Docetaxel hydrate induces apoptosis and suppresses tumorogenesis of oral Burkitt’s lymphoma cells (in vitro and in vivo studies)

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ABSTRACT

Introduction: Burkitt’s lymphoma (BL) is one of the tumours with high malignancy and rapid cell growth, derived from B-cell lymphoma. BL typically found in children at dengue-endemic and HIV-AIDS areas with low socioeconomic levels. This study was aimed to analyse the induction of apoptosis and the suppression of tumorogenesis of oral Burkitt’s lymphoma (Raji) cells using docetaxel hydrate in vitro and in vivo.

Methods: In the present study, the pure experimental laboratory with post-test only control group design was carried out. Raji cell cultures were incubated with docetaxel hydrate by doses of 0, 1.25 \times 10^{-2}, 2.5 \times 10^{-2}, and 5.0 \times 10^{-2} M; and IC50 carboplatin (3.1 \times 10^{-6} M) as a positive control. Induction of apoptotic was analysed by double staining of acridine orange-ethidium bromide. Tumorigenesis assay was performed by inoculating Raji cells in nude mice flanks at 1 \times 10^6 cells/mice. Tumour treatment was delivered by various doses of docetaxel hydrate peroral.

Results: Apoptosis cells were significantly increased in Raji cells treated with docetaxel hydrate by doses of 2.5 \times 10^{-2} and 5.0 \times 10^{-2} M. The tumour volume in mice given doses of 2.5 \times 10^{-2} and 5.0 \times 10^{-2} M was markedly decreasing compared to control (dose of 0).

Conclusion: Docetaxel hydrate has a high antitumour potency by inhibiting tumorogenesis and increasing apoptosis of Burkitt’s lymphoma cells.

Keywords: Docetaxel hydrate, double staining, Burkitt’s lymphoma cell, apoptosis, tumorogenesis

INTRODUCTION

Burkitt’s lymphoma (BL) is one of the aggressive malignant tumours derived from lymphoid and covers 3-5% of all lymphoma tumours. BL attacks 40% of children with non-Hodgkin lymphoma (NHL). The highest incidence of BL is found in malaria endemic and HIV-AIDs areas in the equatorial regions of Africa and Papua-New Guinea, with a 50-70% of all malignancies occurring in the child population. BL is characterised by a chromosome translocation between the proto-oncogene c-myc and one of the immunoglobulins (Ig) loci. It was reported that increased aggressiveness of BL cells was firmly related to the presence of Epstein-Barr virus (EBV). The mortality rate of children...
with BL is growing from year to year, especially in children living in slums with unhealthy nutritional factors. It was reported that Burkitt’s lymphoma patients recorded at the Cipto Mangunkusumo Hospital Jakarta from 2001 - 2006 were as many as 7 patients (6 males, 1 female); ages ranged from 0-15 years old. Data from patients with Burkitt’s lymphoma in Indonesia is still unclear. Based on the reports, development of more effective and potent therapies for oral Burkitt’s lymphoma must be continued, one of them using the docetaxel hydrate agent.

Docetaxel hydrate is a taxoid family antineoplastic agent used for the treatment of several types of human cancers. It is also an anticytostatic agent that has in vitro and in vivo effects. Docetaxel hydrate is reported to have growth inhibition for various types of cancer cells including prostate cancer, breast, oral, and stomach. Docetaxel hydrate suppresses cancer cells by interfering with the microtubule function that is essential for cell survival and leading to apoptosis or cell death.

Apoptosis is the primary physiological process that contributes to the maintenance of cellular homeostasis. Apoptosis is generally characterised by different morphological and energy depending on the biochemical mechanism. Apoptosis plays an essential role in various life processes in response to DNA damage, including normal cell turn over, development of immune system function, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Disruption apoptosis affects many human conditions including neurodegenerative disease, ischemic damage, autoimmune disorders and various types of cancer. Apoptosis is also a natural process that can remove unwanted, excessive or damaged cells from multicellular organisms. Different to cancer cells, the presence of multiple genetic disorders and cellular stress in malignant transformation will activate pro-apoptotic agents. The ability to regulate living cells and/or dead cells is a potentially beneficial therapeutic target. Therefore, the study focused on explanation and analysis of fast-moving apoptotic signalling pathways. Although many of the major apoptotic proteins have been identified, the molecular mechanisms remain unclear.

Tumorigenesis is the formation of tumours derived from healthy cells turned into tumour cells. Its process is characterised by mutations and changes in cellular, genetic, epigenetic and abnormal cell divisions. Generally, there is a balance between proliferation and programmed cell death (apoptosis) to maintain the integrity of cells, tissues and organs. In contrast, there is no balance between proliferation and apoptosis in cancer cells. Cell proliferation process is faster than apoptosis which results in uncontrolled cell division.

In this study, apoptotic induction of Burkitt’s lymphoma cells treated with various concentrations of docetaxel hydrate was analysed by double-staining acridine orange-ethidium bromide (AO-EB), and tumorigenesis assay was performed in nude mice. This study was aimed to analysed the induction of apoptosis and the suppression of tumorigenesis of oral Burkitt’s lymphoma (Raji) cells using docetaxel hydrate in vitro and in vivo.

METHODS

The true experimental laboratory with the post-test only control group design was performed in this study. Burkitt’s lymphoma (Raji) cells was delivered from Department of Paracitology, Faculty of Medicine, Universitas Gadjah Mada, with cell characteristic is ATCC CCL-86 B-lymphocyte (USA).

Cells and cell cultures, Burkitt’s lymphoma (Raji) cells were cultured in Dulbecco’s modified eagle medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (FCS, Moregate BioTech, Australia), 100 μg/ml streptomycin, and 100 units/ml penicillin (Invitrogen Corp., USA). BL cells were incubated in 95% humidity and 5% CO₂ at 37°C.

Apoptosis induction assay using AO-EB staining, Ethidium bromide-acridine orange stock solution was prepared from 50 mg ethidium bromide plus 15 mg acridine orange dissolved in 1 ml 95% ethanol and 49 ml of distilled water. Next, from solution stock was taken 1 ml of solution and diluted in phosphate buffer silane (PBS, Nacalai, Japan) with ratio 1: 100. Microplate 24 wells was prepared and put in the cover glass at each well. Raji cells (2 x 104 cells / wells) from
Docetaxel hydrate induces apoptosis and suppresses tumorigenesis of oral Burkitt's lymphoma cells (Supriatno)

The incubator were cultured in a well contained DMEM 10% fetal calf serum (FCS). Subsequently, cells were incubated for 24 hours at 37°C and CO₂ 5%. After incubation, medium was aspirated and each well was given new DMEM 10% FBS which has been added various concentration of docetaxel hydrate. Cells were incubated for 48 hours. After that the glass cover at the bottom of the well was removed and placed on the object glass according to the docetaxel hydrate doses label. All samples were stained with a combination of 10 μl ethidium bromide-acridine orange solution by dropping on a glass cover for 20 minutes. All preparations were observed under a fluorescence microscope with magnification 40x. Viable cells appeared bright green, while the yellow-red cells showed the apoptotic cells. Apoptosis cells were measured by manual standard procedure (unit: %).

**RESULTS**

The induction of cell apoptosis treated by docetaxel hydrate has been investigated in vitro on oral Burkitt's lymphoma cells through a double staining analysis of AO-EB. Docetaxel hydrate was significantly increased the apoptosis cells compared with that of negative control. Apoptosis cell was also occured in Raji cells induced by carboplatin IC50 which serves as a positive control. Based on Figure 1A, viable cells appeared bright green, while apoptotic cells showed a yellow-orange color. The activity of cells apoptosis was markedly increased in the docetaxel hydrate treatment. The highest dose of docetaxel has the highest apoptotic activity.

It was also found that positive controls had higher apoptotic activity than the highest docetaxel (5.0 x 10⁻² M) dose (Figure 1B). The cell apoptotic activity was started from doses of 1.25 x 10⁻² to 5.0 x 10⁻² M.

**Tumorigenesis assay, Cultured Raji cells (1x10⁶ cells/mice) were inoculated on nude mice flank using 1 ml injection syringe. After growing the tumor (diameter ± 2 cm), mice divided into 5 groups, each group was 3 mice: negative control (dose 0), 1.25 x 10⁻²; 2.5 x 10⁻²; 5 x 10⁻² M, and IC50 carboplatin (3.1 x 10⁻⁶ M) as a positive control. Treatment was started on day 0 at intervals of 3 days. The suppression of tumorigenesis was measured by tumor volume with formula: V = 0.4 x L x W². Statistical analysis, data was analysed using one-way ANOVA followed by Post Hoc (LSD) with 95% significance level. The analysis program was carried out by SPSS 16.00 software (SAS Institute Inc., Cary, NC, USA).**

![Figure 1A](image1.png)

**Figure 1A.** Apoptosis Raji cells were treated with various concentrations of docetaxel hydrate; A. negative control; B. concentration 1.25 x 10⁻² M.; C. Concentration 2.5 x 10⁻² M.; D. Concentration 5.0 x 10⁻² M.; E. Positive control carboplatin IC50 (3.1 x 10⁻⁶ M).

(Green color: viable cell and yellow-orange color: apoptotic cells)
In vivo tumorigenesis assay revealed Raji cells treated with various concentration of docetaxel hydrate had an increasing suppression of tumor growth in accordance with the elevating doses of docetaxel hydrate. Based on Figure 2, the difference in tumor volume size begins on the 6\textsuperscript{th} day of treatment. 

Positive control [carboplatin IC 50 (3.1 x 10\textsuperscript{-6} M)] showed the strongest inhibition of tumor volume, but not significant when compared with concentration of 5.0 x 10\textsuperscript{-2} M. The potential of cell tumorigenesis inhibition treated with docetaxel hydrate 5.0 x 10\textsuperscript{-2} M was detected at 62.5% compared to negative control at day 15.

DISCUSSION

The inhibition of Burkitt’s Lymphoma cell growth using various concentration of docetaxel hydrate through in vitro apoptosis and in vivo tumorigenesis analysis was carried out. The activity of Raji cell apoptosis treated with docetaxel concentration...
of $5.0 \times 10^{-2}$ M and $2.5 \times 10^{-2}$ M through double staining analysis was increased 68.5% and 41.2%, respectively compared with that of negative control. These result suggested that docetaxel hydrate has a strong activity in elevating cell apoptosis, although the potency of apoptosis activity was not stronger than positive controls. This data suggested that carboplatin IC50 used as a positive control was to have stronger sensitive to Raji cells or carboplatin concentrations used were still too high (carboplatin IC50 concentrations used in this study were taken from IC50 head and neck, colon, breast and uterine cancer).

The most important results above indicated an increase in Raji apoptotic cell activity treated with various concentration of docetaxel hydrate (Figure 2) followed by increased the proliferation and cell migratory suppression in accordance with increasing docetaxel hydrate concentration. These results most likely involve a variety of complex protein barrier mechanisms including cyclin-dependent kinase protein, cell-cycle arrest, matric-metalo protein (MMP), Akt/PKB signal transduction, NF-kB transcription factor, and pro-apoptosis protein.\textsuperscript{16} It was reported docetaxel was able to induce KB cell apoptosis through intrinsic mitochondrial pathways and increase in vivo antitumor activity.\textsuperscript{20}

An in vivo tumorigenesis analysis in experimental animal model in this study demonstrated the potential of docetaxel hydrate in suppressing tumor volume. This result was evidenced by the magnitude of tumor growth inhibition by 62.5%. In the present study, there were no burns or necrosis on the flanks of mice due to treatment and no Raji cell metastasis was found in local, regional or distant lymph nodes. This study was discontinued on the 15\textsuperscript{th} day due to an increasingly smaller tumor volume especially tumors treated with concentration $5.0 \times 10^{-2}$ M and a positive control because the tumor tissue will be used for further research like immunohistochemistry that requires cancer tissue nodules. It has been reported that the potential of docetaxel hydrate in vivo is highly effective in several types of human cancers including non-small cell lung cancer,\textsuperscript{24} glioma,\textsuperscript{25} prostate cancer,\textsuperscript{6} breast,\textsuperscript{7} and oral cancer.\textsuperscript{5}

Recent study reported Docetaxel hydrate caused cell cycle inhibition in G2-M phase and induced apoptosis of renal cancer cells by inhibiting the path of mitogen activated protein kinase (MAPK).\textsuperscript{21} The inhibition of cell cycle in G2-M phase was accompanied also by the complex constraints of CDK-1 and cyclin B. Decreased expression of cell cycle proteins is known to activate the cyclin-dependent kinase inhibitor protein p27Kip1 as a negative regulator of cell cycle that can increase cell growth resistance and induction of apoptosis in oral cancers of the squamous carcinoma and salivary gland cancers.\textsuperscript{22,23}

In this study, only concentration difference with the same time (48 hours incubation) was observed. The results revealed the more docetaxel concentration increases the greater the activity of cell apoptosis. The concentrations used for apoptosis analysis had not shown yet that the cells were saturated. It was proofed by increasing apoptotic activity in accordance with concentration elevated. So it is recommended for further research to use larger and broader docetaxel concentrations to produce docetaxel saturation concentrations of oral Burkitt’s lymphoma cells. Obstacles in this study include the provision of materials and research tools that require a long time, the place of research must be queued up and inefficient the bureaucracy of correspondence related to research, as well as limited funding and research time. To address these weaknesses, a research strategy has been carried out by coordinating affiliation with research institutions within the Gadjah Mada University, so that the materials, tools, places and locations of the research that become obstacles are easily solved. In addition, a long-term published correspondence on research permits to be quick published using the personal recommendations of faculty leaders.

**CONCLUSION**

Docetaxel hydrate has a high antitumour potency by inhibiting tumorigenesis and increasing apoptosis of Burkitt’s lymphoma cells.

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