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Cytotoxicity Test of Two Deazaellipticine Compounds against Liver Cancer Cells (HepG2 Cell Line)

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ABSTRACT

Cancer is a genetic disease caused by DNA mutations. Doxorubicin, is one of the liver cancer chemotherapy agents. However, its use may provide cardiotoxic effects, myelosuppression and mucositis in normal cells. Therefore, the alternative of doxorubicin substances is need, one of the potential substances is ellipticine. This study was conducted to test the effect of cytotoxicity of two ellipticine compounds on HepG2 cell liver cancer line. The cytotoxicity test of the compound was carried out by using the MTT test method (3- (4,5-dimethyltiazole-2-yl) -2,5-diphenyltetrazolium bromide). Cell cultures were treated with compounds of 7 variations in concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125). As a control, the treatment of doxorubicin is used. Optical density is measured by microplate reader. The results were analyzed using polynomial regression and displayed as IC50 values. Results of IC50 cytotoxicity of both compounds and doxorubicin were 1,932 μM; 2,990 μM; 0.642 μM.

Key words: *Cancer, Doxorubicin, Ellipticine, Liver Cancer, MTT Assay.*

Introduction

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells (Oolaku *et al.,* 2017). Cancer can also be described in general as cells in the body whose growth and spread is uncontrolled (ATSDR, 2014). The spread of cancer cells can occur either by direct growth in surrounding tissues (invasion) or by remote cell migration (metastasis) (Martin *et al.,* 2020). In 2012 there are 14.1 million cases of cancer and is expected to continue to increase until 2030 to 23.6 million cases annually (WHO, 2014).

Cancer is one of the leading causes of death worldwide. In 2012, about 8.2 million deaths are caused by cancer. Lung, liver, stomach, colorectal, and breast cancer are the biggest causes of cancer deaths each year. Liver cancer is ranked 2nd in the most common cancer of men and is ranked 9th in the most common cancer of women. Liver cancer is a common form of cancer found in the world (Balogh *et al.,* 2016). Chemotherapy is a treatment process using drugs that aim to destroy or slow the growth of cancer cells. However, the side effects of chemotherapy arise because chemotherapy drugs not only destroy cancer cells but also attack healthy cells (Schirrmacher, 2019). Treatment of cancer through chemotherapy, radiation or irradiation and surgery still has a weakness so that the development of new anticancer compounds is better (Huang *et al.,*

2017). Doxorubicin is a chemotherapy agent commonly used for cancer therapy but the effectiveness of the use of chemotherapy agents is still a problem due to the emergence of cancer cell resistance and the presence of toxic effects on normal body tissues (Tacar *et al.,* 2013). Use of doxorubicin may also lead to cardiotoxicity and decreased immune system (Mancilla *et al.,* 2019).

The use of natural ingredients as chemoprevention agents is used to minimize side effects of chemotherapy agents. In addition, natural materials have the ability to bind the harmful target molecules in the body. One type of plant that can be used for anticancer drugs is *Ochrosia elliptica* which contains anticancer activity. Ellipticine (5,11-Dimethyl-6H-pirido [4,3-b] carbazole) is a natural material compound isolated from *Ochrosia elliptica* and is a family plant of Apocynaceae that exhibits anticancer bioactivity. Ellipticine and its derivative deazaellipticine (6,11-dimethyl-5H-benzo [b] carbazole) are (2-bromo-6- (5-bromo-3a, 7a-dihydro-1H-indol-3-yl) -5H-benzol [b] carbazole) as compound one (S1) (Appendix 1) and (6- (3a, 7a-dihydro-1H-indol-3-yl) -5H benzol [b] carbazole) as compound two (S2) 2) has high efficiency against many types of cancer (Miller *et al.,* 2019). Deazaellipticine which has a similar structure with ellipticine is also known to have the ability to inhibit cancer cell activity (Miller *et al.,* 2012).

Based on research (Stiborova *et al.,* 2011) ellipticine has an Inhibitory Concentration 50 or IC50 value of 1.25 µg/ml in breast cancer cells MCF-7, 0.67 µg / ml in cancer cells Leukimia HL-60, 0.27 µmg / ml in cells cancer Neuroblastoma IMR-32 and in cancer cells Neuroblastoma UKF-NB-3 has a value of 0.44 µg/ml. Ellipticine and deazaellipticine derivatives are believed to have good cytotoxicity values. Therefore, this study was conducted to determine the anticancer activity of ellipticine compounds and deazaellipticine derivatives against liver cancer cells HepG2 Cell Line.

Materials and Methods

The research was conducted in June 2016 - February 2017 at Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University, Yogyakarta. While the process of calculating and analyzing the data conducted in the Laboratory of Zoology and Animal Engineering, Department of Biology, Sepuluh Nopember Institute of Technology, Surabaya.

Tools and Materials

The tools used in this study were 96 well-plate (IWAKI), microplate reader, micro pipette, tip, Laminar Air Flow (LABCONCO), centrifuge (Eppendorf 5415-D), incubator CO_2 (HERA Cell), vortex mixer (Eppendorf), Petri dish, Haemocytometer and inverted microscope.

The materials used in this study were cell HepG2 Cell Line suspension from the Parasitology Laboratory, UGM Yogyakarta, Fecal Bovine Serum (FBS) (Sigma) 10%, Tripsin-EDTA 0.25%, Dulbecco's Modified Eeagle Medium (DMEM) medium (Sigma), penicillin-streptomycin 1% (Sigma), 0.5% Amploerizine (Caisson), SDS-Stopper, DMSO (dimethyl sulfoxide), compounds (2-bromo-6- (5-bromo-3a, 7a-dihydro-1H- indol-3-yl) -5H-benzol [b] carbazole) and (6- (3a, 7a-dihydro-1H-indol-3-yl) -5H benzol [b] carbazole).

Ways of Work

This research consists of several steps, namely DMEM (Dulbecco's Modified Eagle Medium), MCF-7 Cell Line propagation, HepG2 Cell Line harvesting, preparation and dilution of test compound and cytotoxicity test by MTT-assay method. The stages will be explained as follows.

Making Complete Culture Media

The complete culture medium used was prepared from 20 ml of Fetal Bovine Serum (FBS) 10%, 2 ml of 1% Penicillin-Streptomycin 1%, 1 ml Amploerizine 0.5% and 200 ml Dulbecco's Modified Eagle Medium (DMEM) medium. The complete culture medium is mixed in the Laminar Air Flow and then stored in the refrigerator at ± 4 ° C (Bowles *et al.,* 2012).

HepG2 Cell Line Propagation

Frozen cell cultures obtained from the Parasitology Laboratory, Faculty of Medicine UGM, were taken from stocks stored in liquid nitrogen tanks at -190 ° C. Cell culture in the frozen state is then melted by immersing a culture container in water at ± 37.7 ° C before transferring to a sterile conical tube filled with 10 ml of complete culture medium. The cell suspension is then centrifuged at a speed of 1,500 rpm for ± 5 minutes. After that, the forming supernatant is removed using a micro pipette. Then given the addition of a complete medium of 1 ml on cell culture and resend slowly to homogeneous. The cell

is then transferred to tissue culture dish and grown to confluent in a CO_2 incubator with 37 ° C and 5% CO_2 flow. CO_2 incubator is used as a pH controller in high concentration cell culture. And media replacement is done every 24 hours incubation (Bowles *et al.*, 2012).

Harvesting HepG2 Cell Line

After the confluent cell culture amount (± 70%), the complete medium was removed from the culture container using a micro pipette. After that, the culture container was rinsed using PBS (Phosphat Buffered Saline) twice. Cell plus Tripsin-EDTA 0.25% as much as 2-5 ml to release the cell. Cells were incubated for 3 minutes in a CO_2 incubator until all the cells released into a single cell. The culture medium is added 5-7 ml into the culture container and the cell is resuspended until it is detached from the culture container wall. The cell suspension was then transferred into a sterile conical tube and sampled for calculated density using a Haemocytometer. This calculation is performed to determine the number of cells to be used later (Bowles *et al.*, 2012).

Preparation and Dilution of Test Compounds

Stock solution of test compound with concentration 100.000 µg / ml using DMSO solvent. This stock solution is diluted to the glow to obtain the test concentration (3.125; 6.25; 12.5; 25; 50; 100; 200 µg/ ml). The serial dilution begins by introducing 2 µl compound 1 stock solution into 998 µl of complete media to prepare a test concentration of 200 µg / ml. After the solution was resolved, the second dilution was carried out by taking 500 µl of solution from the first dilution then added to microtube containing 500 µl of complete culture medium. Next dilution is done in the same way until the seven series dilution obtained.

Test of Cytotoxicity by MTT Assay Method

The HepG2 Cell Line suspension of 100 µl is distributed into wells on a 96-well plate and incubated for 24 hours in a CO_2 incubator. After incubation, the culture medium is removed by way of tap over the tissue until clean. Added 100 µl of new culture medium into every 96 wells. It then incorporated 100 µl of test compound at various concentration series (3.125; 6.25; 12.5; 25; 50; 100; 200 µg/ml) with repetition 3 times each concentration. As a control cell was added 100 µl of cell suspension into a well containing 100 µl of medium. As medium control was added 100 µl complete medium, then incubated for 24 hours at 37 ° C in incubator with 5% CO_2 flow.

At the end of the incubation, complete culture medium that has been added the test compound was removed and 100 µL of MTT solution was added. Then the cells were incubated for 3-4 hours in a 5% CO_2 incubator at 37 ° C. The MTT reaction was discontinued with the addition of 100 µl of Sodium Dodecyl Sulfate (SDS-stopper) reagent. The microplate is then wrapped in paper and incubated for one night at room temperature and dark room. Living cells react with MTT to form a purple color and form formazan fibers. The test results were measured with a microplate reader at a 595 nm wavelength (Borra *et al.*, 2009). The absorbance result of the test solution is converted to percent cell proliferation (%). The value of inhibitory concentration 50 (IC_{50}) or on the amount of concentration of a compound can kill 50% of cell culture, obtained by probit analysis method through polynomial regression between concentration to probit value from percent inhibition of cell proliferation.

Results and Discussion

The cytotoxicity test of compounds 1 and 2 against HepG2 cell line (liver cancer cell) was performed using MTT method (3- (4,5-dimethyltiazole -2-yl) -2,5-diphenyl tetrazolium bromide). This method is based on the reduction reaction of the reductase enzyme characterized by the yellow reagent colour change of MTT reagent into a purplish blue formazane crystals (Riss *et al.*, 2013). Formazan crystal formation is proportional to the viability of cell culture (Septiseyani, 2014). The value of cell viability was measured using a Microplate reader at a wavelength of 595 nm (Borra *et al.*, 2009). The cytotoxicity test was performed as an initial process of toxicity potential analysis of both compounds on the growth of HepG2 cell line (liver cancer cells).

Morphological Analysis

Morphological analysis of liver cancer cells HepG2 Cell Line performed on the control and treatment of S1, S2 and Doxorubicin. Cell morphology analysis using compound microscope with 400x magnification. The normal morphology of HepG2 cancer cells is shown in Figure 1.

In the cell control treatment shown in Figure 2 the HepG2 cell line shows the characteristics of companion cells in which HepG2 liver cancer cells form

Fig. 1. Changes in Morphology of Liver Cancer Cells HepG2 Cell Line After Given Treatment of Test Compounds. HepG2 Cell Line Treatment Compound 1 (a); HepG2 Cell Line Treatment Compound 2 (b); HepG2 Cell Line Treatment Doxorubicin (c); Disconnection occurs between cells (1); and Changes in morphology of rounded cells (2). Observation on a 400x compound magnification microscope.

clusters (Stiborova *et al.,* 2011). Cells attached to the substrate (Khalili *et al.,* 2015). Normal HepG2 Cell Line has a mass form (strong intercellular attachment) (Gerets *et al.,* 2012). After being treated with the test compound, cell morphology showed a change in the absence of cell adhesion to the tissue culture wall, in addition the previously changed cell morphology of the polygonal became more rounded with a smaller size.

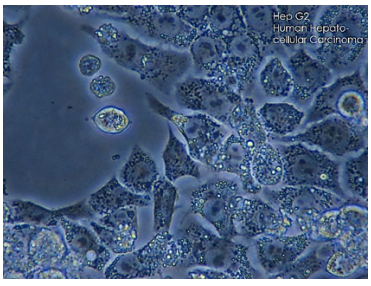

Fig. 2. Morphology of liver cancer cells HepG2 Cell Line normal

After the compound was treated, cell culture at all test treatments showed the intercellular attachment of cells that began to detach while the cultured cell cultures of compound 1 and doxorubicin experienced changes in cellular structure rounded and visible attachment between cells that began to detach. This alteration indicates that the test compound used has induced the structure of the HepG2 cancer cell membrane (Edmondson *et al.,* 2014) shown in Figure 1.

Morphological changes are seen in Figure 1 treated with compounds S1 and doxorubicin. Figure 1 parts a and c, HepG2 with the treatment of compound 1 and doxorubicin seen cells undergoing morphological changes are rounded cells and begin to experience release from each other. In addition to changes in the form of morphology, adherent properties in HepG2 cell culture also began to be unobserved. This indicates that the cell membranes begin to be disrupted resulting in the cell being no longer attached to the surface of the culture container.

Furthermore, for quantitative calculation of the number of viable cells, a test method was performed by giving MTT reagent. The more the number of cells viable, the reaction of MTT reagent reduction by mitochondrial reductase will increasingly occur, so that the concentration of formazan crystals formed will be higher. The formazan as shown in Figure 3 which is formed will have a crystalline

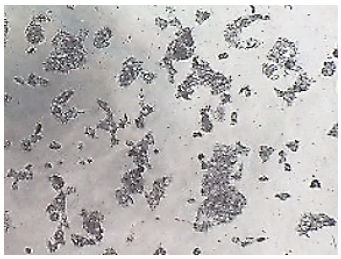

Fig. 3. Cell morphology of HepG2 Cell Line after treatment with MTT reagent. Observation on a 400x compound magnification microscope.

shape that is needle on the cell surface (Riss *et al.*, 2013). According to (Berridge, 1993) NADH and NADPH found in living cell mitochondria is a good substrate to accelerate MTT reduction.

IC50 Value The Fourth Test Compound

Results of cytotoxicity test of two compounds Deazellipticine on HepG2 cell line cancer, obtained IC_{50} value as follows:

Based on Table 1 above, the compound 1 (2-bromo-6- (5-bromo-3a, 7a-dihydro-1H-indol-3-yl) -5H-benzol [b] carbazole) and compound 2 (6- 3a, 7a-dihydro-1H-indol-3-yl) -5H benzol [b] carbazole) shows IC50 values in the high activity category. The best activity by compound 1 with IC50 value, 1.932 µM. The size of the 2 existing results is 2.990 ìM. Doxorubicin as a positive control has the best cytotoxic activity with a value of 0.642 µM. According to (Yang *et al.*, 2015) the limits set for natural materials that can be developed as anticancer are 0-6 ìM. The differences in the results of the cytotoxicity test of the two compounds obtained arise because of the addition of the group to the compound 1. Compound 1 given an additional bromo group, showed an increase in activity when compared to compound 2 without addition of the group. Bromo is one of the elements belonging to the VIIA group. One of the most basic chemical properties of bromo is to have a small electronegativity. As a strong oxidizing agent, bromo has a high reactivity to large elements or other chemicals. Various bromide compounds, both inorganic and organic, can be made relatively easily so that the bromo compound is a class with a very wide application in the industry (Lichota *et al.*, 2018).

Table 1. IC_{50} Value Four Compounds Test Against Cell Line

No	Compound	IC_{50}
1	Compound 1	1,932 µM
2	Compund 2	2,990 µM
3	Doxorubicin	0,642 µM

In compound 1, the H group is replaced by the group Br, in the periodic table, bromo is a reactive group that has a more stable property compared to the H group. The bromo group itself is one of the most commonly used anticancer compounds called cisplatin. Cisplatin or cisplatinum or cis diamminedichloro platinum (II) is a platinum-based cancer chemotherapeutic drug. Above cisplatin in general is a relatively active and straightforward form with all types of active molecules in biological systems including the bases of DNA (Reisz *et al.*, 2014).

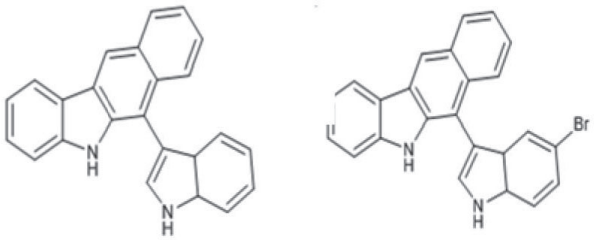

Fig. 4. Chemical Structure of Compound 1 (a) and Chemical Structure of Compound 2 (b)

The 5-Bromo-deoxyuridine compound in cisplatin is a pyruvate halogenation compound which will work by inhibiting hexokinase, which will transfer one phosphate from ATP to glucose molecule and 6-phosphate yield, according to article 6. In addition, 5-Bromo-deoxyuridine also works by inhibiting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by reaction with the nucleophile-SH side of the enzyme (Giacomini *et al.*, 2020).

In compounds 1 and 2 allegedly have the same mechanism in inhibiting the growth of cancer cells in liver cancer cells HepG2 cell line. The thing that distinguishes the two compounds is the ability of both compounds in inducing cancer cell membranes. In compound 1 it is thought to have a better permeability capability compared to compound 2. Therefore, it is suspected that compound 1 with the addition of bromo group can induce cell membranes faster than compound 2.

From several previous studies known ellipticine compounds have several mechanisms that can cause cancer cell death. One of the mechanisms of previously known ellipticin compounds is the ability to resist damaged cell cycles in order not to proliferate

continuously (Feitelson *et al.*, 2015). HepG2 liver cancer is damaged in G2 phase. In case of damage to phase G2 then check point will inhibit the cell to go to the next stage. At stage G2 there is a Mcdk protein. If McDk is active then phase G2 can enter phase M. If mutation occurs in G2, McDk protein will be inhibited by Cdk protein inhibitor so can not go to phase M. However, in liver cancer, cdk protein inhibitor mutation so that Mcdk remain active and cell will enter phase M despite mutations in phase G2 (Bowles *et al.*, 2013).

In addition, ellipticine is believed to be able to regulate the number of levels of cyclin B1 and Cdc2. Cyclin B1 is a regulatory protein that plays an important role in mitosis. Cyclin B1 is a check point in the cell cycle so that when a damage occurs at one stage in the cell cycle, cyclin B1 will perform a check point and the cell cycle will stop and make a correction in the previous stage (Feitelson , 2015). Several studies have also shown that ellipticine may inhibit the phosphorylation of P53 proteins by using Cdk2 kinase. The tumour suppressor protein, P53, is thought to be involved in the induction of apoptosis. Ellipticine is believed to induce P53 protein and induce p21, Waf1 and MDM2. According to (Aleepee, 2015), ellipticine has the ability to damage DNA so that the P53 cell death regulator protein induces and activates the apoptotic pathway. Protein P53 and Akt kinase work together to activate the P53 pathway in apoptosis. Kinase Akt is an enzyme that provides signals in the apoptotic process. In the cell cycle process, ellipticine can hold the cell at the initial G0 / G1 stage so that if an error does not go to the next stage that is G2 / M.

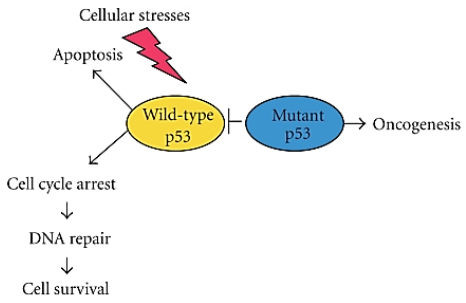

Fig. 5. Regulation of P53 (Aleepee, 2015).

Ellipticine is also capable of inducing apoptosis through an interinsic pathway in apoptosis. Mitochondrial stress induces intrinsic path apoptosis caused by chemical compounds or loss of growth factors, causing mitochondrial disturbance and cytochrome c release from mitochondrial intermembrane. The capcase-8 protein will bypass the Bcl-2 family member Bid. Then the cut bid at the end will induce Bax insertion in the mitochondrial membrane and release the proapoptotic molecules such as cytochrome c, Samc / Diablo, Apoptosis Inducing Factor (AIF), and omi / Htr2 (Zhao *et al.*, 2014). The presence of dATP will form a complex between cytochrome c, APAF1 and caspase 9 called apoptosom. Furthermore, capcase 9 will enable downstream procaspase-3. Active caspase 3 proteins break down a wide variety of substrates, including DNA-repair enzymes such as poly-ADP Ribose Polymerase (PARP) and DNA protein kinases, namely cellular structural proteins and nuclei, including the core mitotic apparatus, lamina nucleus, and actin and endonuclease, such as Caspase- Activated Deoxyribonuclease Inhibitor (ICAD) and other constituents (Aleepee, 2015).

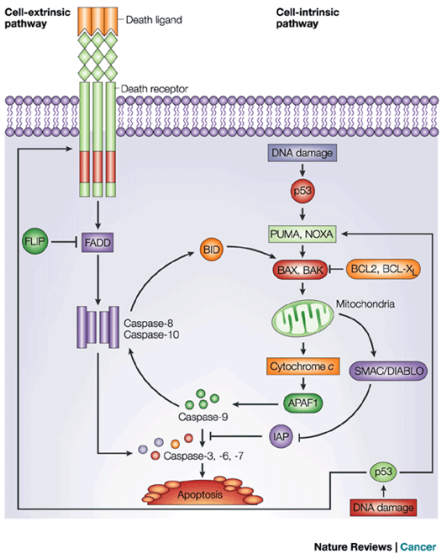

Fig. 6. Ellipticine Mechanisms Through the Intrinsic Pathway of Apoptosis (Aleepee, 2015).

In addition, caspase 3 also has the ability to activate other caspases, such as procaspase-6 and procaspase-7 that amplify cellular damage. The presence of cellular stress increases the expression of p53 protein as shown in Appendix 8 which results in the occurrence of G1 arrest or apoptosis. Members of Apoptosis Stimulating Protein p53 (ASPP) ASPP 1 and ASPP 2 specifically stimulate p53 transactivation function in proapoptotic gene promoters such as Bax and p53 Inducible Gene 3 (PIG 3), but not in the gene promoter causing cell cycle

arrest, ie p21 and MDM2 (Wang *et al.,* 2013).

Based on the results of the cytotoxicity test, it can be said that the addition of bromo groups in compound 1 shows an increase in cytotoxicity activity compared with compound 2 which was not given the addition of clusters. However, the results of the cytotoxicity tests of compounds 1 and 2 have a value that is no better than the chemopreventive agent is Doxorubicin. This is because doxorubicin is a chemopreventive agent that has been frequently used in the treatment of cancer. According to (Lichota *et al.,* 2014), compounds with IC50 values ranging from 1-10 μM have potential as anticancer. This shows that ellipticinehas potency which can be used as alternative anticancer agent of doxorubicin. Doxorubicin is capable of causing cell death by mechanism of stabilizing Topoisomerase II, in which Topoisomerase II functions in supercoiling DNA (maintaining double-stranded DNA) (Lichota *et al.,* 2018).

In this study, Doxorubicin has a better value than the ellipticine compound due to the affinity of the compound from doxorubicin. The affinity of a compound is based on two characteristics: the molecular weight of the compound and the molecular structure of the compound. The molecular weight and complexity of a compound give more toxic effects on cancer cells. Therefore, doxorubicin which has a greater molecular weight and more complex molecular structure has better toxicity than ellipticine (Lichota *et al.,* 2018).

Conclusion

Based on the result of cytotoxicity test that has been done, it can be concluded that two deazaellipticine have the potential of cytotoxicity against HepG2 cell line cancer cell with the value of IC50 of 1,932 (compound 1), 2,990 (compound 2), and 0,642 ìM (Doxorubicin).

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