Article

Modulatory Effects and Action Mechanisms of Tryptanthrin on Murine Myeloid Leukemia Cells

Hoi-Ling Chan[1], Hon-Yan Yip[1], Nai-Ki Mak[3] and Kwok-Nam Leung[1, 2, 4]

Leukemia is the disorder of hematopoietic cell development and is characterized by an uncoupling of cell proliferation and differentiation. There is a pressing need for the development of novel tactics for leukemia therapy as conventional treatments often have severe adverse side effects. Tryptanthrin (6,12-dihydro-6,12-dioxoindolo-(2,1-b)-quinazoline) is a naturally-occurring, weakly basic alkaloid isolated from the dried roots of medicinal indigo plants (Ban-Lan-Gen). It has been reported to have various biological and pharmacological activities, including anti-microbial, anti-inflammatory, immunomodulatory and anti-tumor effects. However, its modulatory effects and action mechanisms on myeloid cells remain poorly understood. In this study, tryptanthrin was shown to suppress the proliferation of the murine myeloid leukemia WEHI-3B JCS cells in a dose- and time-dependent manner. It also significantly reduced the growth of WEHI-3B JCS cells *in vivo* in syngeneic BALB/c mice. However, it exhibited no significant direct cytotoxicity on normal murine peritoneal macrophages. Flow cytometric analysis showed an obvious cell cycle arrest of the tryptanthrin-treated WEHI-3B JCS cells at the G0/G1 phase. The expression of *cyclin D2*, *D3*, *Cdk 2*, *4* and *6* genes in WEHI-3B JCS cells was found to be down-regulated at 24 h as measured by RT-PCR. Morphological and functional studies revealed that tryptanthrin could induce differentiation in WEHI-3B JCS cells, as shown by the increases in vacuolation, cellular granularity and NBT-reducing activity in tryptanthrin-treated cells. Collectively, our findings suggest that tryptanthrin might exert its anti-tumor effect on the murine myelomonocytic leukemia WEHI-3B JCS cells by causing cell cycle arrest and by triggering cell differentiation. *Cellular & Molecular Immunology*. 2009;6(5):335-342.

Key Words: Ban-Lan-Gen, tryptanthrin, myeloid leukemia, cell differentiation

Introduction

An intervention of the sophisticated balance between proliferation and differentiation process during hematopoiesis leads to an accumulation of immature non-functional hematopoietic cells in the blood and bone marrow which conduces to leukemia (1, 2). Complications that arise from the weakened immune system can be fatal. Conventional treatments for leukemia include chemotherapy, radiotherapy, and hematopoietic stem cell transplantation. However, the side effects which are often severe and inevitable, spur on the development of novel tactics for leukemia treatment. The use of Traditional Chinese Medicine has been proposed in the light of discussion as an alternative approach.

Ban-Lan-Gen is a common name referring to the dried roots of indigo plants which include *Polygonum tinctorium*, *Isatis indigotica, Isatis tinctoria* and *Strobilanthes cusia*. Indigo plants have been an important source of dyes for Asian countries since ancient time and they are also commonly used as therapeutics in Traditional Chinese Medicine. In China, water extracts of Ban-Lan-Gen are frequently used as anti-inflammatory and anti-viral medicine for the treatment of hepatitis, influenza, and various kinds of inflammation (3, 4). A number of biologically active ingredients have been identified in Ban-Lan-Gen, which include indigoid alkaloids (e.g. indigo and indirubin) and quinazolinone alkaloids (e.g. tryptanthrin) (5). In fact, indigo, indirubin and tryptanthrin are known to be three marker compounds found in Ban-Lan-Gen (6).

Tryptanthrin,12-dihydro-6,12-dioxoindolo-(2,1-b)-quinazoline, is an active ingredient isolated from Ban-Lan-Gen. It was first isolated from *Strobilanthes cusia* as an anti-fungal agent against dermatophytes (7). Tryptanthrin is a weakly basic alkaloid and has been reported to have various

[1]Department of Biochemistry and [2]Food and Nutritional Sciences Programme, The Chinese University of Hong Kong, Shatin, HKSAR, China;

[3]Department of Biology, Hong Kong Baptist University, Kowloon Tong, HKSAR, China;

[4]Correspondence to: Dr. Kwok-Nam Leung, Department of Biochemistry and Food and Nutritional Sciences Programme, The Chinese University of Hong Kong, Shatin, HKSAR, China. Tel: +852-2609-6802; Fax: +852-2603-5123; E-mail: knleung@cuhk.edu.hk

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biological and pharmacological activities, such as inhibitory activities against a variety of microorganisms and parasites (7-10), immunomodulatory (11-13) and anti-inflammatory activities (13-15), and anti-tumor activity towards human leukemia and breast cancer cell lines *in vitro* (16-18). Moreover, the crude ethyl acetate extract and tryptanthrin extracted from the indigo plant *Polygonum tinctorium* have cancer chemopreventive activity by prevention of azoxymethane-induced intestinal tumor formation in F344 rats (19). Nevertheless, its modulatory effects and action mechanisms on myeloid cells remain poorly understood. In this study, tryptanthrin was examined for its modulatory effects on the proliferation and differentiation of the murine myeloid leukemia WEHI-3B JCS cells.

Materials and Methods

Animals

Inbred female BALB/c (H-2^d) mice, aged 6-8 weeks old and bred at the University Laboratory Animal Services Centre of The Chinese University of Hong Kong under specific pathogen-free condition, were used in this study. The animal experiments were conducted with the license under Animals (Control of Experiments) Ordinance (Cap. 340) issued by Department of Health of the Hong Kong Government and according to the guidelines of Animal Experimentation Ethics Committee, The Chinese University of Hong Kong.

Cell culture

WEHI-3B (D^-) is a myelomonocytic leukemia cell line induced by mineral oil from BALB/c mice. It was originally acquired from Dr. D. Metcalf (Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia) and subsequently subcloned at the John Curtin School of Medical Research, Australian National University, Canberra, Australia. One of the subclones named WEHI-3B JCS (20) was used in this study. WEHI-3B JCS cells were cultured using RPMI medium supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL Life Technologies Inc.) and 1% antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B in 0.85% saline). The leukemia cells were cultured in 25 or 75 cm^2 tissue culture flasks and incubated at 37ºC in a humidified incubator supplied with 5% carbon dioxide (CO_2). Cells in the exponential growth phase were used for all the experiments.

Tryptanthrin

Tryptanthrin (Figure 1) used in this study was purchased from Alexis Biochemicals, USA. The powdered compound (with molecular weight of 248.2 and > 98% purity as determined by ^{1}H-NMR and HPLC) was dissolved in 100% dimethyl sulfoxide (DMSO, Sigma Chem Co.) to give 10 mM stock solutions. The stock solutions were stored in the dark at -20ºC until use.

Isolation and culture of mouse peritoneal macrophages

Inbred female BALB/c mice were first injected intraperitoneally (*i.p.*) with 1 ml 3% (w/v) thioglycollate broth (Difco Lab.) and then sacrificed 3 days later by cervical dislocation. The peritoneal exudate cells (PEC) were harvested from the peritoneal cavities of the mice with plain RPMI medium and were finally resuspended in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (complete RPMI medium) for further use. To obtain the peritoneal exudate macrophages for assays, the PEC (5×10^6 cells/ml) were seeded in a 96-well flat-bottomed microtiter plate and incubated at 37°C in a humidified 5% CO_2 atmosphere for 2 h to allow attachment of the macrophages. The non-adherent peritoneal cells were then removed and different concentrations of tryptanthrin were added to test for its cytotoxicity on the peritoneal macrophages.

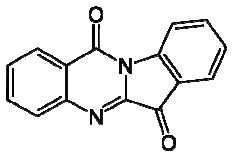

Figure 1. Chemical structure of tryptanthrin.

Determination of cell proliferation by [^{3}H]-TdR incorporation assay

Leukemia WEHI-3B JCS cells (10^4 cells/ml) were either treated with solvent control (0.05% DMSO) or different concentrations of tryptanthrin in 96-well flat-bottomed microtiter plates at 37ºC for different time intervals inside a humidified 5% CO_2 incubator. The cells were then pulsed with 0.5 μCi [^{3}H]-TdR in 20 μl complete medium for 6 h. Cells were undergone a freeze and thaw cycle before being harvested onto a glass microfiber filter. The radioactivity was measured by the liquid scintillation analyzer Packard Topcount. The results were expressed as the percentage inhibition of [^{3}H]-TdR incorporation, using solvent-treated cells as a control (21). The percentage inhibition of [^{3}H]-TdR incorporation was calculated as follows: % inhibition = (cpm of control – cpm of test sample) / cpm of control × 100%

Trypan blue exclusion assay

This assay was employed to determine the number of viable cells in cultures. The cells were incubated with different concentrations of tryptanthrin at 37ºC. The viability of the cells was then determined at the designated time interval. Viable cells were counted and the percentage cell viability was obtained using the Vi-cellTM XR cell viability analyzer (Beckman Coulter). The results were expressed as the mean percentage of cell viability ± S.E. of triplicate cultures.

In vivo anti-tumor assay

Leukemia WEHI-3B JCS cells (10^6 cells) in 1 ml plain RPMI medium were inoculated *i.p.* into each BALB/c mouse on day 1. Starting from day 6 after inoculation of WEHI-3B JCS cells, mice were divided into four groups with five mice in each group and injected *i.p.* either with solvent control or

different dosages of tryptanthrin (0.04 mg/kg, 0.08 mg/kg and 0.16 mg/kg body weight) for 5 consecutive days. At day 12 post-tumor cell inoculation, viable WEHI-3B JCS cells recovered from the peritoneal cavity were counted using Vi-cellTM XR cell viability analyzer (Beckman Coulter).

Analysis of cell cycle profile by flow cytometry

Leukemia WEHI-3B JCS cells (10^4 cells/ml) were first synchronized by culturing in plain RPMI medium for 24 h. After that, they were cultured in RPMI medium + 10% heat-inactivated (HI)-FBS with different concentrations of tryptanthrin at 37ºC for 48 h inside a humidified 5% CO_2 incubator. Then cells were harvested and washed with phosphate-buffered saline (PBS, Sigma Chem. Co.) by spinning at 430 ×g for 5 min. Next, cells were fixed with 1 ml 70% ethanol at 4ºC for 30 min. Afterwards, ethanol was removed and the cells were washed with PBS. One millilitre of freshly prepared propidium iodide (PI) DNA staining solution (50 μg/ml) was used to resuspend the cells. The cells were kept in dark at room temperature for 30 min. Stained cells were analyzed by flow cytometric analysis. By using the fluorescence-activated cell sorter (Becton Dickinson FACSCanto) equipped with an argon laser emitting at 488 nm and the CellQuest software, the fluorescence intensity of the stained cells can be measured. At least 10^4 cells were analyzed for each determination. The percentages of cells in G0/G1, S and G2/M cell cycle phases were calculated by the Modfit 3.0 program (Verity Software House).

Isolation of total cellular RNA

Leukemia WEHI-3B JCS cells (10^4 cells/ml) were cultured in RPMI + 10% HI-FBS with either solvent control (0.05% DMSO) or different concentrations of tryptanthrin at 37ºC for different time intervals inside a humidified 5% CO_2 incubator. Cells were then harvested by centrifugation at 430 ×g for 5 min at 4ºC. TRIZOL reagent (1 ml TRIZOL for 5 × 10^6 cells) was added accompanied by vigorous shaking in order to lyse the cells. The lysates were stood at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes. Followed by the addition of chloroform (200 μl per ml of TRIZOL reagent) and the robust shaking of the cells, the samples were allowed to stand at room temperature for 3 min without disruption. After centrifugation at 12,000 ×g for 15 min at 4ºC, the upper aqueous phase which contained RNA (-400 μl) was collected. Isopropanol (500 μl) was added and the samples were allowed to stand at room temperature for 10 min in order to precipitate the RNA. Next, centrifugation was carried out at 12,000 ×g at 4ºC for another 10 min, RNA pellet obtained was washed with 1 ml 75% ethanol, followed by centrifugation at 7,500 ×g at 4ºC for 5 min and the supernatant was removed and the RNA pellet was air-dried for about 5 min. Finally, the RNA was dissolved in 30 μl diethyl pyrocarbonate (DEPC)-treated water and the RNA content was calculated by measuring the absorbance at 260 nm using a spectrophotometer. Samples were then kept at -70ºC until use. The purity of the RNA was assessed by the ratio of A_{260}/A_{280}, and samples with a range between 1.0 to 2.0 would be considered as pure. The integrity was tested by running 1 μl of RNA sample with a total amount of 5 μg on 1% agarose gel which would then be stained with ethidium bromide.

Reverse transcription

The RNA sample was converted to its complementary DNA (cDNA) before it could be used for the PCR amplification of the target gene. Reaction mixture containing 40 units of $RN_{ASE}OUT^{TM}$ recombinant ribonuclease inhibitor, 1× M-MLV first strand buffer, 0.5 mM of each dNTP, 10 mM DTT, 0.1 μg oligo $(dT)_{12\text{-}18}$ and 200 units of M-MLV reverse transcriptase was prepared, and 2 μg RNA sample was added into 20 μl reaction mixture. A negative control using DEPC-treated water instead of RNA sample was used to check if there was any contamination. The thermocycler, GeneAmp PCR System 9700 (Perkin-Elmer Co.) was used for the reverse transcription. The mixtures were first incubated at 37ºC for 1 h, then at 99ºC for 5 min to inactivate the reverse transcriptase and to denature the template. Finally the samples were cooled down to 4ºC. The cDNA samples were then kept at -20ºC until use.

Real time polymerase chain reaction (RT-PCR)

Real time PCR was performed with 20 μl reaction mixture which contained an amount equivalent to 0.1 μg of cDNA, 2× Brilliant SYBR® Green Master Mix solution, 0.2 μM of both sense and antisense oligonucleotide primers and deionized water with the use of Applied Biosystems 7500 Fast Real Time PCR System. Cycling procedure consisted of initial denaturation for 5 min at 94ºC, followed by 20-40 thermal cycles with denaturation at 94ºC for 30 sec, annealing at 61ºC for 1 min to 1 min and 15 sec and elongation at 72ºC for 1 min. A final extension of 5 min at 72ºC was needed to complete the reaction. The annealing temperature and the number of cycles were varied and optimized for different primer pairs to allow the detection of the amplified products. The threshold cycle (C_T), which represents the PCR cycles at which an increase in reporter fluorescence above a baseline signal, was detected by 7000 SDS 1.1 RQ software. The relative gene expression was then analyzed by the $2^{-\triangle\triangle C}$T Method as described elsewhere (22).

The primers used in real-time PCR and the predicted sizes of the PCR products are listed as follows: GAPDH: sense strand TGA AGG TCG GAG TCA ACG GAT TTG GT; antisense CAT GTG GGC CAT GAG GTC CAC CAC (predicted size of PCR product: 226 bp); cyclin D2: sense strand AAG GAG AAG CTG TCC CTG ATC; antisense GAA CTG CTG CAG GCT GTT CAG (predicted size of PCR product: 273 bp); cyclin D3: sense strand CCG TGA TTG CGC ACG ACT TC; antisense TCT GTG GGA GTG CTG GTC TG (predicted size of PCR product: 397 bp); cdk2: sense strand CAC AGC CGT GGA TAT CTG GAG; antisense TTG CGA TAA CAA GCT CCG TC (predicted size of PCR product: 253 bp); cdk4: sense strand ACG CCT GTG GTG GTT ACG CT; antisense CCA TCT CTG GCA

CCA CTG AC (predicted size of PCR product: 280 bp); cdk6: sense strand ATG GAG AAG GAC AGC CTG AGT CGC; antisense AGT CTT GAT CAA CAT GCT CAA ACA (predicted size of PCR product: 313 bp).

Morphological detection of cell differentiation

The effect of tryptanthrin on the differentiation of WEHI-3B JCS cells was assessed by preparing cytospin smear. WEHI-3B JCS cells (10^4 cells/ml) were cultured in RPMI + 10% HI-FBS with either solvent control (0.05% DMSO) or different concentrations of tryptanthrin at 37ºC for 48 h inside a humidified 5% CO_2 incubator. Treated cells (5×10^4) were fixed onto a microscopic glass slide by cyto-centrifugation at 500 rpm for 5 min using the Shandon Cytospin 3 centrifuge (Shadon Scientific Ltd., U.K.). The cells were air-dried and subsequently stained with Hemacolor staining solutions for 15 sec and destained with deionized water. Finally, the slides were mounted with neutral mounting medium, *Canada Balsam* (Sigma Chem. Co.) and the cell morphology was examined under the light microscope. The stained cells were further scored as the immature blast cells, intermediate stage cells or mature macrophage-like cells as described previously (23).

Detection of differentiated cells by nitroblue tetrazolium reduction assay

Leukemia WEHI-3B JCS cells (10^4 cells/ml) were either incubated with solvent control (0.05% DMSO) or tryptanthrin for 48 h. Then, 100 µl nitroblue tetrazolium - phorbol 12-myristate 13-acetate (NBT-PMA) mix was added and incubated at 37ºC in dark for 4 h. The cells were then harvested and washed twice with cold plain RPMI with vigorous shaking. After that, the cells were resuspended in RPMI + 10% HI-FBS. Treated cells (5×10^4) were cyto-centrifuged onto microscopic glass slides and stained with safranin for 30 sec and washed with running water. The differentiated cells, which had intracellular black-blue formazan deposit, were examined by counting at least 200 cells in triplicate for each determination.

Statistical analysis

The data were expressed as arithmetic mean ± standard error (S.E.). The Student's *t*-test was used for statistical analysis. Normally, $p < 0.05$ was regarded as significantly different. The results shown were representative of at least two to three independent experiments.

Results

Anti-proliferative effect of tryptanthrin on the murine myelomonocytic leukemia WEHI-3B JCS cells

The suppressive effect of tryptanthrin on the proliferation of the murine myeloid leukemia WEHI-3B JCS cells was studied using the tritiated thymidine incorporation assay. WEHI-3B JCS cells were incubated at 37ºC with different concentrations of tryptanthrin for 48 h and cell proliferation was then measured. As shown in Figure 2A, tryptanthrin inhibited the proliferation of the WEHI-3B cells in a dose-dependent manner, with an estimated 50% inhibitory concentration (IC_{50}) of 1.5 µM at 48 h of treatment. Similarly, the growth-inhibitory activity of tryptanthrin on WEHI-3B JCS cells was also examined using the trypan blue exclusion assay. Figure 2B shows that tryptanthrin inhibited the *in vitro* growth of WEHI-3B JCS cells very effectively at all the concentrations tested (1-5 µM). Significant suppression of WEHI-3B JCS cell growth was detected at 48 and 72 h of treatment with tryptanthrin and complete suppression of WEHI-3B JCS cell growth was observed when the concentration of tryptanthrin was ≥ 2.5 µM. Moreover, our results show that tryptanthrin has no direct cytotoxicity on WEHI-3B JCS cells at the concentration which inhibits 50% of the JCS cell proliferation, as measured by the trypan blue exclusion test (data not shown). Similarly, tryptanthrin was

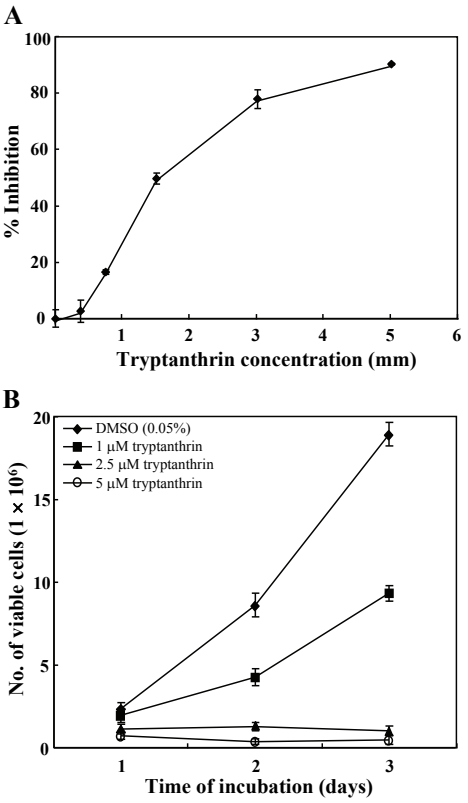

Figure 2. Anti-proliferative effect of tryptanthrin on the murine myelomonocytic leukemia WEHI-3B JCS cells. The myeloid leukemia WEHI-3B JCS cells (10^4 cells/ml) were incubated with solvent control (0.05%DMSO) or different concentrations (0-5 µM) of tryptanthrin at 37ºC. (A) After 48 h of incubation, cultures were pulsed with 0.5 µCi of ^{3}H-TdR for 6 h before harvest. Results were expressed as % inhibition of ^{3}H-TdR incorporation with respect to the solvent-treated cells. Each point represents the mean ± S.E. of quadruplicate cultures. (B) After incubation for various time period (24 h, 48 h and 72 h), the number of viable cells was counted using Vi-cellTM XR cell viability analyzer (Beckman Coulter). Results were expressed as the number of viable cells ± S.E. of triplicate cultures.

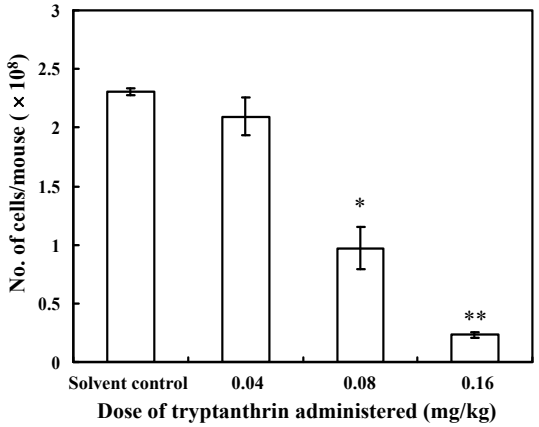

Figure 3. Effect of tryptanthrin on the growth of leukemia WEHI-3B JCS cells in syngeneic BALB/c mice *in vivo*. WEHI-3B JCS cells (10^6 cells /mouse) in RPMI were injected i.p. into BALB/c mice at day 0. At day 6, tumor-bearing mice were divided into 4 groups with 5 mice in each group. They were injected i.p. either with solvent control or with tryptanthrin at three different dosages (0.04 mg/kg, 0.08 mg/kg and 0.16 mg/kg body weight) for 5 consecutive days. At day 12 post-tumor cell inoculation, viable WEHI-3B JCS cells recovered from the peritoneal cavity were counted using Vi-cellTM XR cell viability analyzer. Results were expressed as number of viable cells per mouse. Each bar represents the mean ± S.E. for groups of five mice. (*$p < 0.05$, **$p < 0.005$, significant different from solvent control).

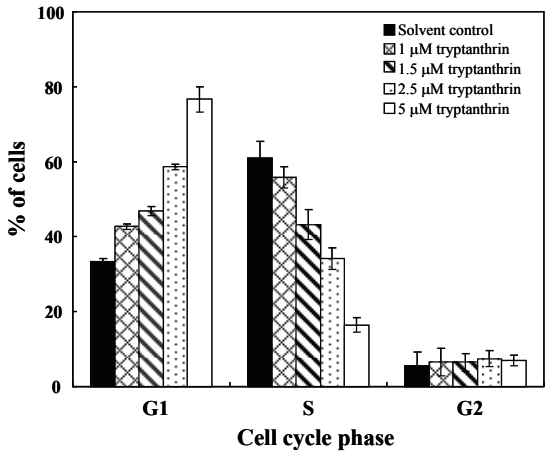

Figure 4. The effect of tryptanthrin on the cell cycle profile of leukemia WEHI-3B JCS cells. WEHI-3B JCS cells (10^4 cells/ml) were either incubated with solvent control (0.05% DMSO) or tryptanthrin at various concentrations for 48 h. Treated cells (10^6 cells) were fixed with ethanol and stained with propidium iodide under hypotonic condition. The fluorescence intensity of the stained cells was measured by using the FACSCanto flow cytometer. Cell cycle distribution was analyzed by using RFIT analysis model of MODFIT program.

found to exhibit little, if any, cytotoxicity on the normal murine peritoneal macrophages at or below 7.5 μM concentrations, as measured by the methythiazoletetrazolium (MTT) cell viability assay (data not shown).

Effects of tryptanthrin on the growth of the murine myelomonocytic leukemia WEHI-3B JCS cells in vivo

Since tryptanthrin has demonstrated potent anti-proliferative effect on the leukemia WEHI-3B JCS cells *in vitro,* it is equally important to show its growth-inhibitory effect on the WEHI-3B JCS cells *in vivo*. In the present study, WEHI-3B JCS cells (10^6 cells/mouse) were first injected *i.p.* into syngeneic BALB/c mice and tryptanthrin at three different dosages was administered *i.p.* 5 days later for 5 consecutive days. At day 12 post-tumor cell inoculation, viable WEHI-3B JCS cells recovered from the peritoneal cavity were counted. As shown in Figure 3, tryptanthrin administered *in vivo* was able to suppress the growth of WEHI-3B JCS cells in BALB/c mice and significant suppression was seen at the dosages of 0.08 mg/kg and 0.16 mg/kg.

Effect of tryptanthrin on the cell cycle profile of murine myelomonocytic leukemia WEHI-3B JCS cells in vitro

One of the possible mechanisms of tryptanthrin leading to its potent anti-proliferative effect is its ability to interrupt with the normal cell cycle progression. To address this issue, WEHI-3B JCS cells were first treated with tryptanthrin for 48 h, and then stained with propidium iodide followed by flow cytometric analysis for the cell cycle profile. As shown in Figure 4, there was a very obvious increase in proportion of cells in G0/G1 phase of the cell cycle, accompanied by a significant decrease in the percentage of cells in S phase. There were little, if any, changes in the percentage of cells in the G2/M phase after 48 h of treatment.

Effects of tryptanthrin on the expression of cell cycle related genes in murine myelomonocytic leukemia WEHI-3B JCS cells in vitro

Since it was shown that tryptanthrin arrested the WEHI-3B JCS cells at G0/G1 phase, the gene expressions of cyclins and Cdk which were involved in the transition of G0/G1 to S phase were examined. They included *cyclin D2*, *D3* and *Cdk 2*, *4*, *6* genes. Using the technique of real time polymerase chain reaction, it was shown that the mRNA level of *Cdk 2*, *4*, *6* decreased in a time-dependent manner after treatment with 2.5 μM of tryptanthrin, whereas optimal suppression of *cyclin D2* and *D3* gene expression was seen as early as 4 h after treatment with tryptanthrin (Figure 5).

Morphological studies on tryptanthrin-treated murine myelomonocytic leukemia WEHI-3B JCS cells

The morphology of WEHI-3B JCS cells was examined after treatment with different concentrations of tryptanthrin for 48 h. The leukemia WEHI-3B JCS cells were then centrifuged onto microscope slides and stained with Hemacolor solution. As shown in Figure 6, tryptanthrin induced morphological changes in the WEHI-3B JCS cells in a dose-dependent manner. The higher the concentration used, the more obvious the changes were. It was found that the cytoplasm versus

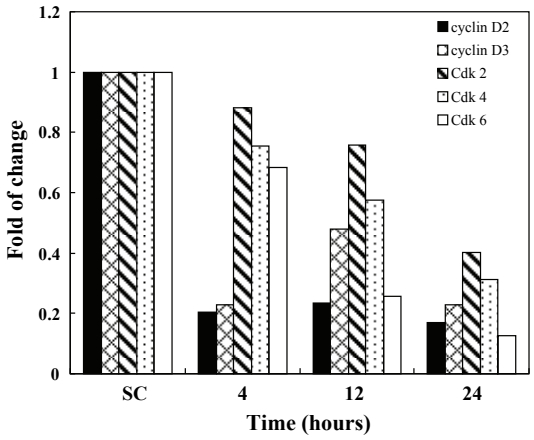

Figure 5. Effect of tryptanthrin on the mRNA expression of cell cycle related genes in leukemia WEHI-3B JCS cells. WEHI-3B JCS cells (10^4 cells /ml) were either incubated with solvent control (0.025% DMSO), or 2.5 μM tryptanthrin at 37ºC for 4, 12 and 24 h. Total mRNA was then isolated by the TRIZOL method, followed by reverse transcription to produce cDNA. Brilliant SYBR® Green QPCR analysis for *cyclin D2*, *D3*, *Cdk 2*, *4*, and *6* were performed according to the manufacturer's instruction. The data were analyzed by the $2^{-\triangle\triangle C}{}_T$ method as described elsewhere (22).

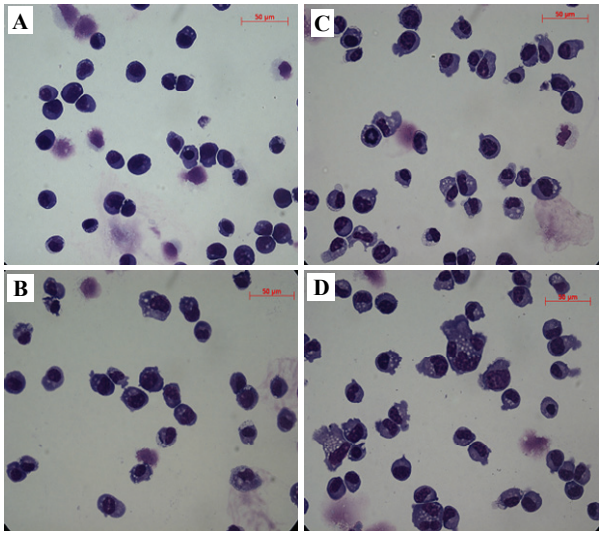

Figure 6. Morphology of the tryptanthrin-treated leukemia WEHI-3B JCS cells. WEHI-3B JCS cells (10^4 cells/ml) were either incubated with (A) solvent control (0.05% DMSO) or with (B) 1 μM (C) 2.5 μM and (D) 5 μM of tryptanthrin for 48 h. Treated cells (5×10^4 cells) were cytocentrifuged onto microscopic glass slides and stained with Hemacolor solutions. (Magnification: 400×; scale bars shown represent 50 μm).

nucleus ratio became larger and vacuolation was observed in the tryptanthrin-treated WEHI-3B JCS cells. These results suggest that tryptanthrin can induce morphological differentiation of the leukemia WEHI-3B JCS cells. The stained cells were further scored as the immature blast cells, intermediate stage cells or mature macrophage-like cells as described previously (23). It can be seen that the percentage of differentiated cells (which include the intermediate stage cells and mature macrophage-like cells) was significantly higher in the tryptanthrin-treated groups (8.97% - 19.2%) when compared to the solvent-treated group (2.64%) (Table 1).

Effect of tryptanthrin on the induction of NBT-reducing activity in the murine myelomonocytic leukemia WEHI-3B JCS cells

The ability of tryptanthrin to induce differentiation of WEHI-3B JCS cells with functional characteristics of normal macrophages was also examined. In this study, WEHI-3B JCS cells were treated with tryptanthrin for 48 h. NBT-PMA solution was added and the mixture was incubated at 37ºC in dark for 4 h. Treated cells were then cytocentrifuged onto microscopic glass slides and then stained with safranin. As shown in Table 2, treatment of WEHI-3B JCS cells with 2.5 μM tryptanthrin significantly increased the percentage of cells with NBT-reducing activity from 0% in the solvent control to 15.3% in the tryptanthrin-treated group, indicating that tryptanthrin can induce WEHI-3B JCS cells to undergo functional differentiation.

Discussion

Uncoupling of the proliferation and differentiation processes resulting in the formation of undifferentiated or poorly-

Table 1. Morphological identification of tryptanthrin-treated WEHI-3B JCS cells at *v*arious stages of differentiation

Treatment of cells with	Immature blast cells	Percentage of different cell types Intermediate stage cells	Macrophage-like cells
Solvent control	97.4 ± 0.69	2.64 ± 0.69	0
1 μM tryptanthrin	78.3 ± 4.4	7.9 ± 3.2	1.07 ± 0.6
2.5 μM tryptanthrin	71.8 ± 6.1	9.6 ± 3.4	3.5 ± 1.8
5 μM tryptanthrin	64.0 ± 0.6	11.9 ± 1.5	7.3 ± 1.9

The leukemia WEHI-3B JCS cells (10^4 cells/ml) were treated with either solvent control (0.05% DMSO) or various concentrations of tryptanthrin. The morphology of the cells was identified by staining the cells with Hemacolor staining solution set. The stained cells were scored as the immature blast cells (myeloblasts), intermediate stage cells (myelocytes and promonocyte-like cells) or macrophage-like cells as described previously (23). At least 200 cells were counted for each determination and expressed in percentage of different cell types.

Table 2. Induction of NBT-reducing activity in the murine myelomonocytic leukemia WEHI-3B JCS cells by tryptanthrin

Treatment of cells with	Percentage of cells which reduced NBT
Solvent control	0%
1 μM tryptanthrin	3.2 ± 0.6%
2.5 μM tryptanthrin	15.3 ± 1.5%

The leukemia WEHI-3B JCS cells (10^4 cells/ml) were either incubated with (A) solvent control (0.05% DMSO), or (B) 1 μM and (C) 2.5 μM of tryptanthrin at 37ºC for 48 h. Then 100 μl NBT-PMA solution was added and incubated at 37ºC in dark for another 4 h. Treated cells (5×10^4) were cytocentrifuged onto micro- scopic glass slides and stained with safranin for 30 seconds and washed with running water. The differentiated cells, which had intracellular black-blue formazan deposit, were examined by counting at least 200 cells in triplicate for each determination.

differentiated non-functional blood cells is commonly seen in leukemia. One possible approach of targeting against leukemia is to specifically suppress this out-of-control proliferation process without causing much toxicity of the normal cells. Phytochemicals are a major source of anti-cancer drugs for centuries and that many phytochemicals possess anti-cancer properties such as induction of tumor cell apoptosis and differentiation, and they represent a promising approach for the prevention and treatment of many cancers (24, 25). Tryptanthrin is a phytochemical found in the medicinal indigo plants such as *Polygonum tinctorium* and *Isatis tinctoria.* It was found to possess a wide spectrum of biological and pharmacological activities, including anti-microbial (7-9), immunomodulatory (11-13), anti-inflammatory (13-15) and anti-tumor (16-19) activities. A previous report has shown that tryptanthrin is able to induce both apoptosis and differentiation in the human leukemia HL-60 cells and U-937 cells *in vitro* (16), however, the underlying action mechanisms remain unclear. In this study, the anti-proliferative and differentiation-inducing activities of tryptanthrin on the murine myelomonocytic leukemia WEHI-3B JCS cells were investigated and the possible anti-tumor mechanisms were elucidated. The WEHI-3B JCS cell line has a short doubling time of around 16 h, and it is also well characterized and subcloned by our group (20, 21). In addition, another advantage of using this cell line is that it can be easily transplanted into syngeneic BALB/c mice without causing any rejection from the mice and is therefore a good model for studying the anti-tumor activity *in vivo.*

Our results clearly showed that tryptanthrin exhibited potent anti-proliferative activity on the murine myelomonocytic leukemia WEHI-3B JCS cells in a dose- and time-dependent manner, with an estimated 50% inhibitory concentration (IC_{50}) value of 1.5 μM at 48 h of treatment. Similar anti-proliferative effect was also demonstrated on other human and murine myeloid leukemia cell lines including HL-60, K-562, EoL-1 and M1 cells (data not shown), indicating the anti-proliferative effect of tryptanthrin on myeloid leukemia is not species- or cell-line-specific. Interestingly, tryptanthrin was found to exhibit little, if any, cytotoxicity on the normal murine peritoneal macrophages at concentrations that are inhibitory to the leukemia WEHI-3B JCS cells. Since tryptanthrin was effective in suppressing the growth of the leukemia WEHI-3B JCS cells *in vitro*, its effect on the *in vivo* growth of the leukemia WEHI-3B JCS cells in the syngeneic BALB/c mice was also examined. It was found that *in vivo* administration of tryptanthrin also inhibited the growth of leukemia WEHI-3B JCS cells in the tumor-bearing mice. These data suggest that tryptanthrin can exert its growth-inhibitory effect on the myeloid leukemia WEHI-3B JCS cells, both *in vitro* and *in vivo*. Nevertheless, the bio-distribution of tryptanthrin in mice should be carried out in order to study its toxicity and the chronic effect of the treatment so as to explore its potential for the treatment against leukemia.

Moreover, tryptanthrin induced cell cycle arrest of the WEHI-3B JCS cells at G0/G1 phase in a dose-dependent manner. Using the technique of real time PCR, the expression of the cell cycle-related genes including *cyclin D2*, *D3*, *Cdk 2*, *4*, *6* were found to be significantly down-regulated, which might affect the formation of cyclin-Cdk complexes and therefore prevent the cell cycle progression from G1 phase into the S-phase, causing cell cycle arrest and resulting in inhibition of leukemic cell growth. On the other hand, indirubin and its derivatives, which are indigoid alkaloids found in the medicinal indigo plants, have been reported to exert potent growth-inhibitory effect on tumor cells through inhibiting the Cdk activities by direct interaction with Cdk's ATP binding site (26, 27). It will be interesting to see if this mechanism would be involved in the case of tryptanthrin, a quinazolinone alkaloid also derived from Ban-Lan-Gen.

Apart from the cell cycle arrest, attempts had been made in studying the ability of tryptanthrin in inducing differentiation in the WEHI-3B JCS cells. Morphologically, an increase in the cytoplasm to nucleus ratio and increased vacuolation were observed after tryptanthrin treatment which showed that tryptanthrin was able to induce differentiation in the WEHI-3B JCS cells. The ability of the tryptanthrin-treated cells to reduce NBT also indicated that tryptanthrin was able to induce functional differentiation in the WEHI-3B JCS cells. Whether tryptanthrin can induce terminal differentiation or functional maturation of the WEHI-3B JCS cells along the monocytic or granulocytic pathway is an intriguing aspect that awaits future investigations.

Collectively, our findings indicate that tryptanthrin might exert its anti-proliferative effect on the murine myelomonocytic leukemia WEHI-3B JCS cells by causing cell cycle arrest and by inducing differentiation of the cells. In the future, investigations should be focused on delineating the molecular events and signaling pathways underlying the anti-leukemia activities of tryptanthrin.

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