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Up-regulation of $p27^{Kip1}$ and Caspase-9 Expression in Oral Malignant Burkitt's Lymphoma Cell Induced by *3,4-dihydro-6-[4-{3,4-dimethoxybenzoyl}-1-piperazinyl]-2(1H)-quinolinone-vesnarinone (Studyon chemotactic migration and apoptosis cell)*

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Abstract: ***3,4-dihydro-6-[4-{3,4-dimethoxybenzoyl}-1-piperazinyl]-2(1H)-quinolinone-vesna rinone is a new and novel inotropic drug that has unique and complex mechanisms of action such as unique anti-proliferating, differentiation-inducing and apoptosis-inducing drug against several human malignancies. The aim of study was to evaluate the up-regulation of $p27^{Kip1}$ and caspase-9 expression in oral malignant Burkitt's lymphoma (Raji) cell induced by vesnarinone. Study on chemotactic migration and apoptosis cell. In the present study, laboratories pure experimental with post-test only control group design was confirmed. Raji cells were incubated with vesnarinone doses of 0, 6.25x 10-3, 1.25x10-2, 2.5x10-2 and 5x10-2 M. After 24 h incubation, chemotactic cell migration was examed by Boyden chamber kit 48 well. Cell apoptosis activity was observed with caspase-9 colorimetric assay. Furthermore, to evaluate the level of $p27^{Kip1}$ protein was detected by Western blot assay. Data were analyzed by Anova one way followed by post-hoc LSD test for significance level of 95%. Results revealed vesnarinone was markedly suppressed the activity of chemotactic migration and significantly induced apoptosis of Raji cell characterized by up-regulation of $p27^{Kip1}$ protein and proteolytic activity caspase-9. In conclusion, vesnarinone increased up-regulation of $p27^{Kip1}$and caspase-9 in oral malignant Burkitt's lymphoma cell chemotactic migration suppression and apoptosis induction targeting this molecule could represent a promising new therapeutic approach to this type of tumor.***

Keywords: vesnarinone, chemotactic migration, apoptosis, Burkitt's lymphoma cell.

1. Introduction

3,4-dihydro-6-[4-{3,4-dimethoxybenzoyl}-1-piperazinyl]-2(1H)-quinolinone-vesnarinone (OPC- 8212) is a newly synthesized positive oral inotropic agent who has been used for the treatment of chronic heart failure.[1] It has multiple biological activities on mammalian cells both *in vitro* and *in vivo*. The mechanisms of action associated with the inotropic properties include a decrease in potassium efflux with an increase in the inward calcium current [2] and an inhibition of phosphodiesterase activity.[3] OPC 8212 also has an immunomodulation effect by inhibiting the production of various cytokines, including TNF-α, IFN-γ, IL-1α, IL-2 and IL-6 in polysaccharide-stimlating peripheral blood mononuclear cells [4], and is shown to inhibit the production of HIV-1 in culture cells [5]. 3,,4-dihydro-6-[4-{3,4-dimethoxybenzoyl}-1-piperazinyl]-2(1H)-quinolinone was reported in relation to its antitumor effect with apoptosis-inducing activity.[2,4] Also, it has been found to suppress the growth of a wide variety of tumor cell line, including human gastric cancer,[6] lung cancer,[7] hepatocelullar carcinoma,[8] adenoid squamous carcinoma,[9] acute myeloid leukemia[10] and pancreatic cancer.[11]

Burkitt's lymphoma (BL) is one of the most aggressive malignancies of lymphoid origins and accounts for 3-5% of all lymphomas. It is a high grade B-cell neoplasm. Usually found in the pediatric population, BL represents 40% of childhood NHL.[12] The highest incidence is found in the endemic form of equatorial regions of Africa and Papua-New Guinea where it accounts for 50-70% of all pediatric malignancies.[13] BL is characterized by chromosome translocations between the proto-oncogene c-myc and one of the immunoglobulin (Ig) loci.[14] It was reported , Epstein-Barr virus (EBV) has been implicated in its etiology and increased the aggressive of BL. BL most often involve the maxilla or the mandible and rarely involves abdominal organs include kidneys, ovaries or retroperitoneal structures. However, the development of a more effective therapeutic method of oral malignant Burkitt's lymphoma must continue.

$p27^{Kip1}$ is a universal cyclin-dependent kinase inhibitor that directly inhibits the enzymatic activity of cyclin-CDK complexes, resulting in cell cycle arrest at G_1.[15] $p27^{Kip1}$ has an important prognostic factor in various malignancies. Recently, decreased expression of $p27^{Kip1}$ has been frequently detected in human cancer.[16] In addition, loss of $p27^{Kip1}$ has been associated with disease progression and an unfavorable outcome in several malignancies.[17] Furthermore, mice lacking the $p27^{Kip1}$ gene show an increase in body weight, thymic hypertrophy and hyperplasia of pituitary intermediate lobe adrenocorticotropic hormon cells, adrenal glands and gonads organ.[18] Moreover, malignant human oral cancer cells transfection with $p27^{Kip1}$ gene leads to inhibition of proliferation, invasion and metastasis. [19,20] These suggest overexpression of $p27^{Kip1}$ protein induced cell growth inhibition and apoptosis.

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Caspase-9 is a member of the caspase family of cysteine proteases that have been implicated in apoptosis and cytokine processing. It is an initiator caspase, encoded by the *CASP9* gene.[21] The aspartic acid specific protease caspase-9 has been linked to the mitochondrial death pathway. It is activated during programmed cell death (apoptosis). Induction of stress signaling pathways (JNK/SAPK) causes the release of cytochrome-c from mitochondria, which then binds to Apaf-1. Activated caspase-9 cleaves downstream caspase-3, -6 and -7 initiating the caspase cascade.[22] In the present study, up-regulation of $p27^{Kip1}$ and caspase-9 expression in oral malignant Burkitt's lymphoma cell induced by *3,4-dihydro-6-[4-{3,4-dimethoxybenzoyl}-1-piperazinyl]-2(1H)-quinolinone,* study on chemotactic migration and apoptosis cell were investigated.

2. Materials and Methods

2.1 Cell and Cell Culture

Raji cell line (ATCC CCL-86 B-lymphocyte, USA) was obtained from Department of Paracytology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. The cell line was cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum, and 100 µg/ml streptomycin, 100 U/ml penicillin (Moregate BioTech, Bulimba, Australia). The cultures were incubated in a humidified atmosphere of 95% air and 5% $C0_2$ at 37^0C.

2.2 Drugs Dilution

A stock solution of OPC-8212 with $1.25x10^{-1}$M concentration was diluted into $6.25x10^{-3}$, $1.25x10^{-2}$, $2.5x10^{-2}$ and $5x10^{-2}$ M in a solution of DMEM 10% FBS. These concentrations were incubated with Raji cells for 24 hours.

2.3 Chemotactic Migration Assay

Chemotaxis (directed migration) was evaluated in the Boyden chamber apparatus (Neuro Probe, Inc., Cabin John, MD, USA). Briefly, subconfluent cells were starved for 24 h and harvested with 0.05% (w/v) trypsin (Invitrogen Corporation, USA) containing 0.02% (w/v) ethylenediamine tetra-acetic acid (EDTA, Invitrogen Corporation), washed twice with PBS, and resuspended to a final concentration of 5 x 10^5 per ml in serum-free medium with 0.1% (w/v) fraction V bovine serum albumin (BSA, Wako Pure Chemical Industries, Ltd). Polyvinyl-pyrrolidone (PVP) filters (Nuclepore Corp, Palo Alto, CA, USA) of 8-µm pore size were precoated with gelatin (Merck KGaA, Frankfurt, Darmstadt, Germany) (0.1 mg/ml) and rinsed in sterile water. Lower chamber was filled with 30 µl of various doses of quinolinone in DMEM 10% FBS and covered with a gelatin-coated membrane. Furthermore, 50 µl of cell suspension, yielding 500 cells/ml of Raji cells were added to the upper chamber. After 24 hours of incubation, the membrane was stained with Giemsa solution (Ted Pella Inc., Redding, CA, USA). The number of cells that had penetrated through the filter was counted under light microscope at 400x magnification. The counting was performed for 12 fields in each concentration.

2.4 Detection of apoptosis using colorimetric assay

Induction of apoptosis was measured using the colorimetric assay kit (caspase-9; BioVision Research Product, CA, USA) according to the manufacturer's directions. Briefly, equal amounts of tissue extracts prepared from Raji treated cells were incubated with the substrate (Ac-LEHD-pNA) in the assay buffer for 2 hours at 37^0C. Absorbance was measured at 450 nm using a microplate reader (BioRad, USA). Each determination was performed in triplicate.

2.5 Western Blot Analysis

Cell lysates were prepared from the Raji-treated cells in Falcon tissue culture for 48 h. Briefly, samples containing equal amounts of protein (50 µg) were electrophoresis on an SDS-polyacryl-amide gel and transferred to a polyvynilidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA). The filters were blocked in TBS containing 5% non-fat milk powder at 37^0C for 1 h, and then incubated with a 1:500 dilution of primary antibodies against the p27 protein (clone 1B4, mouse monoclonal antibody; Novocastra Laboratories, New Castle, UK), For detection of HRP-conjugated antibodies were used the enhanced chemiluminescent (ECL) plus kit (Amersham Pharmacia Biotech, UK). Anti α-tubulin monoclonal antibody (Zymed laboratories, San Francisco, USA) was used for normalization of the western blot analysis.

2.6 Statistical Analysis

Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (Abacus Concepts, Berkeley, CA) using one-way ANOVA and Post-hoc LSD. The significance level was set at 5% for each analysis.

3. Results

3.1 *In vitro* chemotactic migration assay

Cell migration is an essential process involved in tumor invasion and metastasis. The ability of cell migration on each treated cell with the Boyden chamber kit was evaluated for 24 hours incubation. As seen in figure 1, Raji-treated cells with 2.5 x 10-2 and 5 x 10-2 M were markedly showed the low ability of cell migration compared with that of control ($P<0.05$; one-way ANOVA). Raji cells treated with 5 x 10-2 M have the potential barriers to migration 70.2%.

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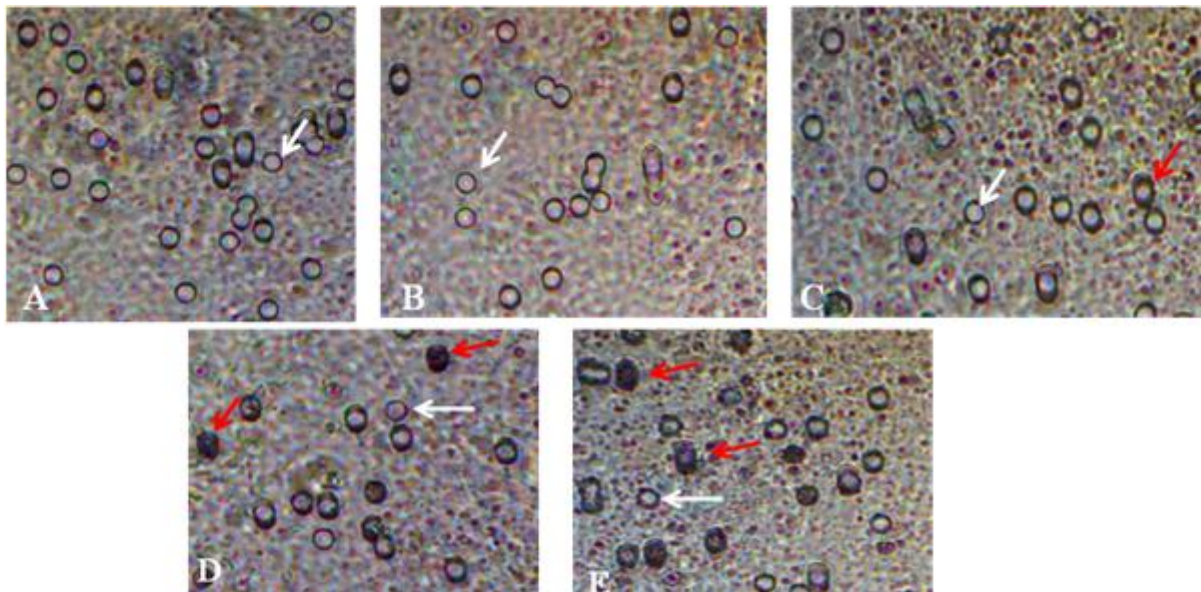

Figure 1: Chemotactic migration of Raji cells were treated by different concentrations of vesnarinone. A. negative control., B. concentration of 6.25 x 10-3 M., C. concentration of 1.25 x 10-2 M., D. concentration of 2.5 x 10-2 M., E. concentration of 5 x 10-2 M ., (White arrows: of viable cells, red arrow: cell apoptosis)

3.2 Proteolytic activity of caspase-9

The activity of caspase-9 in Raji cell treated with various concentrations of OPC-8212 was investigated. Raji cell incubated with 2.5x10-2 M and 5.0 x 10-2 M showed increased caspase-9 proteolytic activities as compared with that of control (Figure 2). Proteolytic activities of caspase-9 in Raji cell incubated with 2.5 x 10-2 M and 5.0 x 10-2 M were detected at 4.1 and 5.9 fold increased, respectively, when compared with that of control.

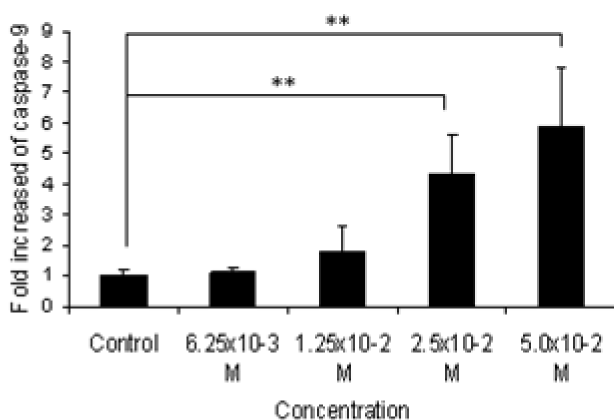

Figure 2: Induction of proteolitic activity of caspase-9. Raji cells were incubated with various concentration of quinolinone (OPC-8212). ** P < 0.001.

3.3 Expresion of p27^{Kip1} and α-tubulin protein

The expression of p27^{Kip1} and α-tubulin protein was examined by Western bloting analysis. We detected the up-regulation of p27^{Kip1} protein in cells treated with concentration 2.5x 10-2 and 5 x 10-2 M when compared with that in control cells. The expression of α-tubulin as an internal control was approximately the same in all of concentration (Figure 3).

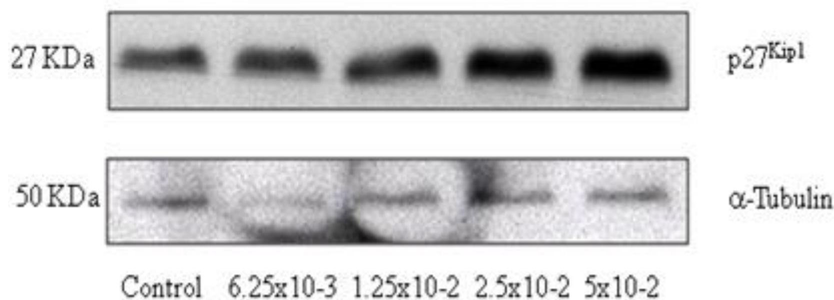

Figure 3: Protein expression of p27^{Kip1} and α-tubulin in Raji cells treated with various concentration of vesnarinone (Concentration unit is M).

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4. Discussion

Increased growth suppression, chemotactic migration, metastasis and apoptosis of cells has become the attention center of researchers in the field of oral cancer, including oral malignant Burkitt's lymphoma, because of the various treatments conventionally, such as radiotherapy, chemotherapy, surgery and a combination of them have still not been satisfactory.[9,15] Similarly, studies by increasing inhibition activity of cell chemotactic migration, proliferation, invasion, metastasis and apoptosis induction is still unexplored in oral malignant Burkitt's lymphoma. However, on the base of the data, it is necessary for ongoing research to find the most effective methods and potential drug agents against oral cancer cells. In the present study, 3,4-dihydro-6- [4- {3,4-dimethoxybenzoyl} -1-piperazinyl] -2 (1H)–quinolinone (OPC-8212) as an anti chemotactic migration and apoptosis induction on oral malignant cell Burkitt's lymphoma was examinated.

3,4-dihydro-6- [4- {3,4-dimethoxybenzoyl} -1-piperazinyl] -2 (1H)–quinolinone-vesnarinone is a synthetic derivative quinolinone as an inotropic drug which used for the treatment of congestive heart failure (CHF). It has a complex biological activities in mammalian cells both *in vitro* and *in vivo* by increasing the immunomodulating effects, anti-inflammatory and cell growth inhibition.[23] It was reported vesnarinone has the cell growth inhibition effect and induction of cell differentiation of the various types of human cancer, including salivary gland cancer,[24] eritroleukemia,[25] pancreatic carcinoma[11] and gastric carcinoma.[26] Furthermore, vesnarinone was reported to have the barrier activity of various types of cytokine TGF-α, TGF-β production and growth factors in the immune system such as TGF-α, TGF-β, amphiregulin, IL-1α, EGF receptor, c-ERBB2 and TGF-β receptor.[5] Our results revealed vesnarinone has a potential antitumor activity against oral malignant Burkitt's lymphoma (Raji) cell through induction activity of chemotactic migration barriers. The potencial of cell migration barriers treated with concentration of 5 x 10-2 M amounted to 70.2%. These data indicated vesnarinone has a strong anti tumor activity proofed by low activity of cell chemotactic migration. In addition, the most important results are increasing cell migration barriers were followed by inducting cell apoptosis in accordance with the raising concentration of vesnarinone. This data is likely to involve a variety of inhibitor protein complex mechanisms include cyclin-dependent kinases inhibitor protein, cell cycle arrest, matric metallo protein (MMP), Akt / PKB signal transduction, NF-kB transcription factor and induction of pro–apoptosis protein expression. Honma *et al.*[7] reported that vesnarinone has the potential to increase the cell cycle arrest at the G1 phase of lung carcinoma cells. Also, it suppressed the cell cycles into G0-G1 phase and decreased to cyclin A, D, E and cyclin-dependent kinase 2 (CDK-2) protein expressions in gastric cancer.[6] Decreased their protein expression were known to activate the cyclin-dependent kinase inhibitor $p27^{Kip1}$ as a negative regulator of the cell cycle that can improve the cell growth inhibition and apoptosis induction into oral cancers included oral tongue squamous carcinoma and salivary gland cancer.[2,27] Moreover, it was reported vesnarinone has the inhibition ability of angiogenesis and tumorigenicity in oral squamous carcinoma cells through the inhibition of growth factor protein VEGF and IL-8 cytokine family.[2] vesnarinone can inhibit the activity of the transcription factor AP-1, TNF-α, NF-κB, c-Jun protein and increase apoptosis of human cancer cells according to the concentration and the contact time of the drug.[23] In this study, the concentration difference was only done with the same time observation (24 hours of incubation). The results revealed the higher concentration of vesnarinone was markedly increased the low ability of cell chemotactic migration followed by induced apoptosis characterized as up-regulation of caspase-9 activity and $p27^{Kip1}$ protein expression. It means that suppression of cell migration and induction of apoptosis by vesnarinone were through the $p27^{Kip1}$ and caspase-9 mechanism targeting this molecule could represent a promising new therapeutic approach for this type of tumor.

In conclusion, *3,4-dihydro-6-[4-{3,4-dimethoxybenzoyl}-1-piperazinyl]-2(1H)- quinolinone-vesnarinone* increased up-regulation of $p27^{Kip1}$and caspase-9 in suppressing the chemotactic migration and inducing apoptosis of oral malignant Burkitt's lymphoma (Raji) cells.

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