FLIP in A549 and H1299 non-small cell lung cancer cells

We examined the expression of TRAIL death receptors and its associated proteins such as DR4 and DR5 by Western blotting. Combined treatment of LA and TRAIL upregulated the expression of DR4, but not DR5 in A549 and H1299 cells compared to LA or TRAIL alone. (Figure 4a). Also, to clarify the role of NF-κB signaling in TRAIL-induced apoptosis, Western blotting was conducted in A549 and H1299 cell treated by combined treatment of LA and TRAIL. Combined treatment of LA and TRAIL attenuated the expression of p-NF-κB and p-IκB in A549 and H1299 cells compared to LA or TRAIL alone (Figure 4b). Next, the effect of TRAIL and/or LA was examined on the expression of TRAIL associated proteins such as FLIP, DcR1 and DcR2 by Western blotting. As shown in Figure 4c, combined treatment of LA (20 μM) and TRAIL (20 ng/ml) synergistically downregulated the expression of FLIP, but not DcR1 and DcR2, compared to LA or TRAIL alone.

Figure 4. Combined effect of LA and TRAIL on the expression of DR4, p-NF-κB, p-IκB and FLIP in A549 and H1299 cells. Cells were treated with LA (20 μM) and/or TRAIL (20 ng/ml) for 24 h and cell lysates were prepared and subjected to Western blotting. (a) Combined effect of LA and TRAIL on the expression of DR4 and DR5 in A549 and H1299 cells. (b) Combined effect of LA and TRAIL on the expression of p-NF-κB and p-IκB in A549 and H1299 cells. (c) Combined effect of LA and TRAIL on the expression of FLIP, DcR1 and DcR2 in A549 and H1299 cells.

2.5. Combined treatment of LA and TRAIL disrupted binding of XIAP with caspase3 and NF-κB in A549 non-small cell lung cancer cells

To confirm the XIAP, caspase3 and NF-κB interaction inhibition of combined treatment of LA and TRAIL, immunoprecipitation was performed in A549 cell treated by combined treatment of LA and TRAIL. Protein-protein interaction (PPI) scores between XIAP and caspase3, NF-κB were found 0.999 and 0.593, respectively (Figure 5a). As shown in Figure 5b, combined treatment of LA and TRAIL interrupted binding of XIAP with Caspase3 and NF-κB.
3. Discussion

TRAIL was known to play an important role in apoptosis as a therapeutic agent in cancers [13, 14]. Nevertheless, chemo-resistance to TRAIL has limited its clinical usage in some types of cancers [15]. To overcome this problem, combination treatment has been proposed as an attractive approach by sensitizing TRAIL-mediated cytotoxicity with less side effects [16, 17]. In the current study, we investigated whether LA was able to augment TRAIL-induced apoptosis in A549 and H1299 NSCLCs that are resistance to TRAIL treatment.

Here, combination of LA and TRAIL enhanced cytotoxicity and induced sub-G1 accumulation, cleavage of PARP and attenuated the expression of pro-caspase8, pro-caspase9 and pro-caspase3 compared to LA or TRAIL alone in A549 and H1299 NSCLCs, implying synergistic apoptotic effect by combination of LA and TRAIL.

It was well documented that DR4 upregulation is a promising molecular target for sensitizing tumor cells to TRAIL-induced apoptosis [18]. In our study, combination of TRAIL and LA activated DR4 in A549 and H1299 cells, implying potent role of death-receptor-dependent pathway by combination of TRAIL and LA.

It was reported that tumor cells acquire TRAIL resistance by upregulation of XIAP, c-FLIP, Bcl2 and Bcl-xL as anti-apoptotic proteins [19, 20], and activation of PI3K, AKT, and NF-κB as proliferation activators [21, 22]. Here, combination of LA and TRAIL attenuated the expression of Bcl-2, XIAP and FLIP along with activation of Bid in A549 and H1299 cells, implying combination of LA and TRAIL inhibits antiapoptotic proteins, leading to apoptosis.

There are accumulating evidences that XIAP, one of IAP family members, contains a C-terminal RING domain and three distinct baculovirus IAP repeat (BIR) domains [23, 24] and plays a critical role in NF-κB activation [25]. The BIR1/TAB1 interaction is crucial for XIAP-induced TAK1 and NF-κB (RelA/p65 and p50 subunits) activation, since the BIR2 domain of XIAP directly blocks the active sites of caspase-3 and caspase-7, while the BIR1 domain directly binds to TAB1 [25-27]. The NF-κB activation of XIAP is essential for cancer cell survival [25]. Thus, we examined the interaction between XIAP, caspase3 and NF-κB by combination of LA and TRAIL in A549 cells. Our results show that combination of LA and TRAIL attenuated the expression of p-NF-κB and p-IκB and also disrupted the binding of XIAP with caspase3 or NF-κB in A549 cells, indicating combination of LA and TRAIL exerts apoptotic effect via interrupted binding of XIAP with caspase3 or NF-κB.

In summary, combined treatment of LA and TRAIL increased cytotoxicity and the sub-G1 population in A549 and H1299 NSCLCs, induced apoptosis by cleavage of PARP and inhibited pro-
caspases 8/9, Bcl-2 and XIAP and activated DR4 in A549 and H1299 cells. Furthermore, the combination of TRAIL and LA suppressed the expression of p-NF-κB and p-IκB. Additionally, combination of TRAIL and LA disrupted the binding of XIAP with caspase3 or NF-κB. Taken together, our findings suggest that combination of TRAIL and LA synergistically induces apoptosis in non-small cell lung cancer cells via inhibition of XIAP/NF-κB as a potent TRAIL sensitizer.

4. Materials and Methods

4.1. Lambertianic acid isolation

*Pinus koraiensis* leaves (3 kg) were pulverized, immersed in 50% MeOH (10 L) for 3 days and distilled to be concentrated for 10 h by using Rotary Evaporator (IKA Korea Limited, Seoul, Korea). Then the MeOH extracts were partitioned with EtOAc / distilled water (1:1) and the water layer was suspended and partitioned with n-butanol/ distilled water. A part of EtOAc fraction was subjected to a celite column chromatography and eluted with CHCl3-MeOH (3:1) to yield 15 fractions. Among these fractions, a distinct and vivid red-purple spot from fr. 6 was isolated purified and identified as lambertianic acid (LA) with over 98% purity based on spectroscopic analyses such as NMR, MS, and IR [28].

4.2. Cell culture

Human non-small cell lung cancers A549 and H1299 were obtained from American Type Culture Collection (ATCC). A549 and H1299 cells were cultured in RPMI1640 supplemented with 10% FBS and 1% antibiotic (Welgene, South Korea).

4.3. Cytotoxicity assay

The cytotoxicity of LA was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, A549 and H1299 cells (1×10^4 cells/well) were seeded onto 96-well culture plate and exposed to various concentrations of LA for 24 h. The cells were incubated with MTT (1 mg/mL) (Sigma Chemical) for 2 h and then treated with dimethyl sulfoxide (DMSO) for 20 min. Optical density (OD) was measured using a microplate reader (Molecular Devices Co., USA) at 570 nm. Cell viability was calculated as a percentage of viable cells in LA treated group versus untreated control.

4.4. Crystal violet assay

For viability and proliferation, crystal violet assay was performed in A549 cells. The cells (2×10^5 cells/well) were seeded onto 35mm culture plate and treated with LA (20 μM) and TRAIL (20 ng/ml) for 24 h. The cells were fixed (4% paraformaldehyde) and stained with crystal violet solution (40% ethanol, 60% PBS and 0.5% crystal violet). Fifteen min later, 1 ml of 10% acetic acid was added to each well, and the absorbance was read at 590 nm using a microplate reader (Molecular Devices Co., USA).

4.5. Cell cycle analysis

A549 and H1299 cells (1x10^6 cells/ml) were treated with various concentrations of LA for 24 h, washed twice with cold PBS and fixed in 75% ethanol at −20 °C. The cells were incubated with RNase A (10 mg/ml) for 1 h at 37°C and stained with propidium iodide (50 μg/ml) for 30 min at room temperature in dark. The stained cells were analyzed for the DNA content by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) using CellQuest Software.

4.6. Western blotting

A549 and H1299 cells (1x10^6 cells/ml) were treated with various concentrations of LA for 24 h, lysed in lysis (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM NaVO₃, 1 mM NaF, and 1× protease inhibitor cocktail) on ice, and spun down at 14,000×g for 20 min at 4°C. The supernatants were collected and quantified for protein concentration by using RC.
DC protein assay kit (Bio-Rad, Hercules, CA, USA). The protein samples were separated on 4–12% NuPAGE Bis–Tris gels (Novex, Carlsbad, CA, USA) and transferred to a Hybond ECL transfer membrane for detection with antibodies for PARP Caspase-8,9,3, DR4, DR5, Bid, p-NF-κB, p-IκB, FLIP, DcR1 and DcR2 (Cell signaling Technology, Beverly, MA, USA) Bcl-2, XIAP (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and β-actin (Sigma, St. Louis, MO, USA).

4.7. Co-Immunoprecipitation

A549 cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM NaF, 1 mM EDTA, 1 mM Na3VO4, and 1× protease inhibitor cocktail), and then were immunoprecipitated with AKT antibody or normal immunoglobulin G antibody. Thereafter, Protein A/G sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were applied. The final precipitated proteins were subjected to immunoblotting with the indicated antibodies.

4.8. Statistical analysis

To statistical analysis of the data, Sigmaplot version 12 software (Systat Software Inc., San Jose, CA, USA) was used. All data were expressed as means ± standard deviation (SD). A Student t-test was used for comparison of two groups. The statistically significant difference was set at p values of <0.05 between control and LA treated groups.

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Author Contributions: Ahn DS and Lee HJ designed and performed several experiments. Han Hk and Shim BS supported experiments. Kim SH supervised this experiments and wrote MS.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
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<tr>
<td>Caspase</td>
<td>Cysteine aspartyl-specific protease</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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<tr>
<td>DR5</td>
<td>death receptor5</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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References


