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Structure and functions of γ-dodecalactone isolated from *Antrodia camphorata* for NK cell activation

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ABSTRACT

The preserved fungal species *Antrodia camphorata* has diverse health-promoting effects and has been popularly used in East Asia as a traditional herb. We isolated a volatile compound from the culture medium of *A. camphorata* and identified it as γ-dodecalactone (γ-DDL). Cytomic screening for immune-modulating activity revealed that γ-DDL can activate human NK cells to express the early activation marker CD69. Further experiments showed that γ-DDL not only can induce NK cells to express CD69 but also stimulate NK cells to secrete cytotoxic molecules (FasL and granzyme B) and Th1 cytokines (TNF-α and INF-γ).

Measuring the distribution of γ-DDL in the subcellular compartments of NK cells revealed that γ-DDL has been converted to 4-hydroxydodecanoic acid (an acyclic isomer of γ-DDL) in a time-dependent manner in the cytoplasm.

Synthetic (*R*,*S*)-4-hydroxydodecanoic acid activated NK cells to express CD69 mRNA within 10 min, in contrast to γ-DDL, which activated NK cells to express CD69 within 50 min. This faster activation suggests that γ-DDL has converted to 4-hydroxydodecanoic acid and to stimulate the NK cells to express CD69.

Optically pure (*R*)-(+)-4-hydroxydodecanoic acid and (*S*)-(−)-4-hydroxydodecanoic acid were obtained via: (1) synthesis of its diastereomeric esters of (*R*,*S*)-4-hydroxydodecanoic (*R*)-(−)-2-phenylpropionate; (2) separation of diastereomers via preparative HPLC, and (3) subsequent hydrolysis of the obtained optical pure ester of (*R*)-(+)-4-hydroxydodecanoic acid (*R*)-(−)-2-phenylpropionate and (*R*)-(−)-4-hydroxydodecanoic acid (*R*)-(−)-2-phenylpropionate, respectively. Further assays of NK cells activation using each enantiomer showed that only the (*R*)-(+)-4-hydroxydodecanoic acid can activate NK cells.

1. Introduction

Antrodia camphorata, a preserved fungus known as 'niu-chang-chih,' is an indigenous and rare mushroom found in Taiwan. *A. camphorata* can only parasitize the inner wood cavity of the species *Cinnamomum kanehiraae*.[1] *A. camphorata* has been utilized in traditional folk medicine to treat a wide variety of diseases, such as diarrhea, abdominal pain, hypertension, skin itching and cancer.[2] *A. camphorata* grows very slow in the wild, and cultivation in greenhouses is difficult. To overcome this problem, a submerged culture method has been applied to obtain useful cellular materials or to produce effective substances from cultured mycelia. Recent studies have reported various biological functions of aqueous extracts of *A. camphorata* mycelia, such as anti-inflammatory, antioxidative, vasorelaxative,[3–7] anti-tumor,[8,9] anti-hepatitis B virus,[10] neuroprotective[11,12] and immune-modulating properties.[13,14] The triterpenoids and polysaccharides are the most widely studied components among the numerous compositions of *A. camphorata.* However, the volatile oils of *A. camphorata* have not been identified, and their functions are still unexplored.

Natural volatile oils are extensively used in cosmetics and in folk medicine to prevent various specific pathologies.[15] For example, tea tree oil possesses antiseptic properties against a variety of bacteria.[16] Both tea tree oil and lavender oil can suppress allergic symptoms by inhibiting histamine release and cytokine production in vitro and in vivo.[17] *Eucalyptus* oil has anti-inflammatory potential.[18] However, little is known about the immune-modulating influence of natural volatile oils on the cellular components of the immune system.[19,20]

Natural killer (NK) cells function in innate immunity and are crucial for its capacity to combat viral infection and destroy cancer cells.[21,22] Recent studies have revealed the memory-like property

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of NK cells and suggested that NK cells have characteristics of both the innate and adaptive systems.[23] NK cells exert direct cytotoxic activity against tumor targets and can regulate the adaptive immune response by cytokine production.[24] Low NK cell activity is associated with increased cancer risk. Therefore, a recent focus of anti-cancer therapeutics is developing drugs that promote NK cell activity.

Here, we identified a volatile oil isolated from culture medium of *A. camphorata* mycelia as γ-dodecalactone (γ-DDL), and the chemical structure and functions of γ-DDL to activate NK cells were studied.

2. Material and methods

2.1. Isolation of DDL from fermented culture of *A. camphorata*

The B71 strain of *A. camphorata* mycelia was cultured according to our previous report[5] with procedures a follows: The mycelia of *A. camphorata* was incubated in a 2 L flask containing 1 L of culture medium at 27–30 °C for 2–3 months. The fermented liquid culture medium was put in a rotavapor (R-3000, Büchi) to collect the evaporated gas. The evaporated gas was condensed at −50 °C by acetone/dry ice and the entrapped volatile oil obtained was stored at 4 °C.

2.2. FID-GC and GC–MS analysis

The volatile oil and commercial γ-DDL were dissolved in hexane (lauric acid as internal standard) and analyzed by gas chromatography (GC) using a Hewlett Packard 6890 system with a capillary column (Agilent DB-1, 300 × 0.25 mm) using nitrogen as carrier gas (0.8 kg/cm^2). The injector temperature was 220 °C. The initial column temperature was 180 °C and was then gradually increased at a rate of 8 °C/min up to 260 °C for 10 min. For detection, a flame ionization detector (FID) was used and set at a temperature of 260 °C. A Shimadzu GC–MS QP2000 system with a Supelcowax 10 capillary column (600 × 0.25 mm) was used for compound identification and detection for FID-GC with an electron impact (EI) analyzer system using ionization energy of 70 eV. The GC oven temperature was initially 150 °C and reached 240 °C with an elevation rate of 5 °C/min. The injector and detector temperature were both 240 °C.

2.3. Cytomic screening of immune-modulating activities

The procedure of cytomic screening was carried out as previously reported.[13] Cells were seeded in 24-well plate (1×10^6 cells per well) and treated with γ-DDL. At the end of the incubation, cell were collected, washed twice with PBS and suspended in 100 μL PBS. Fluorochrome-conjugated monoclonal antibodies (mAb) were added to each cell suspension (10 μL mAb in 100 μL cell suspension) for labeling and incubated at 4 °C for 30 min. In double-staining experiments, two types of mAb conjugated with different fluorochromes were both added to cell suspensions. As negative controls, aliquots of cell suspensions were added with the same fluorochrome-conjugated irrelevant isotype-matched mAb. The cells were washed twice with PBS, fixed with paraformaldehyde (4%) and stored at 4 °C for flow-cytometric analysis. FACSCalibur (Becton Dickinson, San Jose, CA, USA) was used for cytofluorometric analysis. For each sample, 2×10^4 cells were computed and analyzed. The software for data analysis was WinMDI v.2.8 (Windows Multiple Document Interface for Flow Cytometry). Statistical markers were set using the irrelevant isotype-matched controls as reference.

2.4. WST assay

Cell viability was measured by WST assay. Briefly, cells were seeded at a density of 4×10^3 cells/well (100 μL) in 96-well plate one day before γ-DDL treatment. After incubation with γ-DDL for 48 h, WST-1 reagent (10 μL/well) was added, and the cells were further incubated for 4 h at 37 °C in 5% CO_2. Absorbance of WST-1 reagent was measured with a spectrophotometer, using test and reference wavelengths of 450 and 630 nm, respectively. Each experiment was performed in triplicate and repeated at least three times. Results are expressed as the mean ± SEM.

2.5. CD69 detection by flow cytometry analysis

$CD56^+$ NK cells were isolated from human peripheral-blood mononuclear cells (hPB-MNCs) and enriched by a negative magnetic bead-cell separation method (MACS). Aliquots of the sorted cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD56 monoclonal antibody, and the purity of the isolated NK-cells was determined by flow cytometry. Highly enriched $CD56^+$ NK cell suspensions were cultured in a medium supplemented with RPMI-1640. Four different concentrations (100, 250, 375, 500 μM) of γ-DDL were added into cell suspensions, and the control group was treated with PBS only. After 24 h of incubation, the cells were washed twice with PBS, labeled with CD69-PE antibody and analyzed by flow cytometry to detect CD69 in NK cells.

2.6. Cell fractions extract and GC analysis

Primary mononuclear cells (MNCs) or NK cells (10^7 cells) were fed with 1 mL medium containing γ-DDL (1 μL) for specific periods of time, and the incubation medium was collected at certain timepoints. At the end of incubation, cells were washed with ice-cold PBS and pelleted. The cells were lysed by ultrasonication and centrifuged (12,000*g*) for 10 min, and the residual solution and cell pellets were separately collected. Cell nuclei were extracted by hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 mM NaF, and 1 mM Na_3VO_4) and incubated for 15 min on ice; then the cells were lysed by the addition of 0.5% Nonidet P-40, followed by vigorous vortexing for 10 s. The nuclei were pelleted, resuspended in PBS and measured for protein content by the Bradford method using BSA as a standard. The medium and cell fraction solutions were extracted using hexane (20 μL), and the extracts underwent GC analysis to determine the concentration of γ-DDL in the cell fractions and medium.

For GC analysis, five different concentrations of standard solutions were prepared: 500, 1000, 2000, 5000 and 10,000 ng/mL. The calibration curve was obtained by determining the peak areas. The regression equation of the calibration curve was $y = 569.4x + 0.0542$ (x = concentration of γ-DDL in hexane; y = the peak area of γ-DDL; lauric acid as internal standard) with $R^2 = 0.9988$. The content of γ-DDL was obtained by interpolating the calibration curve.

2.7. Q-PCR

Immune cells were treated with γ-DDL or 4-hydroxydodecanoic acid (375 μL) for various incubation periods (10 min to 12 h). RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). RNA (2 μg) was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen Life Technologies). The LightCycler DNA Master SYBR Green I kit (Roche) was used for PCR amplification. The PCR primer sequences (Supplementary Table 2) were designed by LightCycler Primer Design2 software. The mRNA was normalized to GAPDH expression, and results are presented as the fold

induction of mRNA expression relative to the control samples (non-treated cells). The reactions, performed in a LightCycler (Roche), were as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 4 s, with one melting curve analysis of 95 °C and 65 °C for 30 s.

2.8. Fluorescence microscopy

The generated effector NK cells (primary NK cells alone) were harvested and incubated with HepG2 cells for 24 h with or without co-treatment with γ-DDL (375 μM). HepG2 cells were previously transfected with pEGFP-C3 in order to allow fluorescence observation. At the end of incubation, HepG2 cells were examined under a fluorescence microscope (Olympus IX 70). Images were captured with a high-resolution digital camera (Olympus DP 50) and processed using Adobe PhotoShop software.

2.9. Chemical synthesis of 4-hydroxydodecanoic acid

γ-DDL/KOH (1:1 molar ratio) were dissolved in methanol in a flask and stirred overnight at room temperature. The solution was neutralized by adding HCl (2 N) and then evaporated by rotary vapor. The residue was re-dissolved in $CHCl_3$, extracted with water, dried over $MgSO_4$, and then concentrated. The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH = 40/1) as a white solid. The ^1H NMR (400 MHz, $CDCl_3$) results were as follows: 3.63–3.65 (m, 1H, H-4), 2.48 (t, 2H, J = 7.3 Hz, H-2), 1.66–1.85 (m, 2H, H-3), 1.23–1.44 (m, 14H, H-5-H-11), 0.86 (t, 3H, J = 6.5 Hz, H-12).

2.10. Chiral separation by chiral derivatizing agents (CDAs)

Chiral separation of γ-DDL was carried out using the CDA (chiral derivatizing agent) method as described elsewhere.[25] Optically pure (*R*)-(−)-2-phenylpropionic acid was used as a CDA that interacts with γ-DDL. The mixture of diastereomers was separated by preparative HPLC using a normal-phase HPLC column (4.6 × 250 mm, Silica-60) connected to an L-4250 UV–Vis detector and an L-7100 pump (all from Hitachi, Tokyo, Japan). Hexane/diethyl ether (98:2) was used as the mobile phase with a flow rate 4 mL/min and a running time of 30 min. UV absorption of each sample at 220 nm was measured. The chromatographically purified diastereomers were hydrolyzed and recyclized to optically pure γ-DDL stereoisomers. Absolute configurations of the chiral γ-DDL stereoisomers were identified by ^1H NMR and optical rotation analysis.

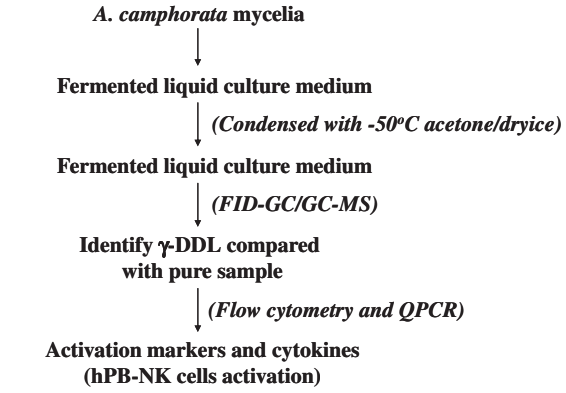
2.11. HPLC analysis

The fermented liquid culture medium (250 mL) from *A. camphorata* was extracted by dichloromethane (CH_2C1_2) three times (medium: CH_2C1_2 = 2.5:1). After the removal of CH_2C1_2, the sample was re-dissolved in acetonitrile (ACN) and separated using a reversed-phase HPLC column (4.6 × 150 mm, C18) with a solvent system (95% ACN, 4% H_2O and 1% phosphoric acid/ 99% H_2O and 1% phosphoric acid = 60/40) at a flow rate of 1 mL/min and analysis duration of 20 min. Samples were detected by UV absorption at 214 nm.

3. Results

3.1. Identification of the major compound (γ-DDL) from volatile oil of *A. camphorata*

Scheme 1 shows the procedures for isolating volatile oils from the submerged culture media of *A. camphorata* mycelia. The B71 strain of *A. camphorata* mycelia was cultured according to a known procedure.[5] The fermented medium (1000 mL) was evaporated by a rotavapor, and the volatile gas was trapped in a condenser with acetone-dry ice at −50 °C. The volatile oils (3.05 mL, separated from condensed liquid) were analyzed by GC and GC–MS.[26] Figure 1 shows the results of GC analysis. The volatile sample had a retention time of 4.8 min (Fig. 1B), and commercial γ-DDL and the internal standard lauric acid had retention times of 4.8 min and 3.7 min, respectively. The structure of the volatile oil was further confirmed by direct comparison of its mass spectral patterns after GC–MS assay (Supplementary Fig. 1). The automated peak assignment showed 16 components from the volatile oil (data not shown). Among the 16 compounds, γ-DDL (98.70%) was the main component, showing well-defined peaks with a retention time of 12.042 min, and commercial γ-DDL showed a retention time of 12.013 min. Co-injection of commercial γ-DDL with volatile oil showed a single peak at ~12 min (Supplementary Fig. 1A). Commercial γ-DDL and the volatile oil both showed a peak at m/z 198 in their GC–MS spectra, confirming the molecular weight of γ-DDL (Supplementary Fig. 1B and C). These results identified γ-DDL as the main component of volatile oil of *A. camphorata* (Fig. 1).



Scheme 1. The isolation of γ-DDL from *A. camphorata*.

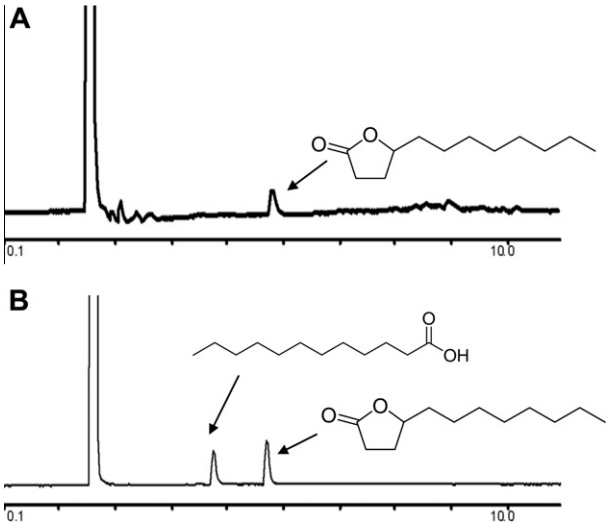


Figure 1. (A) Analysis of hexane extract of volatile oil (1 μL) by FID-GC. (B) Analysis of γ-DDL (0.5 μL) and internal standard, lauric acid (0.5 μL), by FID-GC. The samples were analyzed on a DB-1 capillary column (0.25 mm) using a temperature gradient of 180 °C to 260 °C at a rise rate of 8.0 °C/min. The injector and detector temperature was 220 °C.

3.2. γ-DDL stimulates the activation of NK cells in hPB-MNCs

Cytomic screening was used to monitor the immune-modulating activities of γ-DDL. PMA, which induces CD69 expression in immune cells, was used as a positive control.[27] The hPB-MNCs were treated with various concentrations of γ-DDL for 24 h, and then the cells were double-stained with antibodies against cell type-specific markers and the activation marker CD69. The results indicate that γ-DDL significantly up-regulated CD69 expression in $CD56^+$ NK cells and in hPB-MNCs in a dose-dependent manner (Fig. 2A). Cell viability assays showed that γ-DDL (up to 375 μM) was not cytotoxic to hPB-MNCs (Fig. 2B).

3.3. γ-DDL directly stimulates NK cell activation

Since hPB-MNCs include multiple immune cell populations, cross-talk between different cell populations in γ-DDL-treated hPB-MNCs must be considered. For example, the monocytes and T cells may act in an accessory manner for NK cell activation by producing NK-stimulating cytokines.[28] Therefore, further studies were conducted to elucidate whether the activation of NK cells to express CD69 is directly stimulated by γ-DDL or indirectly stimulated by cytokines secreted by other cells.

$CD56^+$ cell populations were purified from hPB-MNCs by magnetic separation. The NK cell-mediated cytotoxicity markers granzyme B (GraB) and Fas ligand (FasL), and the NK cell activation marker CD69 were analyzed by flow cytometry to evaluate NK cell activation.[29–32] The dose–response of γ-DDL on GraB induction and FasL activation in hPB-NK cells was also investigated. IL-2, which acts as a strong stimulator of NK cell activation, was used as a positive control.[27] As shown in Figure 3, γ-DDL stimulated CD69 expression in hPB-NK cells in a dose-dependent manner. The expression of GraB and FasL gradually increased and reached 1.5-folds and 2.5-folds of the control cells, respectively. Furthermore, the cytotoxic effect of effector hPB-NK cells was tested, in which the tumor cell line HepG2 were used as target cells and the change in number was examined by fluorescence microscopy as shown in Figure 4. The results showed that the HepG2-EGFP cells were killed

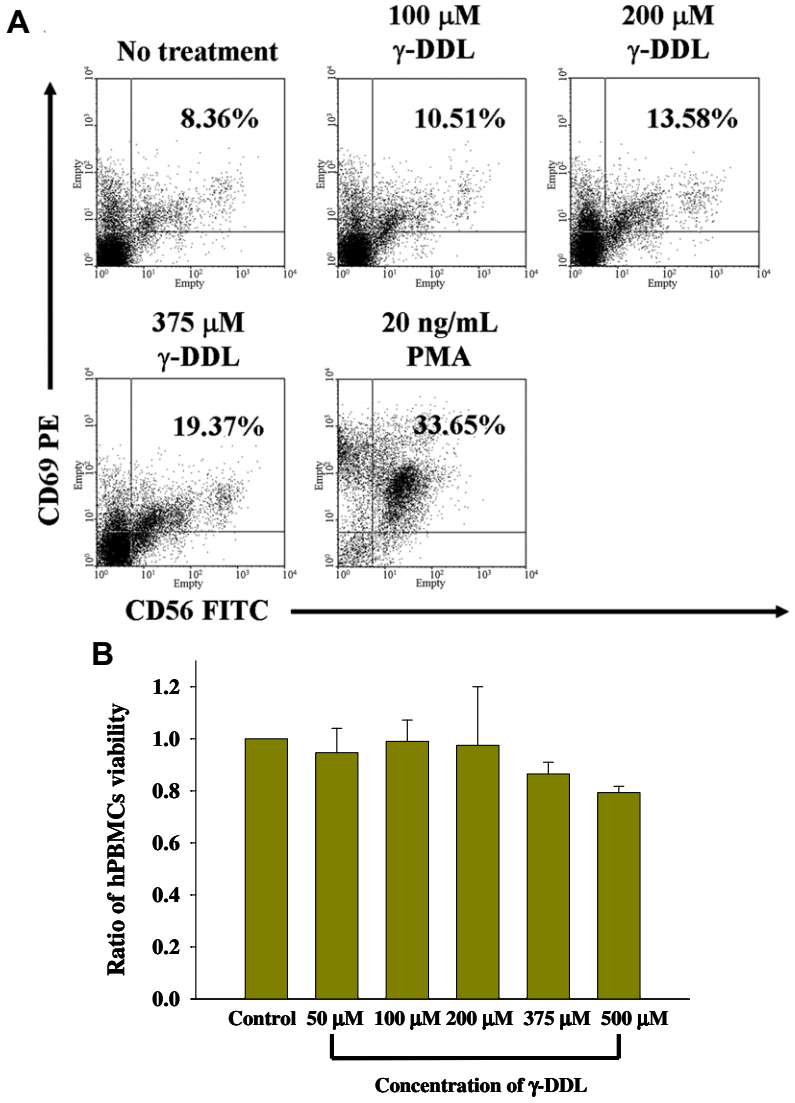


Figure 2. (A) γ-DDL induces CD69 expression of NK cells in PB-MNCs. hPB-MNC cells were treated with γ-DDL at different doses (100, 200 and 375 μM) or PMA (20 ng/mL) for 24 h. Double-staining using monoclonal antibodies against NK cell marker CD56 was performed. The double-positive cells were calculated and normalized to that of the control experiment (without treatment), and the results are expressed as mean ± SEM from three independent experiments. (NT, no treatment.) (B) *The cytotoxicity of γ-DDL in PB-MNCs.* Each culture well of cells was treated with γ-DDL (100–500 μM). For the control cultures, an equal volume of PBS was given. After the addition of WST-1 reagent and further incubation at 37 °C for 4 h, the absorbance was measured with a spectrophotometer using test and reference wavelengths of 450 and 630 nm, respectively.

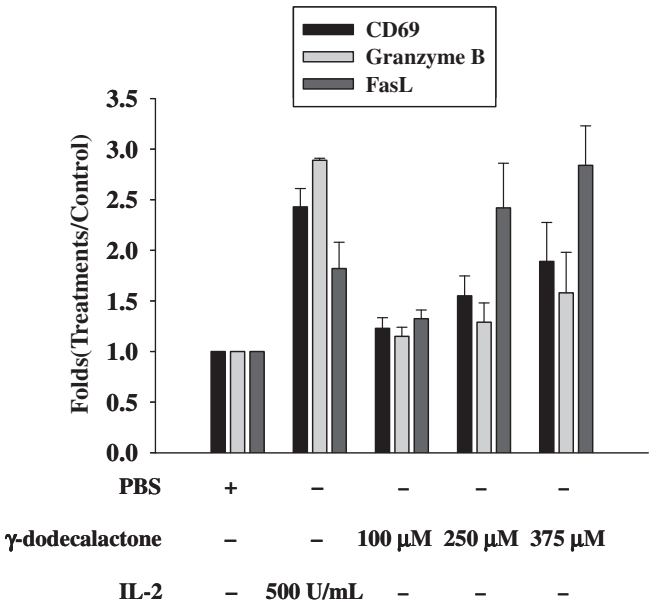


Figure 3. γ-DDL induces CD69, granzyme B, and FasL expression in purified NK cells. hPB-NK cells were isolated from hPB-MNCs by magnetic cell sorting. Flow cytometry was performed on hPB-NK cells after treatment with γ-DDL (100, 250, 375, or 500 μM) or IL-2 (500 U/mL, positive control) for 24 h. The ratio of CD69, GraB, and FasL expression was determined by comparison with the controls (no treatment). The experiments were repeated three times. Results are expressed as mean ± SEM.

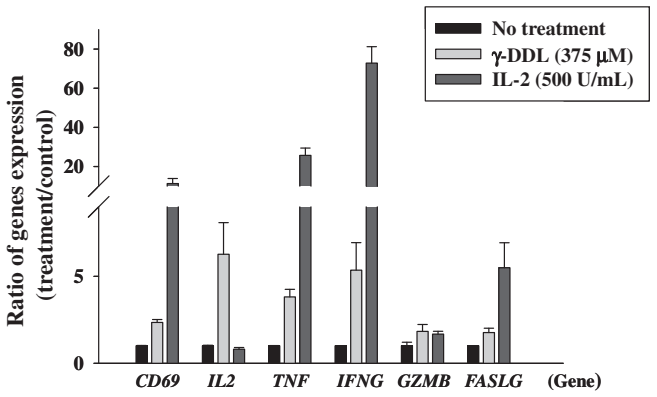


Figure 5. γ-DDL induces the expression of cytokines and activation factors in purified NK cells. Q-PCR analysis of CD69, IL2, TNF, IFNG, FASLG, and GZMB expression in hPB-NK cells after treatment with γ-DDL (375 μM) or IL-2 (500 U/mL) for 4 h. The relative quantification was determined by comparing to the controls (no treatment). The experiments were repeated three times. Results are expressed as mean ± SEM.

by effector hPB-NK cells and the cytotoxicity was raised when co-treated with γ-DDL compared to un-treated control after 24 h. The increased gene expression of these NK cell activation factors was also confirmed. The cells were treated with γ-DDL and IL-2 (positive control) for 4 h, and the expression of six genes, *CD69*, *GZMB*, *FASLG*, *IL2*, and *TNF* and *IFNG* were analyzed by Q-PCR. IL-2 significantly induced the expression of five genes, *CD69*, *TNF*, *IFNG*, *GZMB* and *FASLG*, but not *IL2*. However, all genes were induced after γ-DDL treatment (Fig. 5). These findings suggest that γ-DDL directly affects NK cells and induces their activation.

3.4. Distribution of γ-DDL in NK cells

To determine which cellular components are targeted by γ-DDL, we treated NK cells with γ-DDL and isolated the nuclear, cytosolic, and membrane fractions to analyze the concentration of γ-DDL in each subcellular compartment. The nuclear, cytosolic, and membrane fractions were individually extracted with hexane and analyzed by GC. The concentration of γ-DDL in the medium generally decreased with incubation time (Supplementary Table 1A) and diminished after 3 h of incubation. The highest concentration of γ-DDL in NK cell fractions was found after incubation for 30 min, with 0.175 ng/mg protein in nucleus, 0.019 μg in cytosol and 0.008 μg in membrane. However, much to our surprise, γ-DDL could not be detected after 3 h in all fractions of hPB-MNCs (Supplementary Table 1).

We assumed that the unexpected disappearance of γ-DDL in each fraction of hPB-MNCs might have been due to the opening of its lactone ring under physiological pH in vivo to form 4-hydroxydodecanoic acid, which is insoluble in hexane and could not be

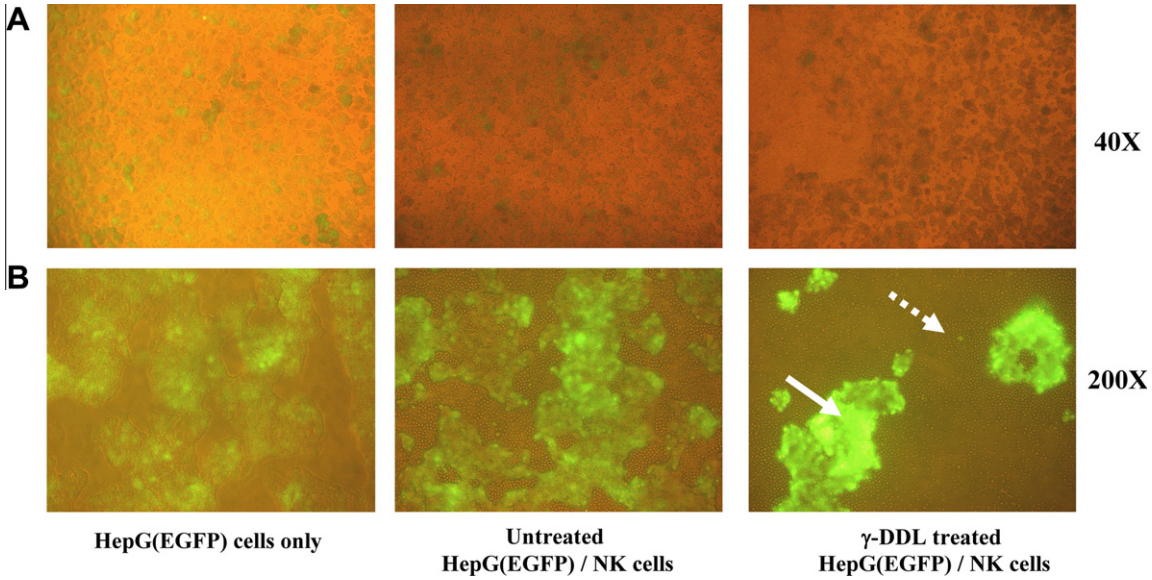


Figure 4. γ-DDL stimulates hPB-NK cells to kill the HepG2-EGFP cells. Effector cells (hPB-NK cells) were co-cultured with target cells (HepG2-EGFP cells) with E:T ratio = 10:1 in the absence or in the presence of the γ-DDL, at a concentration of 375 μM. After 24 h of stimulation, the image of NK cells cytotoxicity was captured with an Olympus DP50 camera. (A) 40× image and (B) 200× image.

extracted. To further confirm this assumption, the time-course (0–60 min) change of γ-DDL concentration in medium and hPB-NK cell fractions was examined. The lowest concentration of γ-DDL in medium was 0.065 μg, whereas the highest concentration of γ-DDL in hPB-NK cells was 18.62 ng found at 20 min (Fig. 6), indicating that γ-DDL did not enter hPB-NK and hPB-MNCs.

3.5. Intracellular conversion of γ-DDL into 4-hydroxydodecanoic acid in NK cells

In the cytosol, the concentration of γ-DDL was highest at 20 min and significantly declined at 30 min. We proposed that γ-DDL may be metabolized by cytoplasmic enzymes. To test this hypothesis, NK cells were treated with γ-DDL in a serial time-course, followed by acidifying the cell lysates to pH 2.0 and extracting the resulting solution with dichloromethane. The extract was analyzed by C18 reversed-phase HPLC (retention time of γ-DDL is 13 min, and of 4-hydroxydodecanoic acid is 4.8 min). The concentration of γ-DDL decreased from 95% to 29% in 45 min, while the concentration of 4-hydroxydodecanoic acid increased with time (Supplementary Fig. 4). The culture medium was also extracted and analyzed to determine whether the conversion of γ-DDL into 4-hydroxydodecanoic acid took place before or after enterance into NK cells. Concentration of γ-DDL in the medium (Fig. 7) decreased

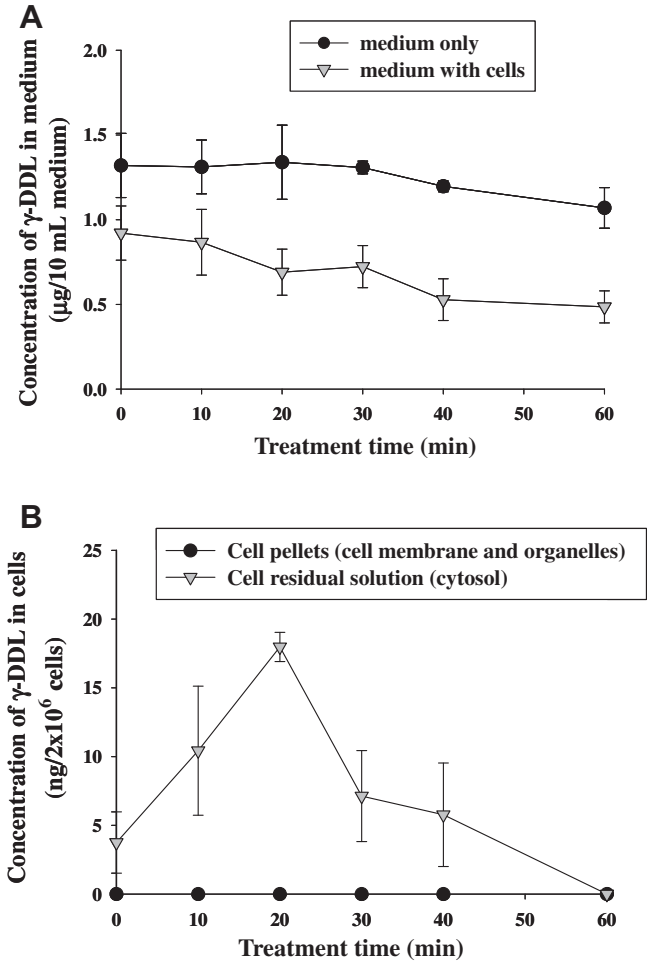


Figure 6. Determination of γ-DDL concentration in medium and hPB-NK cells. NK cells were incubated with medium containing γ-DDL (500 μM) at 37 °C for 0, 10, 20, 30, 40 min or 1 h. Each cell and medium sample was collected and γ-DDL extracted by hexane. The concentration γ-DDL was determined by GC analysis. Each value represents mean ± SEM. (A) Concentration of γ-DDL in medium with or without NK cells. (B) The concentration of γ-DDL in the medium and medium + NK cells.

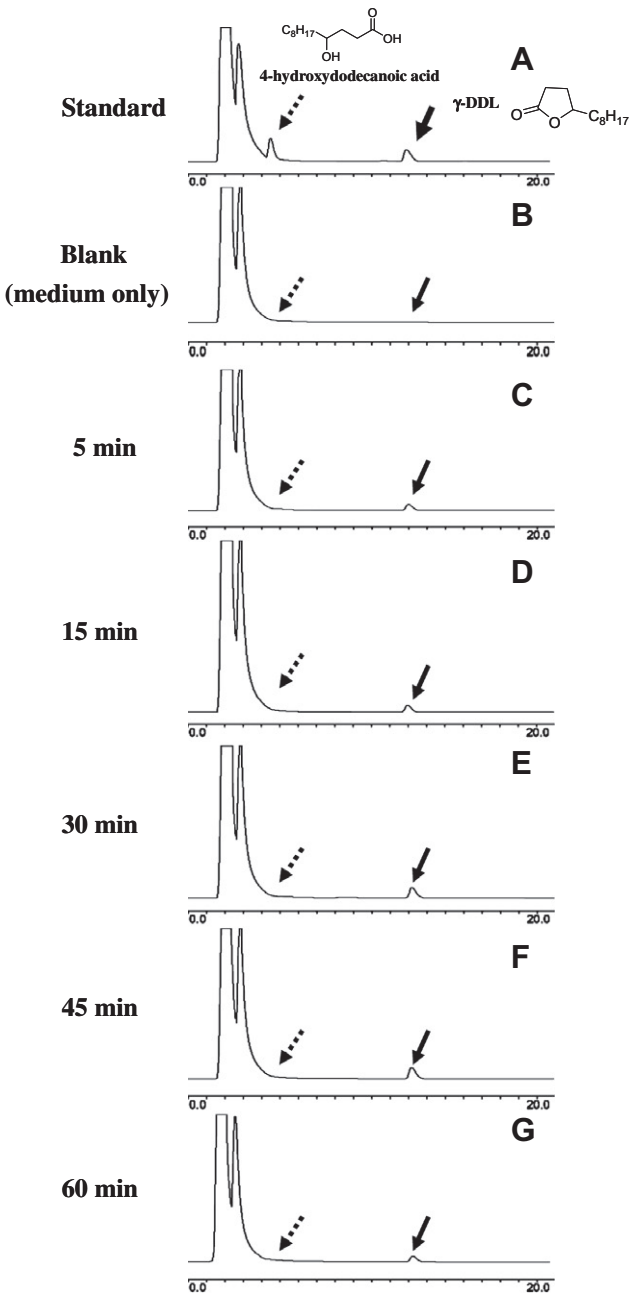


Figure 7. Detection of γ-DDL and 4-hydroxydodecanoic acid in the medium by RP-HPLC analysis. (A) Standard peak of γ-DDL (arrow with solid line) and 4-hydroxydodecanoic acid (arrow with dotted line) in the HPLC fraction. (B–F) are the media collected from cell cultures (1×10^6 NK cells) treated with γ-DDL for different times. (B) Blank. (C) Treatment with γ-DDL for 5 min. (D) Treatment with γ-DDL for 15 min. (E) Treatment with γ-DDL for 30 min. (F) Treatment with γ-DDL for 45 min. (G) Treatment with γ-DDL for 60 min. The samples were analyzed on a reversed-phase HPLC column (4.6 × 150 mm, C18), and for the HPLC condition, an iso-gradient ((A) 95% ACN, 4% H_2O and 1% phosphoric acid; (B) 99% H_2O and 1% phosphoric acid; A/B = 60/40) was used for 20 min running time at a flow rate of 1 ml/min. Samples were detected for UV absorption at 214 nm.

with time, but no 4-hydroxydodecanoic acid was detected throughout 3 h of incubation. To further confirm this, we directly incubated NK cell lysates with γ-DDL for 3 h and extracted the mixture for analysis. 4-Hydroxydodecanoic acid increased with time, similarly to the results of γ-DDL treated NK cells (Supplementary Fig. 4). These results reveal that the structural conversion of γ-DDL into 4-hydroxydodecanoic acid occurs in NK cells and cytoplasmic molecules may be involved in this process.

Because the conversion of γ-DDL to 4-hydroxydodecanoic acid occurred intracellularly, we wanted to determine which compound is biologically active. We obtained 4-hydroxydodecanoic acid by chemical synthesis starting from γ-DDL. NK cells were treated with γ-DDL or 4-hydroxydodecanoic acid for various incubation periods, and *CD69* mRNA expression was analyzed by Q-PCR. Figure 8 shows that γ-DDL induced *CD69* gene expression mostly after 45 min of treatment. However, 4-hydroxydodecanoic acid induced a rapid and efficient activation effect of NK cells within 10 min. The delayed activation time of γ-DDL coincided with the appearance of 4-hydroxydodecanoic acid in the γ-DDL-treated cells. These results suggest that 4-hydroxydodecanoic acid is the major biologically active molecule responsible for γ-DDL-induced NK cell activation.

3.6. Resolution of (*RS*)-4-hydroxydodecanoic acid

4-Hydroxydodecanoic acid is a racemic mixture with a chiral center at its C-4 position. We wanted to determine which enantiomer exhibits the biological activity of inducing NK cell activation. Optically pure (*R*)-(+)-4-hydroxydodecanoic acid and (*S*)-(−)-4-hydroxydodecanoic acid were obtained via the resolution from its diastereomeric esters, (*R*,*S*)-4-hydroxydodecanoic acid (*R*)-(−)-2-phenylpropionate, via the HPLC separation of diastereomers and subsequent hydrolysis of the obtained optically pure esters (*R*)-4-hydroxydodecanoic acid, (*R*)-(−)-2-phenylpropionate, (*S*)-4-hydroxydodecanoic acid, and (*R*)-(−)-2-phenylpropionate. The mixture of diastereomers (*RR* and *RS*) was successfully isolated, and two peaks were shown at rt = 31 min and 35 min in the HPLC spectrum (Supplementary Fig. 2). After collecting these two diastereomers (Supplementary Fig. 3B and C) and removing the chiral derivatizing agents, we confirmed their structure by NMR analysis and measurement of optical rotation, which were $[\alpha]_D^{20} = +37.8$ (MeOH *c* 0.01) for (*R*)-γ-DDL and $[\alpha]_D^{20} = -37.8$ for (*S*)-γ-DDL.[33]

3.7. (*R*)-4-Hydroxydodecanoic acid is the biologically active molecule

The activation of NK cells with (*R*)-(+)-4-hydroxydodecanoic acid and (*S*)-(−)-4-hydroxydodecanoic acid was detected by Q-PCR. As shown in Figure 9, only (*R*)-(+)-4-hydroxydodecanoic acid induced *CD69* gene expression in NK cells. Therefore, we deduce that (*R*)-4-hydroxydodecanoic acid is the major active configuration that activates NK cells.

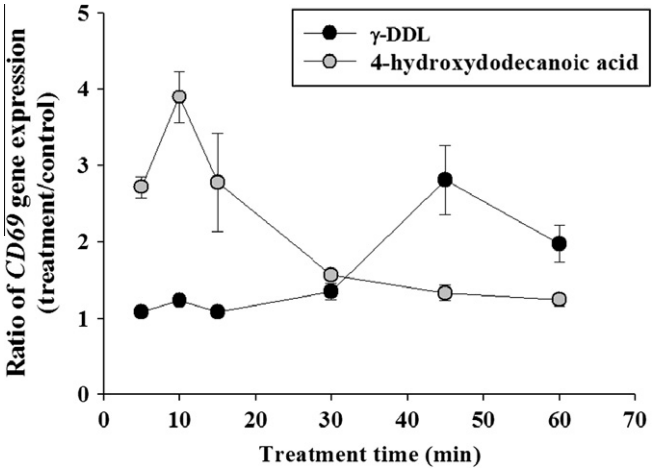


Figure 8. Expression of CD69 in NK cells after treatment with γ-DDL and 4-hydroxydodecanoic acid. The mRNA level of CD69 was analyzed after treatment with 375 μM of γ-DDL or 4-hydroxydodecanoic by Q-PCR analysis at each time point (5, 10, 15, 30, 45 and 60 min). The relative quantification (RQ) represents the ratio of each stimulated gene expression level to that of un-treated cells.

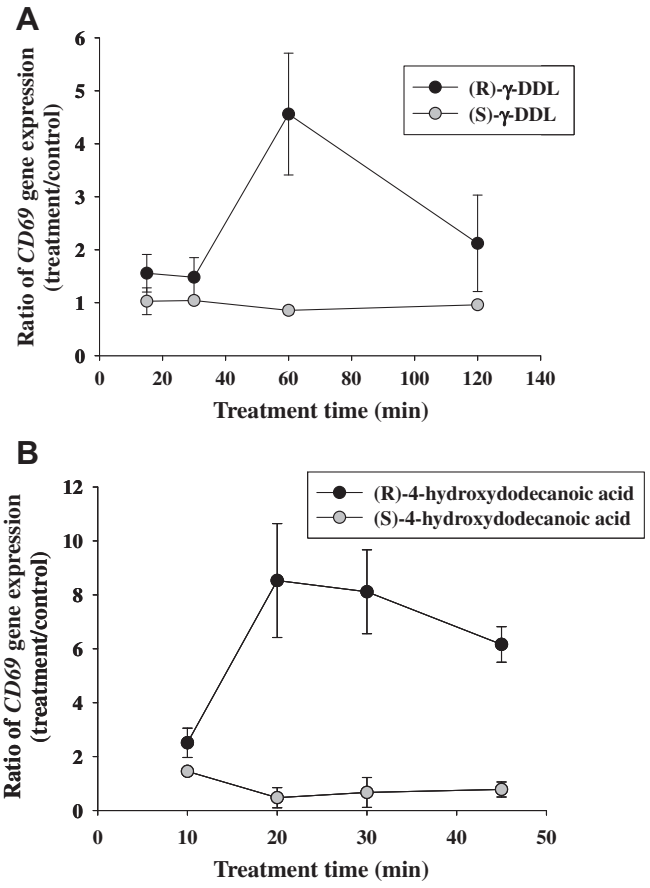


Figure 9. Expression of CD69 in NK cells after treatment with (*R*)- or (*S*)-configured γ-DDL and 4-hydroxydodecanoic acid. (A) NK cells were treated with 375 μM of (*R*)-(+)-γ-DDL or (*S*)-(−)-γ-DDL for 10, 30, 60 or 120 min. (B) NK cells were treated with 375 μM of (*R*)-(+)-4-hydroxydodecanoic acid or (*S*)-(−)-4-hydroxydodecanoic acid for 10, 20, 30 or 45 min. At the end of the treatment, the mRNA level of *CD69* was analyzed by Q-PCR. The relative quantification (RQ) represents the ratio of stimulated each gene expression level to that of un-treated cells.

3.8. Determination of the ratio of γ-DDL to (*R*)-4-hydroxydodecanoic acid in culture medium of *A. camphorata* mycelia and configuration of γ-DDL from *A. camphorata* mycelia

γ-DDL is obtained by trapping the volatile gas during the evaporation of fermented culture medium of *A. camphorata* mycelia and is further converted to 4-hydroxydodecanoic acid by chemical synthesis and separation. To validate the configuration of γ-DDL present in *A. camphorata*, the fermented medium of *A. camphorata* was collected, extracted with dichloromethane, and analyzed with RP-HPLC. We found that 4-hydroxydodecanoic acid was the major component in dichloromethane extracts, with a ratio of γ-DDL:4-hydroxydodecanoic acid of 7:93 (Supplementary Fig. 3). After collecting 4-hydroxydodecanoic acid and confirming its structure by NMR analysis, its optical rotation was measured by a polarimeter, showing a specific rotation of $[\alpha]_D^{20} = +32$ (MeOH *c* 0.01). This result indicates that (*R*)-4-hydroxydodecanoic acid is the major structural configuration present in the medium of *A. camphorata* mycelia.

4. Discussion

By NK cell magnetic separations and CD69 detection, we determined the activation effect of γ-DDL on $CD56^+$ NK cell populations in hPB-MNCs. The interaction between cytotoxic lymphocytes and their target cells can set off two different effector mechanisms: the

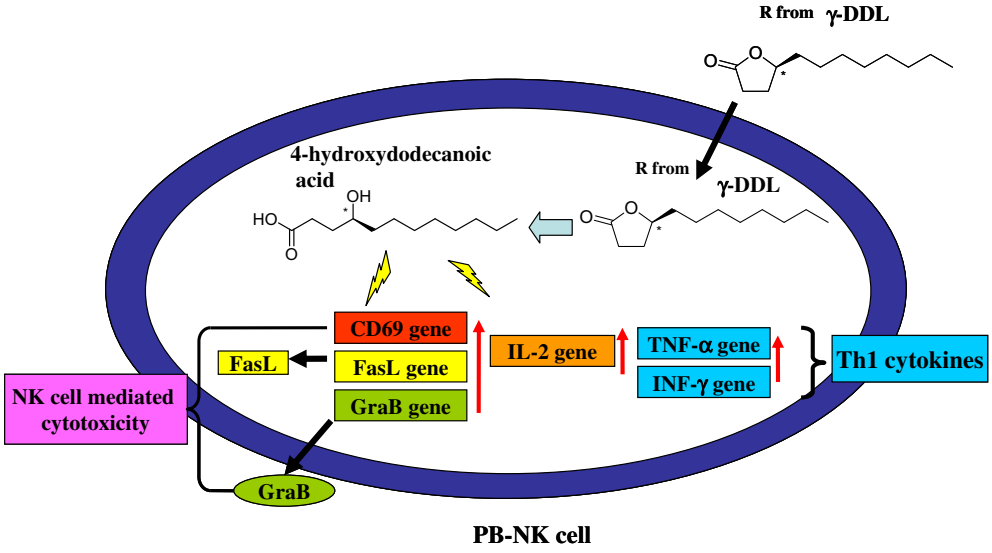


Figure 10. Summary of the γ-DDL activity in hPB-NK cells. Our results demonstrate that γ-DDL extracted from *A. camphorata* was (*R*)-(−)-γ-DDL, which augments NK cell activation through the Th1 pathway. Intracellular structural conversion of (*R*)-(−)-4-hydroxydodecalactone occurred to generate (*R*)-(+)-4-hydroxydodecanoic acid, which is the biologically active molecule that activates NK cells to secrete cytotoxic molecules (FasL and granzyme B) and Th1 cytokines (TNF-α and INF-γ).

perforin/granzymes pathway and the Fas/FasL pathway. The γ-DDL-induced $CD56^+$ NK activation showed increased expression of GraB and FasL compared with unstimulated controls. In addition, the pronounced reduction in the number of HepG2 cells indicated the continuous increase in the cytotoxicity of the hPB-NK cells by γ-DDL induction for 24 h. It is suggested that γ-DDL enhances the cytotoxic activity of NK cells through these two pathways (Figs. 3–5). According to the results in Figures 3 and 5, γ-DDL promotes TNF-α and INF-γ secretion from NK cells, meaning that γ-DDL may promote the Th1 immune response.

Volatile oils generally can be obtained from fermentation, enfleurage or extraction, but our method of steam distillation and condensation by means of acetone under ultra-low temperature was most efficient and resulted in a more natural organoleptic profile in volatile oil production.[34] The method of capillary GC–MS is often used for the isolation, concentration and identification of flavor compounds.[35] It is also applied for the quantitative determination of the volatiles, like volatile oils. However, it is rarely used to quantify compounds in cells. In this study, solvent extraction, GC–MS (Fig. 1, Supplementary Fig. 1) and NMR analysis were used to isolate and identify γ-DDL from the hexane extracts of *A. camphorata* and to quantify the concentration of γ-DDL in cells. The results indicate that due to the hydrophobicity of γ-DDL, it might be able to permeate the cell membrane to enter the cells (Fig. 6, Supplementary Table 1). The difference between the γ-DDL concentration profiles in MNCs and NK cells (Fig. 6, Supplementary Table 1) indicated a difference of enterance efficiency of γ-DDL, and this may also influence the properties and mechanism of immunoactivation. Interestingly, γ-DDL was detected in MNCs and NK cells shortly after treatment but was not found after 3 h and 1 h, respectively. We deduced that γ-DDL might have been converted into compounds with higher polarity and therefore becomes insoluble in hexane.

Lactones are generally formed by acid-catalyzed intramolecular cyclization of hydroxycarboxylic acids.[36] In an aqueous environment, a pH-dependent equilibrium is established between the open-chain hydroxycarboxylate anion and the lactone ring. In basic media, such as blood, the open-chain hydroxycarboxylate anion is favored, while in acidic media, such as urine, the lactone ring is favored. Therefore, γ-DDL might convert into its open ring form, 4-hydroxydodecanoic acid, which is insoluble in hexane. Further, we detected the conformational change of γ-DDL into 4-hydroxydodecanoic acid in NK cells by HPLC analysis and confirmed that γ-DDL could convert into 4-hydroxydodecanoic acid (Supplementary Figs. 4 and 8).

Many authors have reported on the direct enantiomeric separation of some lactones. For example, γ-DDL enantiomers from yellow passion fruit yielded a ratio of 99.9% (*R*) to 0.1% (*S*) after MDGC.[37] Only a few fruit flavors contain optically pure enantiomers, and very often, γ-lactones are found to exist in a distinct enantiomeric ratio in natural products. In most cases, however, high optical excesses in favor of the (*R*)-configured γ-lactones are found, especially for the particularly organoleptically important γ-DDL. The natural occurrence of racemic γ-decalactone has not yet been observed. In our study, the configuration of (*R*)-(−)-4-hydroxydodecanoic acid and (*R*)-(+)-γ-DDL from *A. camphorata* was confirmed by comparison to the literature. Moreover, (*R*)-configured γ-DDL demonstrated more effective NK cell activation than the (*S*)-configured isomer (Fig. 9). This was the reason why NK cells were activated with a higher dose (375 μM) of γ-DDL. Interestingly, the closed-ring form and open-ring form of γ-DDL showed reverse optical rotations.

In conclusion, this study identified the flavor γ-DDL from volatile oil of *A. camphorata* and demonstrated that it can enter hPBMCs and hPB-NK cells to activate them. By combinatorial CD marker detection in hPB-MNCs, we found that γ-DDL can induce the activation of NK cells to kill the tumor cells. The results also suggest the major configuration of γ-DDL in *A. camphorata* volatile oil to be (*R*)-(+), which is converted into the open-ring form (*R*)-4-hydroxydodecanoic acid after entering the NK cells to perform its immunoactivating function (Fig. 10).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.024.

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