Kayadiol exerted anticancer effects through p53-mediated ferroptosis in NKTCL cells

Cuiying He1,2,3, Chengzhao Wang4, Haisheng Liu1* and Baoen Shan2,3*

Abstract

Background: Extranodal natural killer/T cell lymphoma (NKTCL) is a highly aggressive type of non-Hodgkin lymphoma that faces the treatment challenges. Natural compounds are important sources for drug development because of their diverse biological and chemical properties, among which terpenoids have strong anticancer activities.

Methods: The human NK/T cell lymphoma cell line YT and peripheral blood lymphocytes isolated from NKTCL patients were treated with different concentrations of kayadiol. Then, the following experiments were performed: CCK-8 assay for cell viability, reactive oxygen species (ROS) and glutathione (GSH) assay and co-treatment with NAC, reduced GSH, or ferrostatin-1 for ferroptosis, the proteome profiling for elucidating signaling pathways, and western blot for the expression of p53, SCL7A11, and GPX4. siRNA and CRISPR/Cas9 plasmid for p53 knockout was designed and transfected into YT cells to evaluate the causal role of p53 in kayadiol-induced ferroptosis. The synergistic effect was evaluated by CCK8 assay after co-treatment of kayadiol with L-asparaginase or cisplatin.

Results: In this study, we found that kayadiol, a diterpenoid extracted from Torreya nucifera, exerted significant killing effect on NKTCL cells without killing the healthy lymphocytes. Subsequently, we observed that kayadiol treatment triggered significant ferroptosis events, including ROS accumulation and GSH depletion. ROS scavenger NAC, GSH, and ferroptosis inhibitor ferrostatin-1 (Fer-1) reversed kayadiol-induced cell death in NKTCL cells. Furthermore, kayadiol decreased the expression of SLC7A11 and GPX4, the negative regulatory proteins for ferroptosis. We then demonstrated that p53 was the key mediator of kayadiol-induced ferroptosis by SLC7A11/GPX4 axis through p53 knockout experiments. In addition, kayadiol exerted a synergistic effect with L-asparaginase and cisplatin in NKTCL cells.

Conclusion: Taken together, our results suggested that the natural product kayadiol exerted anticancer effects through p53-mediated ferroptosis in NK/T cell lymphoma cells. Hence, it can serve as an effective alternative in the treatment of NK/T cell lymphoma, especially for patients exhibiting chemoresistance.

Keywords: Kayadiol, Ferroptosis, p53, NKTCL

Introduction

Extranodal natural killer/T-cell lymphoma (NKTCL) is a subtype of non-Hodgkin lymphoma derived from mature T cells or natural killer (NK) cells. NKTCL is a highly aggressive neoplasm with a poor prognosis. Currently, treatment for NKTCL mainly relies on chemotherapy and radiotherapy, and L-asparaginase...
(L-Asp)-based treatment regimen is considered as the first-line option. Although most patients with NKTCL can achieve clinical remission after L-Asp-containing regimens and radiotherapy, the long-term overall survival (OS) remains poor, especially for patients with advanced stage and refractory/relapsed diseases [1, 2]. Despite the recent progress in developing new molecularly targeted drugs and antibodies, therapeutic outcomes remain unsatisfactory. Therefore, identifying new anticancer drugs is critical to improve the prognosis of NKTCL patients.

Natural compounds are important sources for drug development because of their diverse biological and chemical characteristics. Many drugs derived from natural compounds have been clinically used, among which terpenoids have strong anticancer activities [3, 4]. By screening some rarely reported natural terpenoids, we found that kayadiol had a significant killing effect on NKTCL cells. Kayadiol is a natural diterpenoid extracted from the pulp of Torreya nucifera. Little of the property of kayadiol has been studied, and its anti-cancer effect has only been reported in some cell lines [5]. The antitumor effects and the mechanism of kayadiol in NK/T cell lymphoma remain unknown.

Ferroptosis is a newly discovered form of programmed cell death, which characterized by accumulation of lipid peroxides-reactive oxygen species (ROS) within the cell [6, 7]. Ferroptosis is usually caused by iron-dependent oxidative damage, and the classical ferroptosis pathway is usually induced by the failure of membrane protective mechanisms against peroxidative damage. Glutathione peroxidase (GPX4) uses glutathione (GSH) as a reductant to catalyze the reduction of lipid hydroperoxides to non-toxic lipid alcohols, thereby protecting cells from lipid peroxidative damage. GSH depletion or GPX4 inactivation leads to the accumulation of ROS, which induces classical ferroptosis [8, 9]. Increasing evidence suggests that ferroptosis plays an important role in various human diseases, including tumorigenesis [10, 11]. Activation of ferroptosis in tumors would be a potential treatment strategy.

In this study, we found that kayadiol exerted a significant killing effect on NKTCL cells without killing healthy lymphocytes. Next, we demonstrated for the first time that ferroptosis was consequent to kayadiol-induced cell death, and p53 was a critical mediator of kayadiol-induced ferroptosis. Furthermore, kayadiol enhanced the sensitivity of NKTCL cells to L-Asp and cisplatin. Taken together, our results suggested that the natural product kayadiol exerted anticancer effects through p53-mediated ferroptosis in NKTCL cells and could be an effective alternative for NK/T cell lymphoma therapy, especially for patients exhibiting chemoresistance.

### Methods

#### Cells and chemicals

The human NK/T cell lymphoma cell line YT was kindly provided by Dr. Mingzhi Zhang, Zhengzhou University First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan, China. Cells were maintained in Roswell Park Memorial Institute 1640 (RPMI1640; Gibco, Tulsa, OK, USA) medium, supplemented with 10% fetal bovine serum. Kayadiol was kindly provided by Dr. Mei Dong, Hebei Medical University, Shijiazhuang, Hebei, China. The following chemicals were obtained from commercial sources: reduced L-glutathione (GSH, HY-D0187; MCE LLC., Monmouth Junction, NJ, USA), N-acetylcysteine (NAC, HY-B0215; MCE LLC.), ferrostatin-1 (Fer-1, HY-100579; MCE LLC.), cisplatin (HY-17394; MCE LLC.), and L-Asp (HY-P1923, MCE LLC.).

#### Peripheral blood lymphocytes (PBLs) isolation

Peripheral blood lymphocytes (PBLs) were isolated from NKTCL patients and healthy donors using a Human Peripheral Blood Lymphocyte Separation Solution (Tbdscience, China). All participants provided their written informed consent and the procedure was accordance with the Ethic Committee of the Forth Hospital of Hebei Medical University.

#### Cell viability assay

Cell viability, expressed as cell proliferation, was measured using a cell counting kit-8 (CCK-8) assay. YT cells or PBLs were added to 96-well microtiter culture plates and stimulated with different concentrations of kayadiol. At the end of each cell culture period, cells were incubated with the CCK-8 solution for an additional 2 h, and the absorbance was detected at 450 nm by a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, Eugene, OR, USA).

#### ROS assay

The ROS levels were detected using a ROS assay kit (Wanleibio, Shenyang, China). After stimulation with kayadiol, YT cells were incubated with DCFH-DA fluorogenic probe for 30 min and fluorescence intensity was measured using a fluorescence microplate reader (Thermo Fisher Scientific, Eugene, OR, USA).

#### GSH assay

The GSH levels were detected using a GSH and GSSG assay kit (Beyotime, Haimen, Chian) and normalized on
the basis of cell numbers, according to the manufacturer’s instructions.

Co-treatment of kadiol and ferroptosis inhibitors
YT cells were added to 96-well microtiter culture plates and stimulated with kadiol (12.5 μM) with or without ROS scavenger NAC (5 mM), reduced GSH (1 mM), or ferroptosis inhibitor Fer-1 (1 μM). After 48 h incubation, cell viability was measured using CCK-8 assay.

Proteome profiling of signaling pathways
To elucidate the signaling pathways, a proteome profiler for human phospho-kinases (ARY003C, R&D Systems, Minneapolis, MN, USA) was used. The levels of phosphorylation or expression were quantified using ImageJ software. The relative protein levels of phosphokinases were quantified using the reference protein spot.

siRNA silencing
Cells were transfected with the designed siRNA oligonucleotides using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The selected sequences of siRNAs were as follows: siCtrl: 5'-TTC TCC GAA CGT GTC ACG T-3', sip53: 5'-CACCATCCACTCAACTCAT-3', and sip53-2: 5'GCACAGAGGAAGAATCT-3'.

CRISPR/Cas9-based p53 knockout
CRISPR/Cas9 plasmid for p53 knockout was designed based on a pSpCas9(BB)-2A-Puro (PX462) plasmid with single-guide RNA (sgRNA). The CRISPR plasmid was electroporated into YT cells and selected using puromycin. The sequences of the sgRNA were used as follows: sgp53: 5'-GCAGTCACAGCAGACGG-3'.

qRT-PCR
Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed into cDNA using a FastQuant RT Kit (Tiangen, China). RT-PCR was performed using the TaqMan® Gene Expression Master Mix (Thermo Fisher Scientific, USA). The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the mRNA input control, and relative mRNA expression levels were computed using the 2^-ΔΔCT method. The primer sequences were shown below: p53-F:5'-TGGAGAATTTTCACCCTTGACGTC-3'; p53-R:5'-TTTTATTGCGGGGAGGTAGACT-3'; GAPDH-F: 5'-CCTGCACCAACCAACTGCTTA-3'; GAPDH-R: 5'-ATGGCATGGACTGTTGTCA-3'.

Western blot
YT cells were lysed with RIPA lysis buffer to prepare whole-cell extracts. Equal amounts of total protein (10 μg) were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membrane (Pall Biotech., Westborough, MA, USA), and then probed with the appropriate primary and secondary antibodies. Immunodetection was performed using a ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, USA). The expression of the target protein was normalized to that of β-Actin (A5441, Sigma-Aldrich, St. Louis, MO, USA). Antibodies against PARP (9542, Cell Signaling Technology, Danvers, MA, USA), LC-3 (12,741, Cell Signaling Technology), phospho-p53 (Ser46, 2521, Cell Signaling Technology), p53 (9282, Cell Signaling Technology), SLC7A11 (ab175186, Abcam Cambridge, MA, USA), and GPX4 (ab125066, Abcam) were used. Full-length blots of Western blot are presented in Supplementary materials WB Figures.

Evaluation of synergy
After drug combination treatment and CCK-8 assay, the combination index (CI) of drug combinations under each experimental condition was calculated using the Compusyn software based on Chou-Talalay’s median effect analysis [12]. CI > 1 indicates antagonistic, CI = 1 indicates additive, and CI < 1 indicates synergistic. The Synergy score was calculated based on ZIP reference model using SynergyFinder analysis (SynergyFinder) [13]. ZIP Synergy scores > 10 indicates synergistic, scores from -10 to 10 indicates additive, and scores < -10 indicates antagonistic.

Statistical analysis
The results are presented as the mean ± standard deviation. Statistical analysis was conducted by the student-t test. A two-sided P-value of < 0.05 was considered to indicate a statistically significant difference. All analyses were performed using the SPSS software version 19.0 (IBM Corp., Armonk, NY, USA).

Results
Kadiol inhibits the proliferation of NKTCL cells
To observe the cytotoxicity and inhibitory effects of kadiol in NKTCL cells, YT cells were stimulated with different concentrations of kadiol at different time points. The chemical structure of kadiol was shown in the Fig. 1A. Cell counting kit-8 assays showed that kadiol suppressed YT cells’ growth in a dose- and time-dependent manner (Fig. 1B, C). Cell proliferation of PBLs from patients with NK/T cell lymphoma was also suppressed by kadiol stimulation (Fig. 1D). However, kadiol had no significant effect on cell growth of PBLs obtained from healthy donors (Fig. 1E). These results indicated that kadiol had a killing effect on NKTCL cells.
Kayadiol-induced cell death in NKTCL cells occurs through ferroptosis

To clarify the form of NKTCL cell death caused by kayadiol, we determined the expression of the apoptosis-related protein (cleaved-PARP) and autophagy-related protein (LC-3) after treatment with kayadiol. No significant effect on cleaved-PARP and LC-3 levels was observed by the stimulation of kayadiol, which indicated that other forms of cell death may have been accountable for the kayadiol-induced cell death.

We then evaluated whether ferroptosis, a newly discovered form of programmed cell death, was involved in kayadiol-induced cell death in NKTCL cells. It is well known that ROS accumulation and GSH depletion are typical iron-dependent ferroptosis events. We examined intracellular ROS and GSH levels following the treatment of kayadiol. As expected, the kayadiol stimulation elevated the ROS levels (Fig. 2A) and reduced the GSH levels (Fig. 2B). In addition, co-treatment with ROS scavenger NAC (Fig. 2C), reduced GSH (Fig. 2D), or ferroptosis inhibitor Fer-1 (Fig. 2E) rescued kayadiol-induced cell death.

The p53 contributes to kayadiol-induced ferroptosis in NKTCL cells

To predict the targets of kayadiol in NKTCL cells, the antibody chip to explore the signaling pathway was used. Kayadiol promoted the phosphorylation of p53, without activating of AKT1, JAK-STAT, MAPK, and other signaling pathways (Fig. 3A). Western blot results confirmed that kayadiol significantly upregulated both phosphorylation of p53 and p53 protein expression (Fig. 3B, C). The role of p53 in ferroptosis was elucidated, and solute carrier family 7 member 11 (SLC7A11) was identified as a direct target gene suppressed by p53 [14]. GPX4 was also an import negative regulator of ferroptosis which could protect cells from lipid peroxidative damage [8, 9]. We found that kayadiol suppressed the expressions of these two negative regulators of ferroptosis, SLC7A11 and GPX4, in NKTCL cells (Fig. 3B, C).

To confirm the causal role of p53 in kayadiol-induced ferroptosis, the expression of p53 in YT cells was silenced. A significant decreased mRNA and protein levels of p53 was observed in YT cells via transfecting sip53 (Fig. 4A, B and C). We then depleted p53 using CRISPR-Cas9 technology in YT cells and found a significant increase in the expression of SLC7A11 and GPX4. Kayadiol suppressed the expressions of SLC7A11 and GPX4 in YT cells, but on depleted p53 cells, no clear effect of kayadiol on SLC7A11 and GPX4 modulation are shown. These results indicated that kayadiol modulated p53, which in turn suppressed
SLC7A11 and GPX4, in the absence p53, kayadiol is not expected to affect SLC7A11 and GPX4 levels (Fig. 4D, E). It confirmed that p53/SLC7A11/GPX4 signaling was a critical regulatory pathway of kayadiol-induced ferroptosis in NKTCL cells.

Kayadiol exerts a synergistic effect with asparaginase and cisplatin in NKTCL cells

It was reported that ferroptosis can reverse tumor cell resistance to cisplatin [15, 16]. We then evaluated the effect of kayadiol on the sensitivity of NKTCL cells to cisplatin and L-Asp, a main drug for lymphoma chemotherapy regimen. The CCK8 results showed that kayadiol significantly increased the sensitivity of YT cells to L-Asp (Fig. 5A) and cisplatin (Fig. 5B). To detect the synergistic efficacy, we used the Chou-Talalay method for analysis, and the combination index (CI) plots of two drugs were calculated using the Compusyn software. The results indicated the synergistic effect of kayadiol with L-Asp or cisplatin (Synergy scores > 10, Fig. 5E, F). These results suggested that kayadiol had a synergistic effect with L-Asp and cisplatin, which may play an important role in reversing chemotherapy resistance in NKTCL cells.

Discussion

The value of natural compounds in the treatment of human diseases has been well documented. The use of natural products and their metabolites in enhancing physiological functions and treating pathological conditions doubled the human lifespan during the twentieth century [17]. Terpenoids, the largest class of natural products, exert strong anticancer activities [4, 18]. For example, paclitaxel, a diterpenoid isolated from Taxus wallichiana, has been widely used in the clinical treatment of breast cancer, ovarian cancer, and non-small cell lung cancer [19, 20]. Although the anticancer effects of terpenoids have been studied for years, there are still many kinds of terpenoids that have not been comprehensively studied. In this study, we screened some rarely reported natural terpenoids and found that kayadiol, a
diterpenoid extracted from *Torreya nucifera*, exerted a significant killing effect on NKTCL cells without killing healthy lymphocytes.

It is well known that T cells and NK cells are key players of the immune system to kill cancer cells. The most prestigious tumor immunotherapy, chimeric antigen receptor-engineered T cells (CAR-T), was also developed based on the tumor-killing function of healthy T cells. NK/T cell lymphoma originated from cancerous NK cells or T cells, and the loss of normal NK/T cell functions make NK/T cell lymphoma more aggressive and difficult to treat than other malignant tumors. So far, there is no therapy format proved best in the treatment of NK/T cell lymphoma [2]. Exploring drugs that can target and kill tumor cells without affecting normal NK/T cells is useful in managing this malignant disease. Kayadiol exactly exerted such a function.

In this research, we reported that p53-mediated ferroptosis occurred in kayadiol-induced cell death in NKTCL cells. It is well known that p53 exhibits tumor suppression through conventional functions such as cell cycle arrest, apoptosis, senescence, and autophagy. Accumulating evidence demonstrated the important role of p53 in ferroptosis, a newly identified form of regulated cell death [14, 21]. SLC7A11, the first identified direct target gene of p53 [22], is an important member of the cystine/glutamate exchange transporter system (xCT system) and promotes the exchange of intracellular glutamate for extracellular cystine [23]. Under the catalysis of γ-glutamylcysteine synthetase and glutathione synthetase, cystine synthesizes GSH. GPX4 uses GSH to

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Fig. 3 The target protein of kayadiol in NKTCL cells. YT cells were treated with kayadiol (12.5 μM) for 24 h. A Proteome profiling of signaling pathways showed p53 activation by kayadiol treatment in YT cells. B The expression of phosphorylated-p53, p53, SLC7A11, and GPX4 were examined by Western blotting, β-actin served as an endogenous control for normalization. C Quantification of the western blot signals was presented as means ± SD from 3 independent experiments, *P < 0.05 vs control group
reduce lipid hydroperoxides to non-toxic lipid alcohols, thereby suppressing ferroptosis [24, 25]. In our study, kayadiol promoted p53 expression, which repressed SLC7A11 and affected the synthesis of GSH, thus decreasing the activity of GPX4 and resulting in ferroptosis cell death. Of note, kayadiol could induce cell death in both YT cells and PBLs from NKTCL patients, but not in PBLs from healthy donors, implying that lymphoma cells are more sensitive to kayadiol than lymphocytes from healthy donor. The molecular mechanism underlying this phenomenon is unclear, one possibility is that kayadiol expert anti-cancer effect on NKTCL cells through p53, and TP53 mutation was reported in 20%–60% of NKTCL cases, which might make the cancerous NK or T cells more sensitive to kayadiol than healthy lymphocytes [26–28]. Although further research is required to test this hypothesis, our findings seem to support the clinical application potential of kayadiol in NKTCL.

Chemoresistance is one of the main treatment problems in NK/T cell lymphoma, especially in advanced disease [29]. Ferroptosis is reported to be of great significance in reversing resistance to chemotherapies in cancers. Sato et al. showed that pretreatment with erastin, an inducer of ferroptosis, strongly elevated the sensitivity to cisplatin in tumor cells [15]. Roh et al. reported that inhibition of Keap1-Nrf2, a key pathway of the cellular oxidative stress response, induces ferroptosis and reverses the resistance of cisplatin-resistant in head and neck tumor cells [16]. Fu et al. developed an Fe(VI)-based nanocomposite system, which can convert H2O2 into active hydroxyl radicals in tumor cells, induce ferroptosis and improve tumor sensitivity to tondoxorubicin in solid tumors [30]. In our research, kayadiol significantly increased the sensitivity of YT cells to L-Asp and cisplatin, which significantly improved its clinical application value.

Fig. 4 The regulatory role of p53 on kayadiol-induced ferroptosis in NKTCL cells, A Silencing of p53 by siRNAs decreased the expression of p53 at mRNA levels in YT cells. B Silencing of p53 by siRNAs decreased the protein expression of p53 in YT cells. C Expression levels of p53 and SLC7A11 were presented as means ± SD from 3 independent experiments, *P < 0.05 vs siCtl group. D Western blotting showed the effect of p53 knockout by CRISPR-Cas9 plasmid on kayadiol-induced ferroptosis in YT cells. E Quantification of the western blot signals was presented as means ± SD from 3 independent experiments, *P < 0.05
In summary, our study reported for the first time that kayadiol had the potential to induce ferropotosis in NKTCL cells without affecting health lymphocytes, which made it a potential compound for NKTCL treatment. Additional in vivo and large-scale clinical investigations should be conducted in the future to substantiate these research findings.

**Abbreviations**
- NKTCL: Extramedullary natural killer/T-cell lymphoma
- L-Asp: L-asparaginase
- OS: Overall survival
- ROS: Reactive oxygen species
- GPX4: Glutathione peroxidase
- GSH: Glutathione
- NAC: N-Acetylcysteine
- PBL: Peripheral blood lymphocytes
- CCK-8: Cell counting kit-8
- siRNA: Small interfering RNA
- sgRNA: Single-guide RNA
- qRT-PCR: Quantitative Real-Time polymerase chain reaction
- SLC7A11: Solute carrier family 7 member 11
- CI: Combination index
- CAR-T: Chimeric antigen receptor-engineered T cells
- xCT system: Cystine/glutamate exchange transporter system
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-09825-5.

Additional file 1.

Additional file 2: Supplement Figure 1. The effect of kayadiol on cleaved-PARP and LC-3 levels in NKTCL cells. (A) The expression of apoptosis-related protein (cleaved-PARP) and autophagy-related protein (LC-3) were examined by Western blotting, β-actin served as an endogenous control for normalization. (B) Quantification of the western blot signals was presented as means ± SD from 3 independent experiments.

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Authors' contributions

HSL and BES designed the study and supervised the project. CYH planned and conducted the experiments and wrote the manuscript. CZW contributed the kind gifts of kayadiol. We are very grateful to Dr. Mingzhi Zhang from Zhengzhou University First Affiliated Hospital for providing YT cells.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved by Ethnic Committee of the Forth Hospital of Hebei Medical University. The patients/participants provided their written informed consent to participate in this study. All the steps/methods were performed in accordance with the relevant guidelines and regulations.

Competing interests

The authors declare that they have no competing interests.

Author details

1Department of Hematology, The Fourth Hospital of Hebei Medical University, NO.169, TianShan Street, Shijiazhuang, Hebei 050035, People’s Republic of China. 2Research Center and Tumor Research Institute, The Fourth Hospital of Hebei Medical University, NO.12, Jiankang Road, Shijiazhuang, Hebei 050011, People’s Republic of China. 3Hebei Medical University, Shijiazhuang, China. 4College of Basic Medicine, Hebei Medical University, Shijiazhuang, China.

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