The andrographolide derivative, AND7, and TRAIL combination attenuates acute lymphoblastic leukemia through P53-regulated ROS accumulation

Highlights

- Structural modification of natural product andrographolide.
- New treatment strategies for acute lymphoblastic leukemia (ALL).
- The combination of andrographolide derivatives and TRAIL is expected to be used for the treatment of ALL.

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In brief

Acute lymphoblastic leukemia (ALL) is a malignant disease of the hematologic system and the current treatment is based on chemotherapeutic drugs, which are becoming less effective due to drug resistance. Based on the easily modified structure of the natural product, andrographolide, we synthesized the derivative, AND7, and used it in combination with TNF-related apoptosis-inducing ligand (TRAIL) as a therapeutic option. The results showed that AND7/TRAIL combination treatment aided in preventing the original TRAIL-resistant cells from activating the caspase-8/caspase-3 pathway through DR4/DR5 and promoted apoptosis via the apoptotic gene P53 to achieve the anti-cancer effect. Therefore, the AND7/TRAIL combination is promising for treating ALL and lays the foundation for clinical research.
The andrographolide derivative, AND7, and TRAIL combination attenuates acute lymphoblastic leukemia through P53-regulated ROS accumulation

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is a malignant disease of the hematologic system. The current treatment is based on chemotherapeutic drugs, which are becoming less effective due to drug resistance. TNF-related apoptosis-inducing ligand (TRAIL) is an apoptotic protein used to treat cancer that does not affect healthy cells. In recent years, however, ALL cells (e.g., U937) have become more resistant to TRAIL. A novel andrographolide derivative (AND7) with high efficiency and low toxicity was synthesized and combined with TRAIL after the optimal combination ratio was screened using U937 cells. We used peripheral blood mononuclear cells (PBMCs) from patients before the initial treatment of ALL as a model and PBMCs from healthy subjects as a control to determine the mechanism underlying ALL treatment. AND7/TRAIL combination treatment was shown to prevent the original TRAIL-resistant cells from activating the caspase-8/caspase-3 pathway through DR4/DR5 and promote apoptosis via expression of ROS and the apoptotic gene, P53, to achieve an anti-cancer effect. Notably, this study demonstrated that the AND7/TRAIL combination enhanced the anti-cancer effect of AND7 and improved TRAIL resistance. Therefore, the AND7/TRAIL combination is promising for treating ALL and lays the foundation for clinical research.

Keywords: TRAIL, Andrographolide derivatives, Acute lymphoblastic leukemia, Apoptosis, ROS

1. INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease with a bimodal age distribution that affects B, T, and NK precursor cells with a higher incidence in the pediatric age range [1]. The pathophysiology underlying ALL is characterized by chromosomal abnormalities and genetic alterations involved in the differentiation and proliferation of lymphoid precursor cells [1, 2]. Although some progress has been made, the treatment of ALL remains dominated by chemotherapeutic agents. Consequently, long-term chemotherapy drug use and drug resistance is a main problem facing ALL [3].

Synthetic drugs used for cancer treatment have side effects that may lead to resistance, which can cause liver, kidney, and cardiac toxicity, infertility, and ovarian failure. Thus, herbal drugs could be used in cancer treatment as an adjuvant therapy [4].

Andrographolide, the major active component of \textit{A. paniculata}, exhibits diverse pharmacologic activities, including anti-inflammation, anti-cancer, anti-obesity, and anti-diabetes effects. Additionally, andrographolide can be metabolized over a short period while exhibiting low toxicity levels to healthy cells [5]. However, the main limitations of drug monomers currently include low viability, short half-life, and lack of specific targeting [6]. Thus, synthesizing safe and efficient andrographolide derivatives is important in clinical applications [7]. It has also been shown that andrographolide enhances tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by affecting P53-mediated upregulation of death receptors [8]. The accumulation
of intracellular reactive oxygen species (ROS) levels is an additional marker reflecting apoptosis [9]. Moreover, andrographolide and its derivatives have been shown to facilitate the accumulation of intracellular ROS, which is associated with induction of apoptosis [9-11].

TRAIL is currently under investigation for the treatment of leukemia and represents a targeted therapy against cancer for its specificity to tumor cells [12, 13]. TRAIL exerts a selective apoptotic effect via its interaction with death receptors (TRAILR1/DR4 and TRAILR2/DR5) in a wide range of cancers, while sparing healthy cells [14, 15]. However, some leukemia cells, such as MOLT-4 and U937, are resistant to TRAIL [16, 17]. Therefore, combining chemotherapeutic drugs with TRAIL for leukemia can alleviate the resistance of tumor cells to TRAIL and achieve a better therapeutic effect [12, 18, 19], especially using some Chinese medicines, the active ingredients of which [20].

This study focused on the optimal use of the andrographolide derivative, AND7, in combination with TRAIL for treating ALL, as well as a preliminary description of the underlying mechanism of action. The properties of andrographolide, a derivative with good efficacy and safety, was modified and synthesized. The andrographolide derivative was shown to improve resistance of the ALL cell line, U937, to TRAIL in vitro based on toxicity testing, indicating the potential for therapeutic significance. It has also been shown that PS3, a gene that regulates apoptosis through which TRAIL induces apoptosis, and the combined drug can activate a regulatory effect of TRAIL [21]. Thus, the combination of the andrographolide derivative (AND7) and TRAIL promoted tumor cell apoptosis and achieved anti-cancer effects.

2. MATERIAL AND METHODS

2.1 Chemicals and reagents

2.1.1 Reagents. The following materials were used in the current study: p-toluenesulfonic acid (Macklin, Shanghai, China); benzaldehyde analogs (Zesheng Technology, Anhui, China); tetrahydrofuran, petroleum ether, ethyl acetate, dichloromethane, and methanol (Sinopharm, Beijing, China); DMSO-d₆ (Heowns Biochemical Technology, Tianjin, China); column chromatography silica gel (100-120 and 200-300 mesh; Gulf Fine Chemicals, Qingdao, China); andrographolide (Macklin); andrographolide derivatives were prepared by the Central Laboratory of Nanjing Combined Chinese and Western Hospital (Nanjing, China); recombinant human TRAIL (1.95 μg/ml; Novoprotein, Shanghai, China); Ficoll-paque™ PLUS (GE HealthCare, Chicago, California, USA); Cell Counting Kit (CCK-8), ChamQ Universal SYBR qPCR Master Mix, and HiScript III RT SuperMix for qPCR (Vazyme, Nanjing, China); RPMI-1640 medium with L-glutamine (Corning, New York City, NY, USA); fetal bovine serum [FBS] (Wisent, Nanjing, China); 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco, CA, USA); rabbit polyclonal anti-DR4 (Proteintech, Jiangsu, China); and rabbit polyclonal anti-DR5 (D4E9), rabbit polyclonal anti-GAPDH, mouse anti-caspase-3 (3G2), and mouse anti-caspase-8 [1C12] (Cell Signaling Technology, Danvers, MA, USA).

2.1.2 Cell lines and patient samples. The human leukemia cell lines (U937 [ALL] and THP-1 [AML]) were purchased from ATCC (Shanghai, China). Cells were maintained in RPMI-1640 with L-glutamine and supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

Peripheral blood mononuclear cell (PBMC) samples were collected from healthy donors and ALL patients who were receiving care at Jiangsu Province Hospital (Jiangsu, China). PBMCs were isolated from heparinized blood by density gradient centrifugation using histopaque according to standard protocols.

Peripheral blood samples from ALL patients were obtained from Nanjing Drum Tower Hospital (acceptance number: 2022-157-01; Nanjing, China) after receiving informed consent and prior approval from the Institutional Ethics Committee. A total of 10 peripheral blood samples were obtained following clinical diagnosis (ALL n=5) and healthy donors (n=5). The details of the patients and healthy donors are listed in Table 1.

2.2 Synthesis of andrographolide derivative

Aldehydes, tetrahydrofuran, and p-toluenesulfonic acid reacted at 60°C for 4 hours, as shown in Scheme 1. All reagents and solvents were of analytical grade and used

<table>
<thead>
<tr>
<th>S. no</th>
<th>Type of leukemia</th>
<th>Age</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B-ALL</td>
<td>29</td>
<td>M*</td>
</tr>
<tr>
<td>2</td>
<td>B-ALL</td>
<td>58</td>
<td>F*</td>
</tr>
<tr>
<td>3</td>
<td>B-ALL</td>
<td>55</td>
<td>F*</td>
</tr>
<tr>
<td>4</td>
<td>B-ALL</td>
<td>6</td>
<td>M*</td>
</tr>
<tr>
<td>5</td>
<td>B-ALL</td>
<td>60</td>
<td>F*</td>
</tr>
<tr>
<td>6</td>
<td>Healthy control</td>
<td>23</td>
<td>F</td>
</tr>
<tr>
<td>7</td>
<td>Healthy control</td>
<td>23</td>
<td>M</td>
</tr>
<tr>
<td>8</td>
<td>Healthy control</td>
<td>25</td>
<td>F</td>
</tr>
<tr>
<td>9</td>
<td>Healthy control</td>
<td>26</td>
<td>M</td>
</tr>
<tr>
<td>10</td>
<td>Healthy control</td>
<td>23</td>
<td>F</td>
</tr>
</tbody>
</table>

*Peripheral blood samples from ALL patients were obtained from Nanjing Drum Tower Hospital (acceptance number: 2022-157-01; Nanjing, China) after receiving informed consent and prior approval from the Institutional Ethics Committee.
without further purification. Reactions were monitored via thin-layer chromatography (TLC) using Qingdao TLC silica gel GF254 plates (city, China). $^1$H NMR spectra were recorded on a Bruker DRX 500 or Bruker DRX 600 NMR instrument (city, state, country).

(4S,E)-3-(2-(((4aR,6aS,7R,10aS,10bR)-3-(3-bromo-5-chloro-2-hydroxyphenyl)-6a,10b-dimethyl-8-methylenedecahydro-1H-naphtho[2,1-d][1,3]dioxin-7-yl) ethylidene)-4-hydroxydihydropyran-2(3H)-one

The detailed reaction process is as follows: 3-Bromo-5-chloro-2-hydroxybenzaldehyde (47 mg [0.2 mmol]) and p-toluenesulfonic acid (50 mg [0.3 mmol]) were added to a solution of andrographolide (70 mg [0.2 mmol]) in tetrahydrofuran (20 mL). After stirring at 60°C for 4 h the solvent of the reaction mixture was evaporated to dryness. Then, the residue was separated and purified by column chromatography to obtain AND7 (22 mg [20%]).

$^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 8.34 (s, 1H), 7.47 (d, $J$ = 2.4 Hz, 1H), 7.20 (d, $J$ = 2.5 Hz, 1H), 6.95 (t, $J$ = 7.0, 1.8 Hz, 1H), 5.91 (s, 1H), 5.04 (d, $J$ = 6.1 Hz, 1H), 4.93 (s, 1H), 4.64 (s, 1H), 4.46 (m, 1H), 4.27 (m, 2H), 3.75 (dd, $J$ = 11.5, 1.5 Hz, 1H), 2.96 (d, $J$ = 1.3 Hz, 1H), 2.88 (d, $J$ = 1.4 Hz, 1H), 1.45 (s, 3H), 0.86 (s, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta$ 150.74, 148.66, 146.24, 133.01, 128.28, 127.26, 125.11, 111.56, 109.73, 103.57, 94.67, 74.44, 72.01, 69.93, 66.40, 55.78, 54.88, 48.34, 38.94, 37.58, 37.16, 35.97, 26.11, 24.85, 22.85, 21.65, 15.48.

HRMS (+ESI) $m/z$ calcd for C$_{27}$H$_{33}$BrClO$_6$ [M-H]$^-$: 567.0987, found 567.0989. Calcd for C$_{27}$H$_{33}$BrClO$_6$: C, 57.10; H, 5.68; O, 16.90. Found: C 57.15; H: 5.71; O, 17.00.

### 2.3 Isolation of PBMCs

According to the manufacturer’s instructions, PBMCs were isolated from whole blood samples using Ficoll Paque gradient separation with Ficoll-paque™ PLUS. Isolated PBMCs were cultured in RPMI-1640 medium with L-glutamine supplemented with 10% FBS, 2 mM L-glutamine, 100 μM of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified 5% CO$_2$ atmosphere.

### 2.4 Cytotoxicity testing

The CCK-8 assay was performed to evaluate cell viability after andrographolide and andrographolide derivative treatment. U937 and THP-1 cells were seeded into 96-well plates with 5000 cells per well and incubated for 24 h at 37°C under 5% CO$_2$. Then, culture medium in each well was replaced with 100 μL of culture medium containing continuously diluted drugs at a concentration range of 1.5625–100 μM. After a specified period of time, 100 μL of CCK-8 reagent (100 μg/mL in PBS) was added to each well. The absorbance at a wavelength of 450 nm was measured after incubation at 37°C for 2 h. More effective derivatives were selected to measure toxicity to healthy human PBMCs.

### 2.5 Combination index analysis of synergy

Combination index (CI) analysis was performed to determine whether the combination of curcumin with TRAIL had a synergistic anti-cancer activity based on the median effect. Additionally, CI analysis was used to determine an optimal combined concentration ratio. Compusyn software (ComboSyn, Inc., Paramus, NY, USA) was used to calculate the CI, which determined the type of interaction between the chemicals, as follows: CI = 1 indicates an additive effect; and CI > 1 indicates antagonism if CI < 1 represents the interaction as synergistic.

### 2.6 Apoptosis by annexin V-FITC assay

Cell apoptosis detection was performed using the Annexin V-FITC/PI cell apoptosis detection kit (Solarbio, Beijing, China). Briefly, U937 cells and PBMCs from healthy volunteers and ALL patients were cultured on 6-well plates at a density of 5 × 10$^4$ cells/mL and incubated with andrographolide, AND7, andrographolide + TRAIL, and AND7 + TRAIL for 12, 24, and 48 h. The concentration of andrographolide and AND7 was 5 μM and the concentration of TRAIL was 4 ng/mL. The cells were collected and washed 3 times with PBS and resuspended in 100 μL of binding buffer from a kit containing annexin V-FITC and PI for 15 min. Finally, cell apoptosis in all the
samples were analyzed using a flow cytometer (ACEA NovoCyte™; ACEA Biosciences Inc., Hangzhou, China).

2.7 ROS production
DCFH-DA was hydrolyzed across the cell membrane to DCFH, which was detected as green fluorescence under the fluorescence microscope. U937 cells treated with 4 ng/mL TRAIL and 5 μM of AND7 were collected and washed with PBS after the treatment of drugs for 4 h. Fluorescence was measured with flow cytometry after the cells were re-suspended in fresh PBS and incubated with 10 μM DCFH-DA-containing serum-free medium for 20 min.

Intracellular estimation of intracellular reactive oxygen species (ROS) was detected using flow cytometry (ACEA NovoCyte™) via DCFH-DA (ROS Assay Kit; Beyotime, Shanghai, China).

2.8 Western blot analysis
U937 cells were collected and the total protein was extracted after treatment of the above groups for 12, 24, and 48 h. The culture media was removed after treatment and the cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). The total protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Assay kit (Beyotime). Equal amounts of protein extracts were resolved on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). As an internal reference, primary antibodies were used as follows: caspase-8; caspase-3; DR4; DR5; and GAPDH. Then, the membranes were immunoblotted with the corresponding primary antibodies at 4°C for 8 h. After incubation with the appropriate secondary antibodies, the signals were detected using the SuperSignal West Pico substrate (ThermoFisher Scientific, Hangzhou, China).

2.9 Real-time PCR analysis
Total RNA was extracted from U937 cells using TRIzol extraction kits (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol after the treatment of drugs for 24 h. Following reverse transcription of extracted RNA to cDNA using HiScript III RT SuperMix (Vazyme), qRT-PCR was performed with ChamQ Universal SYBR qPCR Master Mxi (Vazyme), 1 μM of each primer, and 6 μL of diluted cDNA using a Roche LC480II instrument (Roche, Basel, Switzerland). GAPDH was used as an internal control. The relative amounts of mRNAs were calculated using the comparative threshold cycle (Ct) method. Furthermore, all reactions were performed in triplicate. The details of sequences of primers used for qRT-PCR are listed in Table 2.

Table 2 | Sequences of primers used in the qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Assay-ID</th>
<th>Primer(5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR4</td>
<td>Homo</td>
<td>ENST00000221132_8</td>
<td>F: GTGTCCACAAGAAATCAGGCAA*&lt;br&gt;R: GAGCCGATGCAACAACAGACVV*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>Homo</td>
<td>ENST00000269305_9</td>
<td>F: TGACACGGCTCCCTGGATTG*&lt;br&gt;R: GGCAAGGGGGACAGAAGC*</td>
</tr>
<tr>
<td>FADD</td>
<td>Homo</td>
<td>ENST00000301838_5</td>
<td>F: CGAGCTCAAGTTCCTATGCT*&lt;br&gt;R: AAAGTGCTGCAACAGGTCTTC*</td>
</tr>
<tr>
<td>NADPH</td>
<td>Homo</td>
<td>ENST00000220764_7</td>
<td>F: TCGCTTCTCCGGAGGTTTCA*&lt;br&gt;R: CTTAGGCATCACGACTAGG*</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Homo</td>
<td>ENST00000647810_1</td>
<td>F: GTTCACAGACCTGGCATCCGT*&lt;br&gt;R: GAAGGTATGGGCCATCTGCTGT*</td>
</tr>
<tr>
<td>P50</td>
<td>Homo</td>
<td>ENST00000226574_9</td>
<td>F: CCCCAGCCCGCTTAGGA*&lt;br&gt;R: GAAGGTATGGGCCATCTGCTGT*</td>
</tr>
<tr>
<td>P65</td>
<td>Homo</td>
<td>ENST00000406246_8</td>
<td>F: CACCACCACTCGGACTCGT*&lt;br&gt;R: GTCCCACGCTGCTTTCTA*</td>
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<tr>
<td>GAPDH</td>
<td>Homo</td>
<td>ENST00000229239_10</td>
<td>F: TGACAGTCAGGGATTTGCC*&lt;br&gt;R: TGGACTCCACGAGCAGTACTCA*</td>
</tr>
</tbody>
</table>

*All primers were synthesized from GenScript (Nanjing, China).
2.10 Statistical analysis
The data from all experiments are represented as the mean ± SD and tests were used for statistical analysis, as follows: (*), (**), (***), and (****) signify a P<0.05, P<0.01, P<0.001, and P<0.0001, respectively. All the analyses were done using GraphPad Instat (GraphPad Software, San Diego, CA, USA).

3. RESULTS
3.1 Synthesis of andrographolide derivative and cell cytotoxicity
The anti-cancer activity of andrographolide and its derivative (AND7) against leukemia cell lines in vitro is shown with IC\textsubscript{50} in Table 3 and Figure 1. Andrographolide

Table 3 | Values of andrographolide and AND7 in U937 cells, THP-1 cells, ALL-PBMCs, and healthy PBMCs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>U937</th>
<th>THP-1</th>
<th>ALL-PBMCs</th>
<th>Healthy PBMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AND7</td>
<td>6.63±0.68****</td>
<td>18.85±2.79**</td>
<td>23.74±14.11***</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Andrographolide</td>
<td>73.03±12.40</td>
<td>81.62±7.95</td>
<td>70.50±15.29*</td>
<td>&gt;100</td>
</tr>
<tr>
<td>TRAIL (ng/mL)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Means and standard deviations are calculated from triplicates of three independent experiments. ALL-PBMCs: peripheral blood leukocytes from ALL patients. Healthy PBMCs: peripheral blood leukocytes from healthy donors. Compared with andrographolide, *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 1 | Cytotoxic effects of AND7 and andrographolide on human leukemic cells (U937), THP-1 cells, and PBMCs from ALL patients and healthy controls.
THP-1 cells, U937 cells, or PBMCs were treated with andrographolide and its derivatives at various concentrations (0-100 μM) for 24 h, then the CCK-8 assay was used to determine cell viability. Error bars represent the SE.
demonstrated resistance to the anti-cancer effect of leukemia cell lines. At the same time, the lethal effect of TRAIL on leukemia cell lines was no longer effective. The results showed that andrographolide and TRAIL alone exhibited poor therapeutic effects on ALL. Compared to the acute myeloid lymphoid leukemia (AML) cell line, THP-1, the andrographolide derivative (AND7) exhibited significant anti-cancer effects in the ALL cell line (U937), as shown in Table 3.

We next compared cell cytotoxicity of AND7 combined with TRAIL in U937 cells, THP-1 cells, and healthy human PBMCs (Figure 1). Both andrographolide and AND7 showed good cytotoxicity against U937 cells. In contrast, the cytotoxicity of AND7 to healthy human PBMCs was not significantly higher than andrographolide, indicating that AND7 is likely to be more safe. Thus, we selected AND7 as a therapeutic drug in combination with TRAIL for treating ALL in the follow-up experiments.

3.2 Validating synergy of AND7 and TRAIL and optimal dosages

We verified that the combination treatment of an andrographolide derivative (AND7) and tumor necrosis factor-related apoptosis-inducing ligand (TNFSF10 [TRAIL]) increased cell death in the ALL cell line (U937) without affecting healthy PBMCs. The highest single-agent approach was used to determine the optimal combination ratio of AND7 and TRAIL based on CI values. Notably, the CI evaluates drug interaction synergistic or antagonistic effects, in which CI = 1 indicates an additive effect and CI > 1 indicates antagonism if CI < 1 represents the interaction as synergistic.

The highest single-agent approach compared the efficacy index of the combination group with the best single-agent group, indicating that the combination was more effective than either single agent and confirmed the rationality based on the P value. Four joint ratios of TRAIL combined with AND7 (1:10, 1:5, 2:5, 4:5 [ng/mL: µM]) were tested. The CI values for each combination at EC₅₀, EC₅₀, EC₇₀, and EC₉₀ from cytotoxicity data were tested using Compusyn software (Figure 2A, B). The results showed that at a TRAIL-to-AND7 ratio of 4:5 (ng/mL: µM), the CI value was 0.417 at EC₅₀, showing the best synergistic effect (Table 4). This combination of drugs would be a potentially less toxic and more effective treatment option. As shown in Figure 2C, the AND7/TRAIL combination showed a significant synergistic effect compared to separate use alone at the same control concentration. This finding indicated that the combination was feasible for subsequent experiments.

3.3 AND7/TRAIL combination enhances apoptosis in U937 cells and ALL-PBMCs

The distinctive feature of patients with ALL is an increase in PBMCs. Additionally, the effect on PBMC apoptosis becomes a more apparent indicator to evaluate the drug effect on ALL treatment. The apoptotic effects of andrographolide and its derivative, AND7, in combination with TRAIL on the ALL cell line (U937) and PBMCs from ALL patients (Figure 3A, C) indicated that AND7
in combination with TRAIL had a more pronounced pro-apoptotic effect on U937 cells and significantly promoted the apoptosis of PBMCs from ALL patients. The toxicity of the AND7/TRAIL combination to PBMCs from healthy humans was insignificant (Figure S4). Moreover, there was a time lag in collecting blood samples from ALL patients and healthy individuals, and the mortality rate of control cells reached 20% when the cells were processed uniformly. Hence, the error was taken into account when processing the data.

AND7 and TRAIL alone or in combination were less toxic to healthy human PBMCs (Figure 3). Andrographolide and TRAIL alone had poor effects on apoptosis in the ALL cell line (U937) and PBMCs from

Figure 3  | Apoptosis analysis of AND7-treated U937 cells and ALL-PBMCs in the presence or absence of TRAIL.
(A, C) Apoptosis analysis of U937 cells and ALL-PBMCs was performed using flow cytometry after treating andrographolide or AND7 in the presence or absence of TRAIL for 24 h. (B, D) Statistical analysis of A and C plots, respectively. The concentration was taken as the IC$_{20}$ value of the drug. The concentration of andrographolide and AND7 was 5 μM and the concentration of TRAIL was 4 ng/mL. "+" indicates that the processing group uses TRAIL processing. Error bars represent SE. * indicates a significant difference (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).
ALL patients. In contrast, AND7 had good effects on apoptosis in U937 cells and PBMCs from ALL patients. The apoptotic effect on PBMCs from U937 cells and ALL patients significantly increased when combined with AND7-grade TRAIL without toxic effects on normal human PBMCs (Figure 5A). The apoptotic effect on PBMCs was significantly increased in U937 and ALL patients (Figure 3). TRAIL, in combination with AND7 (4 ng/mL [5 μM]), demonstrated a highly effective and low-toxic therapeutic effect in ALL.

3.4 The AND7/TRAIL combination increases the level of ROS in U937 cells
The accumulation of ROS levels correlates with apoptosis. Therefore, the DCFH-DA probe was used to detect changes in intracellular ROS levels. Intracellular DCF fluorescence levels were higher under fluorescence microscopy after 4 h of AND7/TRAIL combination treatment, implying higher levels of ROS accumulation (Figure 4A). After loading the probes, the cells were subjected to flow cytometry to detect fluorescence intensity and perform statistical analysis (Figure 4B-D). NADPH, a marker of ROS, also showed significant differences at the RNA level, indicating that the AND7/TRAIL combination-regulated apoptosis was associated with elevated ROS (Figure 5H).

3.5 The AND7/TRAIL combination affects levels of apoptosis pathway-related gene mRNA expression
The levels of apoptotic pathway mRNA and the increased levels of NADPH mRNA suggested an increase in ROS with the combination of AND7 and TRAIL. This finding is consistent with the above results, demonstrating that the AND7/TRAIL combination is pro-apoptotic by increasing the accumulation of ROS levels. Neither NF-κB nor its associated protein, P50, was significantly changed, which shows that the combination was not useful for cell survival. The results showed that compared with controls, the apoptosis receptor gene, DR5 (Figure 5B), was significantly different from DR4 (Figure 5A). Activation of death receptors, such as Fas, DR4, DR5, and TNF-α, by their respective ligands induced apoptosis. I found that the AND7/TRAIL combination activates the poptotic pathway via death receptors DR4 and DR5. Figure 5C shows that there was no significant change in the expression of FADD, the activation receptor of Fas. The expression of DR4 and DR5 was increased (Figure 5A, B), which indicated that the modified combination induced apoptosis through the downstream death pathway of DR4/5. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway regulates cell growth and death, and its complexes (RelA [p65] and p50/p105 [NF-κB1]) were not significantly altered (Figure 5D, E). High expression of the tumor apoptosis gene, P53 (Figure 5F), led to a focus on the P53 apoptosis pathway. Moreover, expression of the ROS-related gene, NADPH (Figure 5H), showed that AND7 in combination with TRAIL increased intracellular ROS accumulation.

3.6 The AND7/TRAIL combination induced apoptosis via upregulation of the death receptor, DR5
The pro-apoptotic activity of TRAIL acts mainly through TRAIL-R1 (DR4) and TRAIL-R2 (DR5). First, we detected the level of DR4/DR5 mRNA expression in U937 cells after a 24-h treatment with the drug by Q-PCR (Figure 5A, B), which showed that DR4 and DR5 had significantly different mRNA levels compared to controls. Then, we determined DR4 and DR5 expression in U937 cells at the protein level after the above treatments. The results showed that DR5 protein expression was significantly increased in U937 cells after a 24-h AND7 combined with TRAIL treatment (Figure 6A-C). This finding indicated that AND7 sensitized TRAIL-induced apoptosis via upregulating the death receptor, DR5. Then, DR5 protein expression was determined in U937 cells after exposure to AND7/TRAIL combination treatment for 12, 24, and 48 h. The most significant difference in the level of DR5 protein expression occurred after 24 h of treatment (Figure 6D, E).

3.7 The AND7/TRAIL combination activates the caspase-8/3 apoptotic pathway via DR5
Caspase activation is a hallmark of cell apoptosis. The apoptotic protein, caspase-8, is a downstream apoptotic protein of DR5, which can be shared with CL-caspase-8. CL-caspase-8 promotes the activation of caspase-3, which in turn allows apoptotic cell death. Several studies have shown that caspase-8 is involved in TRAIL-mediated apoptosis in cancer cells. The AND7/TRAIL combination was first investigated at the gene level to activate the apoptotic pathway at the receptor DR5 and was shown to be associated with the apoptotic gene P53 (Figure 5F). It was initially hypothesized that caspase-8/3, which regulates apoptosis, would be activated. To confirm whether TRAIL-induced apoptosis resulted from caspase activation, caspase activation was measured in U937 cells treated with 4 ng/mL of TRAIL by western blot analysis. As revealed by the intensities of the immune-positive bands, caspase activation was more potent after treatment with AND7 combined with TRAIL than AND7 or TRAIL alone (Figure 7A, B). The caspase was shown to be activated by sheared after the combination of andrographolide derivatives and TRAIL, which is consistent with the previous hypothesis.

Additionally, the levels of caspase-3 accumulation were markedly reduced, indicating that caspase-8 and -3 apoptosis were activated by AND7 combined with TRAIL compared to andrographolide alone. Notably, the above findings indicated that AND7 sensitized TRAIL-induced apoptosis by activating the caspase-8/3 apoptotic pathway through DR5.
3.8 Anti-cancer enhancement through activation of the apoptotic protein, P53

It has been previously demonstrated that the AND7/TRAIL combination promotes cancer cell apoptosis by activating the apoptosis gene, P53, through increased ROS accumulation, resulting in the treatment of ALL. The effect of the combination on relevant apoptotic proteins was verified by protein measurement. The results showed no significant difference in the stock-associated protein, NF-κB (Figure 8E). However, the expression of
Figure 5 | Effect of AND7 and TRAIL on gene expression in the apoptotic pathway.
(A-H) Expression of DR4, DR5, P53, FADD, NF-κB, P65, P50, and NADFH, as assessed by quantitative polymerase chain reaction after 24 h of drug treatment. The concentration of andrographolide and AND7 is 5 μg/mL and the concentration of TRAIL is 4 ng/mL. “+” indicates that the processing group uses TRAIL processing. Data are displayed as the mean ± SD; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n=3.
Figure 6 | Effect of andrographolide and AND7 on DR4 and DR5 expression in U937 cells in the presence or absence of TRAIL.

(A-C) Quantitative analysis of protein expression of DR4 and DR5 in U937 cells after 24 h treatment with andrographolide and AND7 in combination with TRAIL. The results showed that there was a significant change in DR5 expression. (D, E) Quantitative analysis of the changes in the apoptotic receptor DR5 under a time gradient showed that the most significant changes were observed at 24 h post-treatment. The concentration was taken as the IC_{20} value of the drug (TRAIL: 4 ng/mL; andrographolide and AND7: 5 μM). “+” indicates that the processing group uses TRAIL processing. Error bars represent the SE. * indicates a significant difference compared with control (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

Figure 7 | Effect of AND7/TRAIL combination on caspase-8 and -3 expression in U937 cells.

Quantitative analysis of caspase-3 (A) and caspase-8 (B) expression changes in U937 cells after a 24-h treatment with andrographolide and its derivatives in combination with TRAIL. The concentration of andrographolide and AND7 was 5 μM and the concentration of TRAIL was 4 ng/mL. “+” indicates that the processing group uses TRAIL processing. Error bars represent the SE. * indicates a significant difference compared with control (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).
the apoptosis receptor, FADD, and the terminal apoptosis-regulating protein, P53, was significantly increased (Figure 8B, D), which is in agreement with the previous gene validation. Thus, the AND7/TRAIL combination activated expression of the apoptosis gene, P53, to promote cancer cell apoptosis and achieve anti-cancer effects.

4. DISCUSSION

Like other cancers, leukemia develops mainly due to mutations in oncogenes that lead to failure of the oncogenic effect. The development of a malignant hematologic disease probably involves a mutation in a critical gene of cell proliferation, differentiation, and survival in a hematopoietic progenitor [2]. ALL is characterized by abnormal proliferation of T- or B-lymphocytes, and PBMCs are usually used to evaluate the effectiveness of treatment for ALL. Some mutations suppress gene function and occur in tumor suppressor genes, such as P53 [22]. Several studies have shown that P53 expression has a stronger apoptotic effect in leukemic cell lines and primary cells of patients [21].

TRAIL is currently under investigation for the treatment of leukemia, which can represent a targeted therapy against cancer for its specificity to tumor cells [12, 13]. TRAIL exerts a selective apoptotic effect via its interaction with the death receptors, TRAILR1/DR4 and TRAILR2/DR5, in a wide range of cancers while sparing healthy cells [14, 15]. However, some leukemia cells are resistant to TRAIL, such as U937 cells [16, 17]. Therefore,

Figure 8 | Effect of AND7/TRAIL combination on expression of relevant apoptotic proteins.
Quantitative analysis of FADD, NF-κB, P53, PARP-3, and MMP-9 expression changes in U937 cells after a 24-h treatment with andrographolide and its derivatives in combination with TRAIL. The concentration of andrographolide and AND7 was 5 μM and the concentration of TRAIL was 4 ng/mL. “+” indicates that the processing group uses TRAIL processing. (A) Quantitative analysis of protein expression of FADD, NF-κB, P53, PARP-3, and MMP-9 in U937 cells after a 24-h treatment with andrographolide and AND7 in combination with TRAIL. Compared with control, the expression of apoptosis-regulating proteins FADD and P53 was significantly increased, while there was no significant difference in NF-κB. Error bars represent the SE. *indicates a significant difference compared with control (**P<0.01; ***P<0.001; ****P<0.0001).
combining the andrographolide derivative, AND7, with TRAIL for leukemia can potentially achieve a better therapeutic effect [12, 18, 19].

The unique property of TRAIL triggers both extrinsic and intrinsic apoptosis, making it a potentially effective anti-cancer agent [23]. Resistance of cancer cells to TRAIL-mediated apoptosis represents a major limitation to its clinical application [12, 13, 24]. Thus, identifying agents capable of enhancing TRAIL-induced anti-cancer effects is important.

The andrographolide-derived compound, AND7, induced apoptosis and improved TRAIL resistance in the ALL U937 cell line and PBMCs derived from ALL patients. While enhancing the anti-cancer effect of andrographolide, the combination with TRAIL showed a better potentiation effect.

The mRNA level showed that the tumor suppressor gene, P53, expression was significantly elevated, which promoted apoptosis (Figure 5F) [21, 25]. The literature suggests that the anti-cancer ability of andrographolide against leukemia cancer cells is associated with the activation of the apoptotic gene, P53 [6]. Additionally, TRAIL exerts its activity by binding to and activating death receptors. However, a lack of or defects in the expression of death receptors can lead to TRAIL resistance. Furthermore, the expression of the death receptor, DR5, is regulated by p53 [26].

Andrographolide also inhibits inflammation by inhibiting NF-κB and signal transducer activation in peri-odontal ligament fibroblasts [6, 27]. The NF-κB gene was shown not to be activated at the mRNA level, thus inhibiting cell survival, which was consistent with the expected results [6] (Figure 5D).

AND7/TRAIL and increased levels of ROS, DR5 overexpression, and caspase-8 activation, which are all downstream responses to P53. The AND7/TRAIL combination alleviated TRAIL resistance and enhanced the anti-cancer efficacy of andrographolide to achieve the effective treatment of ALL.

DR5 and caspase-8 are downstream proteins of the apoptotic gene, P53 [28]. The mRNA level showed that the P53 gene was indeed overexpressed in U937 cells after the AND7/TRAIL combination treatment. Thus, we tentatively concluded that the AND7/TRAIL combination affected U937 apoptosis by affecting the P53 gene and was enhanced by ROS (Figure 9).

Figure 9 | Mechanism of apoptosis pathway activation by AND7/TRAIL combination.
5. CONCLUSIONS

In this study the andrographolide derivative, AND7, with TRAIL achieved a potent and safe effect against drug resistance in ALL. The optimal combination ratio of 4:5 (ng/mL [μM]) of AND7/TRAIL combination was selected for subsequent experiments, and the combination of AND7/TRAIL increased the accumulation of ROS in cancer cells and activated P53, which promoted apoptosis in ALL cancer cells through the caspase pathway. This study has provided a basis for combining chemically synthesized and natural protein drugs and investigating the underlying mechanism of action. However, in vivo investigations need to be further refined.

ABBREVIATIONS

ALL, Acute lymphoblastic leukemia; AML, Acute myeloid leukemia; CK-8, Cell Counting Kit-8; DMSO, Dimethyl sulfoxide; AND7, Andrographolide derivatives 7; Andro, Andrographolide; PBS, Phosphate-buffered saline; BCA, Bovine serum albumin; NMR, Nuclear magnetic resonance spectrum.

ASSOCIATED CONTENT/SUPPORTING INFORMATION

Supplementary data to this article can be found online at https://amm-journal.org/wp-content/uploads/2024/05/amm20240008_Suppl.pdf. Additional experimental data, including synthesis, characterization, photophysical properties, cytotoxicity, cell imaging and experimental details, contents of the material supplied as Supporting Information.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES


