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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2017.01.001

Inhibition of Mahkota Dewa (*Phaleria macrocarpa*) bioactive fraction on proliferation of human retinoblastoma tumor cells Y-79 through suppression of mRNA level of cyclin E

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ARTICLE INFO

Article history:
Received 29 Jul 2016
Received in revised form 8 Sep, 2nd revised form 20 Sep 2016
Accepted 10 Nov 2016
Available online 5 Jan 2017

Keywords:
Phaleria macrocarpa
Mahkota Dewa
DLBS1425
Retinoblastoma
Cyclin E
Cell cycle

ABSTRACT

Objective: To prove the molecular mechanisms of Mahkota Dewa (*Phaleria macrocarpa*) in suppressing proliferation of human retinoblastoma cells through suppression of cell cycle's gene-regulators expression.

Methods: In this study, the molecular mechanism of anti-tumor effect of fractioned extract of *Phaleria macrocarpa* (DLBS1425) in human retinoblastoma cells Y-79 was investigated by measuring the tumor cells viability, the assessment of population profiles of tumor cells in the cell cycle, and the mRNA concentration of p16, p21, p53, cyclin D, cyclin E, and E2F.

Results: DLBS1425 showed an inhibition effects towards proliferation of Y-79 cell line. Inhibition of proliferation was shown by suppression of cell cycle progression. DLBS1425 downregulated cyclin E, a G1 phase regulator gene of cell cycle, in dose-dependent manner without affecting p53–p21 pathway. In the other word, DLBS1425 inhibits cell proliferation through suppression of cyclin E independently towards conventional proliferation pathway.

Conclusions: Our results suggest that DLBS1425 is a potential anticancer agent which targets genes involved in cell proliferation in human retinoblastoma cells which make it pharmacologically ideal for the prevention and/or treatment of retinoblastoma cancer.

1. Introduction

Retinoblastoma is a malignant tumor, which develops on young children, and it is a primitive neuroectodermal tumor. According to Knudson 1971, retinoblastoma is initiated by allelic inactivation of putative tumor suppressor gene. Genetic factor takes parts in allele inheritance which is prone to genetic mutation of retinoblastoma tumor suppressor gene. Retinoblastoma incidences vary a lot among many parts in the world with the higher incidences to babies and children. Retinoblastoma incidences in the world are between 1:16000–1:18000 live birth. Retinoblastoma incidences per 1 million children aged 0–4 years in Asian continent are 6.3–19.6 and in African continent is 10.6–42.5. On the other hand, the incidences in North America are 6.3–22.5 and in European continent are 3.4–12. The highest retinoblastoma mortality rate is in African continent (70%) and the lowest is in North America and Japan (3% each) [1–5].

Most of retinoblastoma treatment is still on therapeutic aspect because early retinoblastoma detection is difficult to do. Generally, retinoblastoma therapy modality is given in combination to reach maximum effect in eradicating tumor. However, the rate of secondary tumor development along chemotherapy and a relapse after chemotherapy is quite high. Accumulation of new tumor incidences after chemotherapy to patients who get medication of two or three standard regimen (6–8 chemotherapy cycle) is 10%–24%

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Foundation Project: Supported by The Dexa Laboratories of Biomolecular Sciences (DLBS), PT. Dexa Medica, Cikarang, West Java, Indonesia, Grant No. 135/MP/DLBS/2016.
Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

[6,7]. The recent standard of retinoblastoma therapy, that are carboplatin, etoposide, and vincristine, shows a quite satisfying therapy result to early disease (> 90%), but on the advanced stage of the disease the therapy result is still not satisfying (< 50%). Some chemotherapy effects like alopecia, allergy, pain, constipation, trouble hearing, and acute myelogenous leukemia, are still frequently repeated [1,7–9]. Therefore, nowadays many efforts are done to improve therapy outcomes and to minimize the toxic effects. One of the efforts is using natural plant substances (herbals) as an anticancer medicine for the main therapy or the adjuvant therapy.

Phaleria macrocarpa (*P. macrocarpa*), an original Indonesian plant which is more popular as Mahkota Dewa, is a plant which has been studied repeatedly and known as a plant with anticancer benefit. The DLBS1425, fractionated extract of fruit flesh (endocarp) of *P. macrocarpa*, has strong anticancer activity which can suppress proliferation, migration, and invasive potential on human breast cancer cell (MBA-MB-231) which depends on the dose level through track suppression mechanism of signaling phosphoinositide (phosphatidyl-inositol)-3-kinase (P13-K/AKT), a protein kinase B, with suppressing transcription level of P13-K and AKT phosphorylation. Expression of pro-apoptotic genes, such as BAX, BAD, and PUMA, are also induced by DLBS1425, and finally will induce cellular death signal through caspase-9 activation, PARP [poly (ADP-ribose) polymerase] breaking, and DNA fragmentation [10,11]. Besides, DLBS1425 also regulates BAX and Bel-2 on mRNA level in inducting apoptosis. Antiproliferative effect and DLBS1425 pro-apoptotic effect happens through eicosanoid inhibition track with reducing the expressive regulation of COX-2, cPLA2, VEGF-C mRNA, c-fos, HER2/Neu mRNA to human breast cancer cell induced by TPA (12-O-Tetradecanoylphorbol-13-acetate) or fatty acid [11]. Some of the findings show that DLBS1425 has antiproliferative, anti-inflammatory, and anti-angiogenic activity which has pharmacologic potential as anticancer.

DLBS1425 anticancer effect has been proven for breast cancer cell but has never been studied for retinoblastoma cancer cell. Although there are some differences to some parts of the pathogenesis chain, as in the genes which experience mutation (BRCA1/BRCA2 on breast cancer, and Rb1 on retinoblastoma), generally the pathogenesis are similar, so it is hoped that DLBS1425 has the same anticancer effect to retinoblastoma. DLBS1425 effect to retinoblastoma cell cycle regulation, that is the main machine of cell proliferation, has not been studied before. Cell cycle regulation by the regulator needs to examine because cell cycle regulation determines whether cell proliferation works normally or not. The research will prove the effect of DLBS1425 on proliferation and proliferation regulation to retinoblastoma tumor cell cycle *in vitro*.

2. Materials and methods

2.1. Chemicals

2.1.1. Tumor cell culture

The human retinoblastoma cells were cultured in media that consist of RPMI 1640 (Gibco, 30-2001), fetal bovine serum (Gibco, 26140-079), penicillin-streptomycin solution (Gibco, 15140-122). Trypsin-ethylenediaminetetraacetic acid (Gibco, 25300-062) was needed in thawing of frozen cells.

2.1.2. Tumor cell viability count (hemocytometer)

The tumor cells were stained with Trypan blue solution (Invitrogen) to indentify the dead cells in cell viability assay using hemocytometer procedure. Ethanol solution 70% (MDK) was needed for washing the Neubauer improved cell counter glass.

2.1.3. Fluorescence activated cell sorter (FACS)

Phosphate buffer saline solution (PBS) (Merck) was used for washing and dissolving the tumor cells that have been fixed in molecular grade 100% ethanol (Sigma, E7023). Solution of propidium iodide (Calbiochem, 537059) was added into tumor cells solution for flow cytometric analysis by using FACS.

2.1.4. Real time PCR

Real time PCR assay needed some chemicals in the preparation stages. In homogenization stage, tumor cell suspension required TRIzol® reagent (Invitrogen, 15596-018), and chloroform (MP Biomedical, 194002) to get tumor suspension aqueous phase containing RNA. The 100% isopropanol was then added into the aqueous phase to obtain RNA pellet. RNA pellet was washed with 75% ethanol, and after washing, the RNA pellet was dissolved in nuclease free water (Promega, 821 932). Furthermore, the RNA solution was ready to be analyzed for electrophorese gel PCR and real time PCR assays.

2.1.5. Electrophorese gel PCR and real time PCR assays

The chemical reagents such as MMX (Master Mixed) PCR (Promega), primer forward (F) (IDT), primer reverse (R) (IDT), BAM 289 forward (F) (IDT), BAM 289 reverse (R) (IDT), actin 138 forward (F) (IDT), actin 138 reverse (R) (IDT), Eva green (Biorad, 172-5201AP) were required for manufacture of PCR master mix.

2.2. Preparation of extract and fractionation

The raw material of *P. macrocarpa* was attained from farmers in Salatiga, Central Java. Plant identification was performed by Herbarium Bogoriense, Pusat Riset Biologist, Institut Sains Indonesia. Dry fruit of *P. macrocarpa* was minced and macerated with methanol, filtered, and dried with rotapavor in Dexa Laboratories of Biomolecular Sciences (DLBS) Cikarang, West Java. Liquid–liquid extraction was conducted with ethyl acetate and water to get organic phase. Then, the organic phase was dried with rotapavor. The extract was named DLBS1425 and was used for the next experiment. Fractionation process was monitored with thin layer chromatography analysis. The used concentration in the experiment based on the dry weight of the extract (μg/mL).

2.3. Cell culture

Y-79 cell culture (human retinoblastoma cell line) used in *in vitro* model was for retina and the development of retinal tumor. Y-79 was also used in the experiment of proliferation regulation and cell differentiation and the role of type 1 pituitary adenylyl cyclase-activating polypeptide receptor in cell signaling. Y-79 cell was derived from ATCC with product code ATCC® HTB-18™.

2.4. Cell viability assay

The number of viable tumor cells was counted by using cell count method with Neubauer improved booth count and trypan blue staining. Cytoplasm of viable tumor cells looked bright and transparent, but cytoplasm of dead tumor cells looked livid stained by trypan blue.

The viable tumor cells were counted in five big boxes (four big boxes at the corner, one box in the middle). The counting was conducted in duplo from the top and bottom booth count. The average number of living tumor cells was gained by dividing by two of the duplo count result. The above steps were conducted for each treatment group.

2.5. Flow cytometric analysis

After DLBS1425 treatment in many dose levels (0.0 μg/mL, 0.5 μg/mL, 5.0 μg/mL, 50.0 μg/mL) to tumor cells for 24 h, cells were washed with PBS and centrifugation at 3000 r/min for 5 min was conducted until pellet formed. After fixation with ethanol, cells were resuspended with PBS and colored with propidium iodide for 30 min in room temperature. Cell distribution in cell cycle was determined with FACS Calibur machine (Benton Dickinson, San Jose, CA).

2.6. RNA isolation and reverse transcription-PCR

Total RNA was extracted with TRIzol® reagent in accordance with extraction procedure as described in the reagent instructions leaflet. After incubation for 10 min at room temperature, the suspension was centrifuged at 12000 × *g* for 10 min at 4 °C until RNA pellet formed a gel in the bottom of the tube wall. DNA pellet was washed with 75% ethanol and then underwent gentle vortex until the pellet detached from the tube wall and then centrifuged at 7500 × *g* at 4 °C for 5 min.

After the pellets were dried perfectly, RNA was resuspended by adding nuclease free water until homogeneous. Then the tube was incubated in a temperature of 55–60 °C for 10–15 min and the solution was ready for further RNA processing. RNA concentration was measured with a spectrophotometer (NanoDrop) at 260 and 280 nm.

For PCR reverse transcription checking, RNA mixture solution was made by adding nuclease free water, oligo (dT), buffer, Go Script, RNAsin, PCR nucleotide, and MgCl. Mixture RNA was then inserted into the machine RT-PCR with GO program (25 °C: 5 min, 42 °C: 1 h, 70 °C: 15 min, 4 °C: infinite) to form a cDNA sample. PCR was performed for specific primers p16, p21, p53, cyclin D, cyclin E, and E2F according to Table 1. The PCR products were analyzed by semiquantitative real time PCR chemidoc (BioRad, Hercules, CA).

3. Results

3.1. Counting the number of Y-79 human retinoblastoma tumor cells

The number of Y-79 human retinoblastoma tumor cells was decreased dose-dependently after 24 h of DLBS1425 exposure. The tumor cell viability was the lowest in the group DLBS1425 dose of 50.0 μg/mL (D50), while the highest was in the group of DLBS1425 dose 0.0 μg/mL [(C) Control]. The number of viable tumor cells in D50 was 47.11% of the number of tumor cells in C. One-way ANOVA test showed differences in the number of viable tumor cells after DLBS1425 exposure in various dose levels (P = 0.020). Differences between the mean number of viable tumor cells were tested with least significant difference *post hoc* test which showed significant differences between group C with group D5 and D50 group and the group D0.5 with group D50. The mean number of viable tumor cells of group C was higher than the D5 group (P = 0.039) and D50 group (P = 0.007), whereas the mean number of viable tumor cells in group D0.5 was higher than the D50 group (P = 0.011). This shows that DLBS1425 lowered the number of Y-79 human retinoblastoma tumor cells corresponding to dosage levels.

3.2. Population patterns of tumor cells in cell cycle phase

Population patterns of tumor cells based on the number of tumor cells in each phase of the cell cycle, measured using FACS generally indicated that the pattern of the number of cells of each phase at each dose level was almost the same as presented in Figures 1 and 2. Furthermore, the pattern of the number of cells in each phase of the cell cycle would be described sequentially starting from G0 phase, G1 phase, phase S, and G2/M phase. The tumor cell populations increased in accordance to dosage level of DLBS1425 in G0 phase and decreased in G1 phase but did not show significant differences of tumor cell population pattern.

3.3. Concentration of RNA genes regulator of retinoblastoma cell cycle

The expressions of mRNA of several genes regulator of retinoblastoma cell cycle such as p16, p21, p53, cyclin D, cyclin E, and E2F, were measured using the real time PCR. The unit used was the relative concentration of mRNA compared with group control which is worth 1. The results of measurements of the relative concentration of mRNA gene p16, p21, p53, cyclin D, and E2F showed no significant difference between the various DLBS1425 dose levels. While the relative concentrations of gene cyclin E showed the significant

Table 1
Primers used in research.

Primer	Origin	Forward	Reverse	Size (bp)	Brand
p16 (CDKN2A)	–	5′-GCA CCA GAG GCA GTA ACC AT-3′	5′-TGG TTC TGC CAT TTG CTA GC-3′	501	IDT
p21 (CDKN1A)	Homo sapiens	5′-TTA GCA GCG GAA CAA GGA GT-3′	5′-ATT CAG GCA TTG TGG GAG GGA G-3′	337	Invitrogen
p53	–	5′-TCT AGG TAG GGG CCC ACT TC-3′	5′-CAA GAT CAC GCC ACT CCA CT-3′	530	IDT
Cyclin D (CCND1)	–	5′-TGT GAT GCT GGG CAC TTC AT-3′	5′-GAC AGA CAA AGC GTC CCT CA-3′	549	IDT
Cyclin E (CCNE1)	–	5′-GTG GCG TTT AAG TCC CCT GA-3′	5′-AAG GCC GAA GCA GCA AGT AT-3′	198	IDT
E2F	–	5′-GAC CTG TTC TTC TGC CCC AT-3′	5′-GCG AGG AAG CTG ACC TTT CT 3′	528	IDT

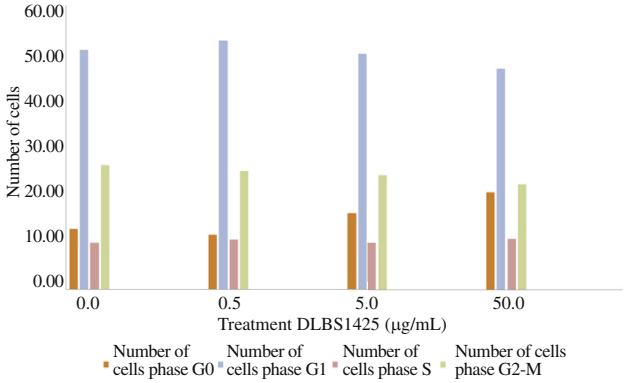

Figure 1. Mean (% total) number of Y-79 tumor cell population in the cell cycle in accordance to DLBS1425 dose level.

Relationships between the various variables were analyzed with multivariate analysis multiple regression test which showed a correlation of various variables and the Y-79 human retinoblastoma tumor cell viability as well as the level of mRNA concentrations of various cell cycle regulatory genes. DLBS1425 dose levels strongly correlated with the number of viable Y-79 human retinoblastoma tumor cells. The higher DLBS1425 dose level, the lower the number of viable Y-79 retinoblastoma tumor cells (P = 0.000 1, Pearson r = −0.898). DLBS1425 dose levels also correlated very strongly with cyclin E mRNA concentrations (P = 0.000 1, Pearson r = −0.851) and cyclin E mRNA concentration correlated very strongly with the number of viable Y-79 tumor cells (P = 0.001, Pearson r = 0.777).

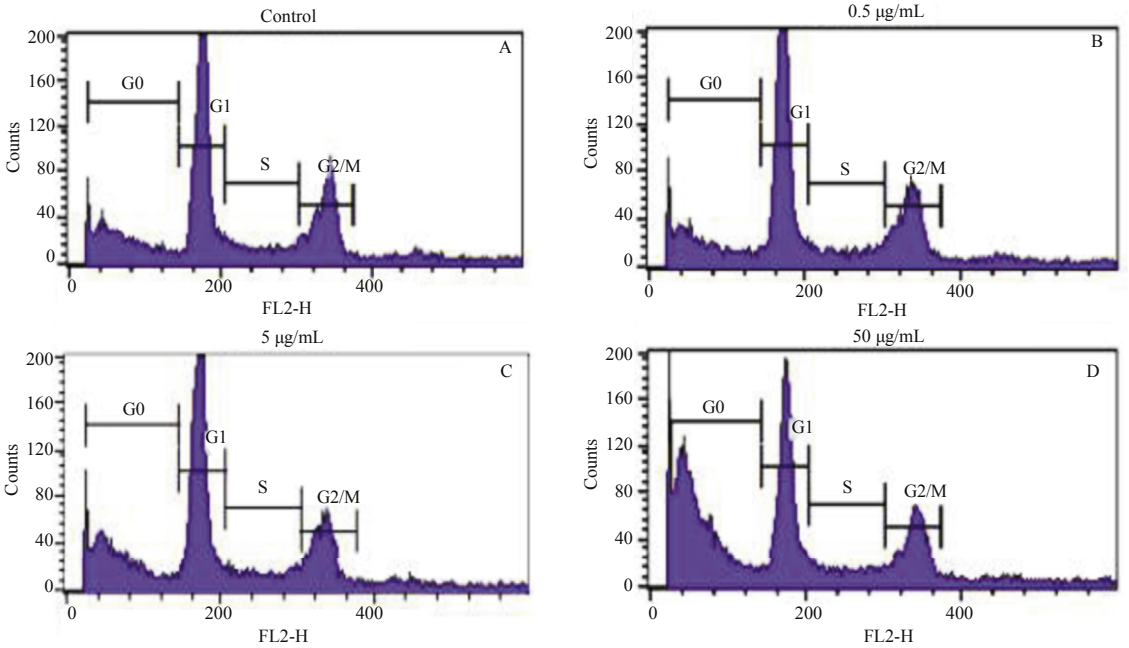

Figure 2. Analysis of the Y-79 cell cycle after DLBS1425 exposure in various dose levels.
A: DLBS1425 at the dose of 0 μg/mL; B: DLBS1425 at the dose of 0.5 μg/mL; C: DLBS1425 at the dose of 5 μg/mL; D: DLBS1425 at the dose of 50 μg/mL. Proportion of cells each phase of the cell cycle was determined by propidium iodide staining samples using flow cytometric analysis. The proportion of G0 phase of the cell cycle of cells increased as the dose level DLBS1425 showed that the rate of apoptosis increased as DLBS1425 dose escalation.

difference (a decrease) corresponding to DLBS1425 dose levels (P = 0.022), *i.e.* between group C with group D5 (P = 0.015) and group D50 (P = 0.005).

3.4. Differences between DLBS1425 dose rates compared with variable control and inter-variable relations

The bivariate analysis using unpaired t-test (independent t-test), which examined differences in a population of cells in each phase of the cell cycle between DLBS1425 dose level compared to controls showed no significant differences either in phase G0, G1 phase, S phase, and G2 phase. Meanwhile, in the bivariate analysis, the result of unpaired t-test that tested different concentrations of mRNA gene regulator of cell cycle showed no significant differences between the concentration of mRNA controls and the concentration of mRNA cyclin D cell tumor dose of 50 μg/mL (P = 0.029) and the concentration of mRNA cyclin E cell tumor dose of 0.5 μg/mL (P = 0.013), a dose of 5 μg/mL (P = 0.043), and a dose of 50 μg/mL (P = 0.003).

DLBS1425 dose level also had a significant strong negative correlation to the concentration of mRNA cyclin D (P = 0.032, Pearson r = −0.550) but the concentration of mRNA cyclin D had no correlation with the number of viable Y-79 human retinoblastoma tumor cells (P = 0.105, Pearson r = 0.390). The mRNA concentration of cyclin D also had a significant strong correlation with the concentration of mRNA E2F (P = 0.012, Pearson r = 0.641). The mRNA concentration of cell cycle regulatory genes, namely p16, had a significant strong correlation with the concentration of mRNA E2F (P = 0.010, Pearson r = 0.657).

4. Discussion

4.1. Number of tumor cells in Y-79 human retinoblastoma

DLBS1425 effect on the inhibition of cell proliferation of Y-79 was evaluated by hemocytometer. The measurement result of hemocytometer showed a decrease in the number of cells Y-79

compatible with dosage levels of DLBS1425. A decrease in tumor cell viability can be caused by tumor cell death and a decrease in tumor cell proliferation activity [10–16]. Naturally, cells are always faced with the decision to proliferate, differentiate, or undergo apoptosis process. All the three have an impact on the number of viable cells to undergo normal cell life processes. Activity of oncogenes and tumor suppressor gene is associated with the three above-mentioned process so that the three processes have the potential to be a therapeutic target of tumor disease. In addition to the three factors, the genes playing a role in DNA repair are also the target of oncogenes activity and tumor suppressor genes [12].

The result of this study indicates that the dose level of DLBS1425 influences on the decline in the number of viable human retinoblastoma tumor cells Y-79. As explained earlier, the viability of tumor cells was associated with the process of proliferation, differentiation, apoptosis, and DNA repair. Viability loss of tumor cells in this study is in accordance with the result of previous studies that also investigated the effect of exposure to DLBS1425 on decreasing the various tumor cells viability. The viability of tumor cells MDA-MB-231 (human breast cancer), MCF-7 (human breast adenocarcinoma), HCT116 (human colon carcinoma), HepG2 (human hepatocellular carcinoma), PC3 (human prostate cancer), and AGS (human gastric adenocarcinoma) that are cultured and exposed to DLBS1425 decreases under the appropriate dose level of DLBS1425 [11]. The results show that the viability of human retinoblastoma tumor cells in the group D50 was amounted to 47.11% as compared to the group C. These findings are close to the findings of mean viability of tumor cells MDA-MB-231, MCF-7, HCT116, HepG2, PC3, and AGS after DLBS1425 exposure at the same dose in 24 h, although the effect of suppression viability is still weaker than the DLBS1425 effect on cells breast tumor MDA-MB-231. Thus, the results of this study are in accordance with the findings of previous studies that DLBS1425 can reduce tumor cell viability significantly [10,11]. Nevertheless, the decline in the number of Y-79 tumor cells which is viable after DLBS1425 exposure in this study cannot explain factors which are more or the most instrumental suppressing on tumor cell viability. One factor that is hypothesized to relate to a decrease in the number of tumor cell viability is apoptosis [10,11,15]. DLBS1425 has the effect of increasing apoptosis of cancer cells through multiple mechanisms of DNA fragmentation, activation of caspase-9, regulation of Bcl-2 protein, protein BAX at mRNA level, inhibition of COX-2 that is associated with mitochondrial apoptosis pathway and suppression of pathway PI3-K/AKT [10,11,15,17–19].

Another factor that is hypothesized to play a role in decreasing the cell viability is the process of cell growth and proliferation. The main system that regulates the growth and proliferation of cells is cell cycle. Cell cycle regulation is performed by various cell cycle regulator genes that express some cyclin protein kinase and protein regulators which both suppress and increase the rate of cell cycle activity. Besides, the progression of the cell cycle is also influenced by the protein products of tumor suppressor genes such as p21. DLBS1425 has been proven to increase level of p21 gene transcripts in the tumor cells MDA-MB-231, resulting in inhibition of cell cycle rate and declining population of cells in the S phase of the cell cycle [10]. In contrast to the results of previous studies, the concentration of mRNA p21 after exposure to DLBS1425 in human retinoblastoma cells Y-79 decreases corresponding with the increase in dose of DLBS1425, but the differences in mRNA p21 concentration are not significant. Decreasing the concentration of mRNA p21 after DLBS1425 exposure with various dose levels may be due to the death toll of tumor cell. Decreased expression of p21 after exposure inhibitor of PI3-K also occurs in ovarian cancer cells accompanied by increased expression of p16 inhibitor proliferation. Both of these findings suggest that the inhibition of tumor cell proliferation Y-79 and ovarian cancer cells may not be through the mechanism of inhibition of cyclin E-Cdk2 activity by p21 [20]. DLBS1425 is considered to inhibit gene transcription of cyclin E human retinoblastoma tumor cells Y-79 in the level of mRNA without going through the p21 gene regulation line.

The other role of DLBS1425 in suppressing tumor cell proliferation is through the suppression mechanism of upstream proliferation gene expression such as NF-κB, c-fos, and COX-2 [10,11]. Suppression of tumor cell proliferation by DLBS1425 is also demonstrated in this study through the suppression of a gene transcription of cell cycle regulators, namely cyclin E gene, that forms a complex with Cdk2 and causes phosphorylation of pRb. Suppression of cyclin E gene transcription in the mRNA levels results in inhibition of phosphorylation of pRb, so the transcription factor E2F remains bound to the pRb in the inactive form. Inactivating E2F will further decrease the progression of the cell cycle and transcription of gene cyclin E itself, which will ultimately reduce proliferation of tumor cell [21,22]. Further details will be discussed in another part of the discussion below.

Suppression of concentration of mRNA cyclin D and the number of Y-79 tumor cells after exposure can be associated with the suppression of DLBS1425 cellular signaling pathway of PI3-K/AKT which plays an important role in various cellular functions such as growth, differentiation, and cell survival. Exposure to DLBS1425 in tumor cells MDA-MB-231 leads to suppression of pathway of PI3-K/AKT, but does not occur in normal cells MCF10A [10]. Suppression of activation of PI3-K/AKT pathway plays an important role to activate apoptosis through induction of several pro-apoptotic genes such as BAX, BAD, and PUMA which will activate caspase-9 and then induce the signal of cell death [10,12,18]. PI3-K/AKT pathway also plays a role in regulating cell growth and proliferation through activation of mammalian target of rapamycin (mTOR). Suppression of PI3-K/AKT by DLBS1425 will affect mTOR activation, so the growth and proliferation of cells will also be hampered. Activity of cyclin kinase inhibitors such as p16 will increase as a result of suppression of the pathway PI3-K/AKT/mTOR resulting in suppression of cyclin D that interacts with Cdk4. Suppression of cyclin D activity will inhibit the phosphorylation of pRb so the transcription factor E2F remains bound to the pRb and causes E2F in inactive condition. E2F inactive state will inhibit activation of the cell cycle, particularly the G1 phase, which will eventually suppress tumor cell proliferation ability [10,11,17,22–24]. The PI3-K/AKT/mTOR is an important signaling pathway for DLBS1425 in suppressing the number of tumor cells through suppression of cyclin D gene expression.

The bioactive compound of *P. macrocarpa*, namely gallic acid, is also proven to inhibit the cell cycle, especially in the G1 phase and G2/M phase cell adenocarcinoma [18]. Like DLBS1425, gallic acid in some studies has also shown to be pro-apoptotic to activate and inhibit caspase cycle cell through

the emphasis on protein cyclin dependent kinase (Cdk) and cyclin [10,18,25–27].

Based on the findings of the studies, a significant decrease in the number of tumor cells Y-79 in this study is likely to be through the mechanism of suppression of cell cycle activity and the increase of apoptosis. DLBS1425 suppressive effect on the pathway PI3-K/AKT would increase p16 activity that suppresses the activity of cyclin D1-Cdk4 which is a cell cycle regulator in the G1 phase. The suppression of cyclin D1-Cdk4 activity will hold down phosphorylation of pRb that binds E2F transcription factor to prevent tumor cell proliferation [10,20,28]. The proposed hypothesis of the action mechanism of DLBS1425 in suppressing tumor cell viability is through the mechanism of suppression on tumor cell proliferation, apoptosis increase, and inflammation suppression.

4.2. Cells population patterns in cell cycle

Analysis of flow cytometry using FACS showed no pattern of increasing or decreasing the proportion of a population of cells that was significant in each phase corresponding to the dose levels of DLBS1425. The pattern of the proportion of the number of cells at each dose level also showed a similar pattern. The findings of flow cytometric analysis in this study indicated that the total population of tumor cells in the G0 phase increased with dose levels after exposure to DLBS1425 for 24 h.

The population of tumor cells in G1 phase in this study experienced a decrease in the non-significant number, while the population of tumor cells in the G0 phase increased. It may occur where some of tumor cell population of G1 goes into a resting phase specific (G0 phase) to undergo DNA repair or apoptosis. This finding is in line with the research result of the previous anticancer DLBS1425 effects which showed that 24 h exposure to DLBS1425 would increase tumor cell apoptosis of MDA-MB-231 through activation of Bcl-2 and BAX and by suppression pathway PI3-K/AKT which in turn would activate BAD and Caspase-9 which induced apoptosis [10,11].

4.3. mRNA concentration of cell cycle regulator genes

Several hypotheses that are arranged to determine the action mechanism of DLBS1425 in suppressing the number of viable human tumor cell retinoblastoma Y-79 are under the mechanism of cell proliferation regulation suppression pathway and pro-apoptotic pathway. In the regulation of tumor cell proliferation pathway, DLBS1425 allegedly pressures cyclin E gene transcription in the mRNA level under the mechanism of suppressing post-transcriptional process of E2F. Expression of mRNA E2F itself is not affected significantly by DLBS1425 as found in this study; however, DLBS1425 may regulate post-transcriptional process of E2F in that E2F protein is not quite adequate to activate cyclin E gene transcription in the mRNA level. Cyclin E has an important role in controlling the cell cycle rate, especially in the phase G1/S by regulating the transcription factor E2F. In contrast, cyclin E is also a transcriptional target of E2F, so activation of cyclin E causes a positive feedback to gene transcription and protein expression of E2F itself.

Research on mouse fibroblast cells that express Cdk inhibitor p16INK4a retroviral (NIH-3T3) shows that cyclin E can intercept protein dephosphorylation of pRb family and suppression activity of E2F by p16. Thus, cyclin E has a function that does not depend on E2F in the process of cell cycle regulation, particularly in the phase G1/S. Action mechanism of cyclin E in cutting the E2F pathway is not simple, but it involves the gene expression and proliferation of regulatory proteins such as c-Myc, cyclin A, CDC2, and p27 [21,29].

Expression of mRNA in p16 genes and E2F genes after DLBS1425 exposure is not significantly different with the control group, but the expression of cyclin E mRNA decreases significantly with the DLBS1425 dose levels. Decreasing expression in mRNA cyclin E is correlated with the decreasing number of tumor cells after DLBS1425 exposure. These results support findings that DLBS1425 lowers the number of Y-79 tumor cells by regulating the cell cycle through the mechanism of suppressing cyclin E without affecting the regulation of p16 and E2F pathway.

Cyclin E gene transcription is also influenced by other transcription factors such as the LRH-1. In the pancreas cells and liver cells, cyclin E is a transcriptional target of LRH-1, so it can be hypothesized that DLBS1425 may also suppress the activity of LRH-1.

Regulation of mRNA cyclin E is also determined by the joining (splicing) process of mRNA strand which will produce various isoforms of protein cyclin E, but the relevance of function and physiology is still unknown [21,30]. Cyclin E-Cdk2 participates in regulating cell cycle activation mechanism of suppression of pRb which later will activate E2F and the rate of phase G1/S cell cycle. This shows the important role of cyclin E in increasing the rate of cell cycle independently without preceding activation of cyclin D-Cdk4. Decreasing expression in mRNA cyclin E corresponding to dose levels that occurs after DLBS1425 exposure will reduce protein expression of cyclin E. Decreasing expression in cyclin E protein, that forms a complex with Cdk2, will reduce the phosphorylation of pRb in binding E2F so that E2F transcription activities on the rate of phase G1/S cell cycle also experiences suppression [21,31,32]. In normal cells, the expression of cyclin E will decline when entering the S phase of the cell cycle, but instead in the tumor cells, such as MDA-MB-231 (breast tumor), HT29 (colon tumors), and U1690 (lung tumor), the expression of cyclin E remains high in the S phase of the cell cycle [21,29,33]. Decreasing expression in mRNA cyclin E after DLBS1425 exposure will reduce expression of cyclin E protein in the S phase of the cell cycle like in normal cells, and later inhibit the cell cycle especially at the end of the G1 phase.

Evidence suggests that the regulation of apoptosis is associated with some cell cycle regulatory genes such as p53 gene, Rb1, myc, and others. The function of p53 and Rb1 gene products is closely related to processes in the cell cycle at the G1 phase. The working mechanism and function of the p53 gene is very complex because p53 can regulate cell proliferation and apoptosis depending on the situation and the environment around the cell. The wild-type p53 gene can compensate for loss of Rb1 gene function so that it can prevent the occurrence of malignant transformation, but cells losing p53 gene function will lose the apoptosis ability. Activation of E2F and p53 can trigger apoptosis through a series of degradation processes of Rb1 tumor suppressor gene and apoptosis factor of Mdm2 inhibitors catalyzed by upstream caspase. Expression of Rb1 mutant, which is resistant to Caspase recasting, protects E2F and prevents degradation of Mdm2, so death effector activation of caspase is inhibited and apoptosis does not occur [12,34]. E2F and

p53 high expression on Y-79 cell allows the higher activation of caspase death effector to get increasing apoptosis [34]. Despite the high expression of E2F at Y-79, the mRNA expression of cyclin E does not increase due to suppression by DLBS1425 so that the rate of cell cycle will be hampered especially in the final phase of the G1/S which result in decreasing cell proliferation rate. Thus, a decrease in Y-79 tumor cell viability after DLBS1425 exposure is at least caused by the decrease in cell proliferation due to cyclin E suppression and the increase in apoptosis due to the high activation of E2F and p53 on the Y-79 cells.

As cyclin E plays a role in regulating the cell cycle in pRb pathways, cyclin D forming a complex with Cdk4, also plays a role in regulating pRb pathways together with cyclin E-Cdk2 complex, that is by regulating phosphorylation of pRb binding E2F. Cyclin D-Cdk4 complex, together with cyclin E-Cdk2 complex, phosphorylates pRb and then inactivates pRb by binding of the complex to pRb. Activity of cyclin D-Cdk4 complex is inhibited by cyclin Inhibitor Kinase (CIK) p16 that competes with Cdk4 to bind cyclin D [22]. In this study, the expression of mRNA p16 increases and expression of cyclin D mRNA decreases after DLBS1425 exposure. Although it is statistically insignificant, the suppression of the number of Y-79 tumor cells by DLBS1425 is still possible through the mechanism of increasing activity of p16 and suppressing activities of cyclin D in regulating the cell cycle. DLBS1425 exposure degrades expression of mRNA cyclin D corresponding to the dose levels, but the hypothesis test showed no significant difference. Exposure of DLBS1425 higher doses is required to prove the significant effect of DLBS1425 to suppress cyclin D. The proposed hypothesis of this study is that DLBS1425 reduces the number of human retinoblastoma tumor cells through suppressing the expression of cyclin E thus inhibited tumor cell proliferation. On the other hand, the high expression of p53 and E2F on the Y-79 cells will increase tumor cell apoptosis. Suppression of tumor cell proliferation and increased apoptosis of tumor cells at the same time will increase the tumor cell death and decrease the number of viable tumor cells.

DLBS1425 has a suppressive effect on mRNA concentrations in cyclin D and cyclin E. Bivariate analysis using unpaired *t*-test showed that DLBS1425 significantly suppressed mRNA concentration in cyclin D at the dose of 50.0 μg/mL and cyclin E at doses of 0.5 μg/mL, 5.0 μg/mL, and 50.0 μg/mL. On the other hand, the multivariate analysis that assessed relationships and roles between variables indicated that the dose level of DLBS1425 correlated with the mRNA concentrations of cyclin D and cyclin E, and the number of viable human retinoblastoma tumor cells Y-79. DLBS1425 dose levels were very strongly negative correlated to the significant number of human retinoblastoma tumor cells Y-79. This suggests that exposure to DLBS1425 can suppress tumor cell viability of human Y-79 retinoblastoma under appropriate dosage levels. DLBS1425 dose level also has a very strong negative and significant correlation with the concentrations of mRNA in cyclin D and cyclin E so that the higher dose of DLBS1425, the lower concentrations of mRNA in cyclin D and cyclin E. On the other side, cyclin D concentration did not correlate significantly to the number of viable Y-79 tumor cells, and the concentration of mRNA in cyclin E had a very strong and significant positive correlation to the viable Y-79 tumor cells. These findings suggest that exposure to DLBS1425 can reduce the number of viable human retinoblastoma tumor cells Y-79 through the mechanism of suppression of mRNA expression in cyclin E and not through regulation of cell cycle regulatory genes such as p16, p21, p53, cyclin D, and E2F. Suppression of mRNA concentration in cyclin E by DLBS1425 may occur directly and not under the regulation of pathway of p53, p21 and E2F.

A strong and significant correlation between the concentration of mRNA in p16 and mRNA concentration in cyclin D with mRNA concentration in E2F showed that the activity of p16, cyclin D, and E2F interconnected. Increasing p16 activity would reduce the activity of cyclin D, which in turn would reduce the activity of E2F to activate the cell cycle system, especially G1 phase so that the cell proliferation would reduce [12,21,28,29].

DLBS1425 shows the effect of suppressing Y-79 human retinoblastoma tumor cell proliferation through the mechanism of gene regulation of cyclin E. Identification of the molecular mechanism of action of DLBS1425 in regulating tumor cell proliferation through the regulation of cyclin E showed that DLBS1425 has anticancer potential against human retinoblastoma tumor cells Y-79. DLBS1425 shows the potential to be developed as an anticancer therapy. A research on the effects of anticancer DLBS1425 against retinoblastoma tumors in experimental animals is necessary in order to find out and explain the *in vivo* anticancer effect of DLBS1425 level. In addition, the study on the effects of standard chemotherapy combination for retinoblastoma (cisplatin, etoposide, vincristine) with DLBS1425 needs to be done to identify and explain the synergistic effect of DLBS1425 as retinoblastoma chemotherapy adjuvant.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This study was supported by the Dexa Laboratories of Biomolecular Sciences, PT. Dexa Medica, Cikarang, West Java, Indonesia, Grant No. 135/MP/DLBS/2016. The authors would like to thank Mr. James Sinambela and Asep Aripin for preparing DLBS1425, Olivia Mayasari, Guntur Berlian and Rehmadanta Sitepu for guiding cell culture, performing PCR analysis, and assisting research report, and Agung Heru Karsono for performing flow cytometric analysis.

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