International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2015): 6.391

Inhibition and Anti Proliferation Human Tongue Cancer Cells Supri's Clone-1 Invasion with Cyclooxygenase-2 Inhibitor

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Abstract: ***The aim of this study was to examine inhibition and proliferation of tongue cancer cells by using the SP - C1 chemopreventive drug, Celecoxib.In the method of invasion, tongue cancer cells treated with SP - C1 Celecoxib at concentrations of 5 µm, 10 µm, 25 µm, 50 µm, 75 µm, 100 µm and 125 µm, and 0 as the control group. Tests were carried out for 24 and 48 hours with the calculation of the mean tongue cancer cell invasion SP-C1. Invasion tests used Boyden Chamber Assay and polycarbonate membrane. In proliferation test, Tongue cancer cellsSP-C1 were treated with various concentrations ofCelecoxibandtime.192 cancer cells was grouped in 4 different groups of observation time, each group have 8cancer cells, one without Celecoxib treatment (control), 5 µm, 10 µm, 25 µm, 50 µm, 75 µm, 100 µm and 125 µm. Mean growth inhibition of cancer cells SP-C1 was calculated using BioradMicroplate Reader 540 nm. The results of the study showed the average number of tongue cancer cell invasion SP-C1 after the administration Celecoxib based on the concentration and time with ANOVA gives F = 60.46, which statistically significant. In zero concentration of Celecoxib(control) was 24.4 with Celecoxib concentrations ranging from 5 to 125 had decreased from an average of 11 to 2.3. In a proliferation test, results were statistically significant. Time factor (calculated F = 45.78), concentration factor (F count = 17.77), and time and concentration interaction (F count = 46.64) had showed the effect of different views of the average cell growth inhibition. The conclusion of this research Celecoxib delivery on tongue cancer cells showed SP - C1 barriers invasion and proliferation. Greater concentration of Celecoxib administered will provide a greater effect on the barrier invasion and proliferation of tongue cancer cells SP-C1.***

Keywords: Invasion, Proliferation, Cancer Cells tongue SP-C1, Celecoxib

1. Introduction

Squamous cell carcinoma is a malignant tumor derived from epithelial tissue with a cell structure in groups. Squamous cell carcinoma is able to infiltrate through the bloodstream and lymphatic spread throughout the body.[1] Squamous cell carcinoma is the most common type of cancer that occurs in the oral cavity which is about 90-95% of the total malignancy in the oral cavity. Location squamous cell carcinoma of the oral cavity is usually located on the tongue (ventral, and lateral), lips, floor of the mouth, buccal mucosa, and retromolar area.[2,3]

Squamous cell carcinoma of the tongue is a malignant tumor derived from epithelial mucosa of the oral cavity and is largely a type of carcinoma of the tongue squamous cell carcinoma epidermoid.[3]Ranged from 25 to 50 % of all malignant cancers in the mouth, squamous cell carcinoma of the tongue has a poor prognosis, so early diagnosis is necessary especially if there has been a metastasis to other areas (neck and cervical). Carcinoma of the tongueis often found together with syphilis and premalignant diseases such as leukoplakia. According to research oferythroplasia byFrazell and Lucas, tongue cancer cases occurred at dorsum of the tongue was only 4%, but were more malignant (Undifferentiated epidermoid carcinoma).[4]

Cell proliferation is an increase in the number of cells with a process involved cell cycle. Cell cycle in normal cells is strictly controlled. Cyclins, CDK, and CKI's workis mutually sustainable. Cyclins, CDK, and CKI together are responsible for controlling various phases of cell cycle control mechanism through the check point. Basicsettings of cancer cell's damage mechanisms, which control the regulation of growth of normal cells disruption, are resulted in uncontrolled cell proliferation. Celecoxib's effect as an anticancer intervention contained in the cell cyclecause a decreased in the expression of cyclin A, B and D, increased expression of $p21^{cip1}$ and cell cycle inhibitors $p27^{kip1}$ and loss of CDK activity. This condition led to inhibiting proliferation in cancer cells.[5]

Squamous cell carcinoma of the tongue is occured due to loss of control of the cell cycle, which control cell survival (loss of apoptosis), and the control of cell motility (increased activity of invasion and metastasis).[6,,7]The process of formation of squamous cell carcinoma is a gradual process, which is due to the growth regulators malfunction (protooncogene and tumor inhibitor gene) resulting in increased production of growth factors and the number of cell surface receptors, stimulate intercellular signal transduction, and increasing production of transcription factors.[6,7,8]Lethal traits of cancer is the ability to invade the tissues around, spread throughout the body, and metastasize to other region in the body.[9, 10]

There were two important facts on the use of a combination of Non-Steroid Anti-Inflammatory Drugs (NSAID) with radiotherapy in the treatment of cancer. First aspect is prevention (preventive cancer) which then affects the long-term effects. The second aspect is to be used in cancer treatment (curative). Celecoxib (one class of NSAIDs) can inhibit the proliferation and invasion of cancer cells and kill

cancer cells (in some percentage). Celecoxib as an anti-cancer play role on intervention in the cell cycle.[11,12]

Celecoxib has anti-cancer effects by altering protein expression that stopping the cell cycle and lead to cell death. Cells move through three phases before they divide, the G1 phase, the cell grows and makes the proteins, the S phase, cells make DNA and chromosomes copies, and G2 phase, in which cells is prepared to divide. The cell cycle also has several checking points that function in deciding that the cell is ready to move to the next phase. Celecoxib is able to alter the expression of the protein at the G1 check point and significantly decreases the activity of a protein called E2F1. These activities are necessary for the cells to move through the cell cycle and are also important for the activity of S phase in DNA repair. Inhibition of E2F1 activity by Celecoxib is a mechanism of S phase -specific toxicity to cancer cells.[13]

COX-2 is involved in the change of procarcinogens into carcinogens, and has an important role in initiating tumor formation. COX-2 is a key regulatory enzyme in the synthesis of prostaglandin E2 (PGE2), which is important to increase tumorigenesis. Increased expression of COX-2 inhibits apoptosis and is involved in angiogenesis. COX-2 inhibitors inhibit intestinal polyps and colorectal tumours.[13,14]

2. Materials and Methods

The whole study was conducted byexperimental laboratory using cultured human tongue cancer cells Supri's-Clone (SP-C1). The study was conducted at the Laboratory of Integrated Research and Testing (LPPT) GadjahMada University in Yogyakarta from July to October 2014. The study of cancer cell invasion test SP-C1 commenced using *boyden chamber assay* and membrane polykarbonat preceded by dilution of the drug Celecoxib (Celebrex) to adjust the number of research samples of tongue cancer cell SP-C1 then with the activation and propagation of cancer cells SP - C1 in culture media (DMEM , FBS and Fungizone).

Using a Boyden chamber invasion assay, polycarbonate membrane layer discs were placed in cell disc culture (petri dish), given the mill-Q solution and incubated at 37^0C. Boyden chamber was opened and the lower chamber (holes) was inserted media experiment/control (Celecoxib) with each concentration of 5, 10, 25, 50, 75, 100, and 125. Cancer cells inserted into the upper wells Boyden chamber as much as 50 µL, respectively. Both *Boyden chamber* for 24 and 48 hourswas being put back into the incubator with a wet paper towel to get the moisture at a temperature of 37°C. Cell count was commenced at polycarbonate membranes (24 and 48 hours). Membranes were mounted on a glass object to do the calculation.

Cellproliferationbarriertest with MTT Assay was performed by preparing 4 plates containing 96 hole. MTT assay testing hours to 0, 24, 48, 72 hours. In each plate, tongue cancer cells SP-C1 were inserted as much as 5 X 10^3 cells/holes in 100 µL DMEM (*Dulbelco's Modified Eagle Medium*) in accordance with the concentration of Celecoxib. Based on the calculation, total numbers of cells required were 12.8 X 10^5 cells for all of the holes and the amount of DMEM solution was as much as 25.6 ml. Cancer cell's calculation was determined using a hemocytometer. 96 holes plates were measured with a *Bio-radMicroplate Reader* OD at 540 nm wavelength. The same test was done at 24, 48 and 72 hours.

3. Results and Discussion

The calculation results of tongue cancercellsSP-C1commenced in 24houronpolycarbonatemembranesunder a light microscope appeared to have changed significantly tocancercellbarriersSP-C1, rangingfromconcentrations5 test concentrations125. In addition, when compared the control group at24hoursand48hours, the number oftonguecancercellsSP-C1 was still experience an increased number of reinvasion after the calculations done on the research data span of48hours.

Table 1. Invasion analysis of Cancer Cell SP-C1

Time	Concentrations (B)								Total	Mean
	control	5%	10%	25%	50%	75%	100%	125%		
24 hours	95	65	40	24	22	18	16	13		
	96	52	39	25	19	18	14	12		
	94	54	40	23	23	17	13	9		
Total	285	171	119	72	64	53	43	34	841	
Mean	95,0	57,0	39,7	24,0	21,3	17,7	14,3	11,3		35,0
48 hours	98	30	27	22	19	18	11	7		
	99	32	24	20	20	16	15	6		
	103	32	26	21	19	17	13	7		
Total	300	94	77	63	58	51	39	20	702	
Mean	100,0	31,3	25,7	21,0	19,3	17,0	13,0	6,7		29,3
Total	585	265	196	135	122	104	82	54	1543	

When the amount oftonguecancercellsSP-C1 in control group was calculated, the control group was also compared after 24hours. The results of the 48hours' group calculation showed an increased percentage of 5%. The result indicated

cancer cells without Celecoxib will yield an increased in the number of SP-C1tongue cancer cells.

Table 2. Comparison of the number of cell invasion by time and drug concentration

Time	Concentration									F count	p
24 hours	Mean (SD)	95,0 (1,0)	57,0 (7,0)	39,67 (0,577)	24,0 (1,0)	21,33 (2,082)	17,67 (0,577)	14,33 (1,528)	11,33 2,082)	311,920	<0,001
	Range	94-96	52-65	39-40	23-25	19-23	17-18	13-16	9-13		
48 hours	Mean (SD)	100 (2,646)	31,33 (1,155)	25,67 (1,528)	21,0 (1,0)	19,33 (0,577)	17,0 (1,0)	13,0 (2,0)	6,67 (0,577)	1209,220	<0,001
	Range	98-103	30-32	24-27	20-22	19-20	16-18	11-15	6-7		

From the results of this study, we found the resistance of cancer cell invasion by a given concentration of 5µm to 125µm showed significant changes, seen in the decrease in the number of cancer cells in the Boyden chamber based on the test barrier invasion of cancer cells' calculations, either in the span of24hoursandin the span of48hours. Based on the results of research on barriers tongue cancer cell invasion on the SP-C1 treated with Celecoxib concentrations, respectively5, 10, 25, 50, 75, 100, 125, and without celecoxib (control) were conducted during the next 24hoursand48hours, we concluded that time, concentration, and the interaction between time and concentrations showed different effects.

The results of time factor using ANOVA showed F = 80.504countwhich was statistically significant. The result showed time factor's influenced the activity of Celecoxib. The longer time passed, the decreased cell invasion could be seen from the average of cancer cell invasion (35.042 at24 hours showed a difference from the average of48hours' value =29.250). The result of concentration factor with using ANOVA gives Fcount=984.880 was statistically significant meaning the average occurrence of induction was seen due to Celecoxib usage compared with certain concentrations of Celecoxib showed there were no difference. The result from group without Celecoxib(control) was 97.50 with Celecoxib's concentration from 5to125had undergone a change with average values of44to 9.00. The interaction between time and concentration showed calculated F= 27,790whichwasalsosignificant.

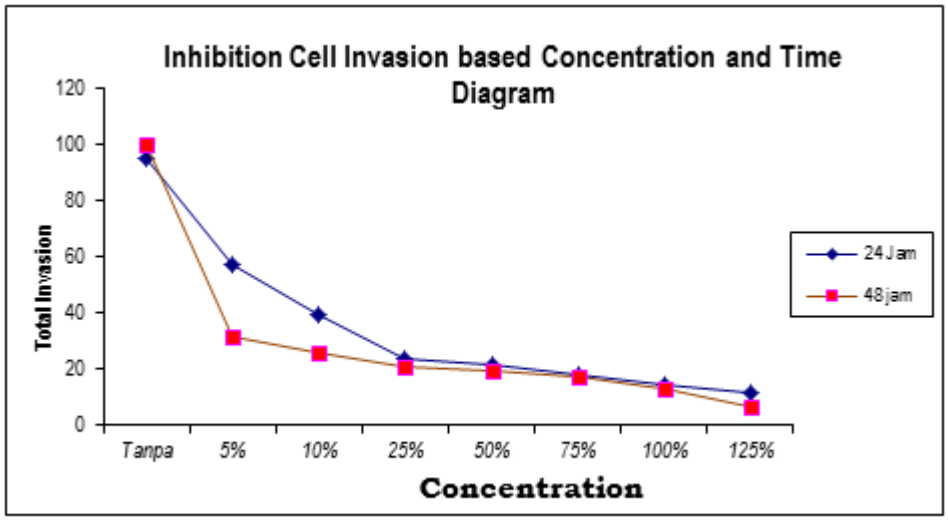

Figure 1. Mean tongue cancer cell SP-C1's invasion.

The results of this study was supported by the research of Lucille et al (2007) which stated 10 µm Celecoxib inhibited cell invasion or migration through collagen type 1 matrix approximately 40 % within 24 hours. Zymography results stated the presence of 10 µm Celecoxib showed enzyme activity of MMP - 2 and MMP-8 decreased about 30-40 %. This vitro study also showed that there were barriers' proliferation and invasion of squamous cell carcinoma by specific COX-2 inhibitor. Celecoxib produced anti-cancer effects through variation in cellular and molecular mechanisms.[15]

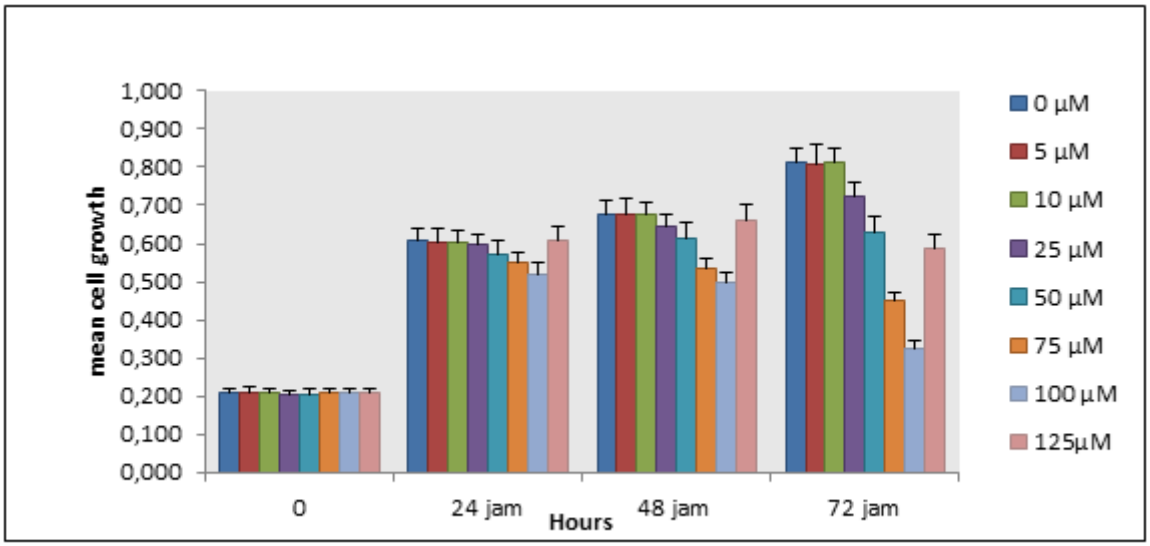

Figure 2. Mean cell growth rate based on the time and concentration of Celecoxib.

The inhibition of SP-C1 cancer cell invasionbased on the concentration and time showed Celecoxib would slow the proliferation and invasion of cancer cells and killed cancer cells. Celecoxib has an anti-cancer role in intervention on cell.[16]Cycle enzyme cyclooxygenase (COX) which is the target of action of non-steroid anti-inflammatory drugs (NSAIDs) has two isoforms, namely COX-1 and COX-2. Both of these enzymes catalyze the reaction and produce the same product, namely prostaglandins, but with different biological functions. [16.17]

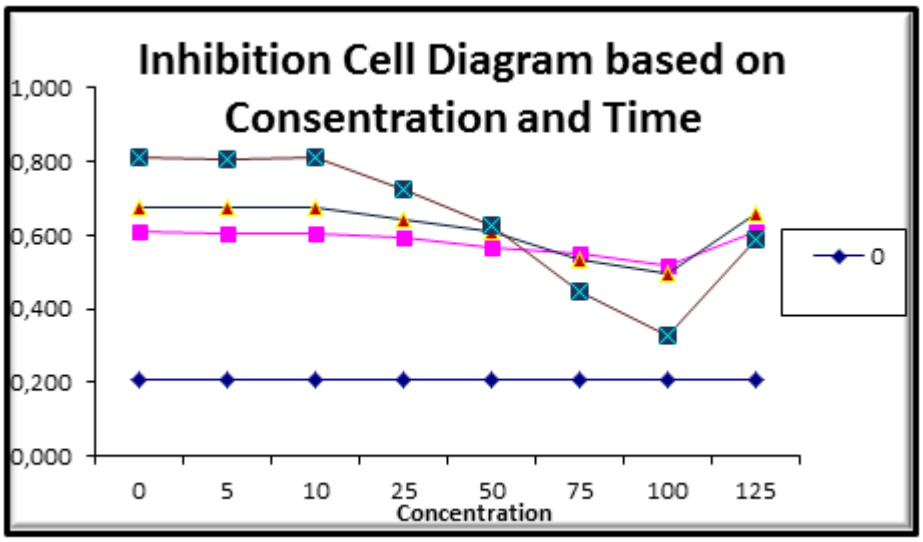

Figure 3. Cell growth rate based on the time and concentration

In proliferation barriers's test, the effective cell growth inhibition concentrations of Celecoxib began at 25 µm and 100 µm. It was the most effective concentration. This study was similar with Wang et al showed the decreased expression of pancreatic cancer cells was decreased to 50 % at concentrations of 25 µm and reached maximal inhibition at concentration of 100 µM.[18] At the concentration of 125 µm growth inhibition at concentrations was lower than 100 µm. This condition was happened because of the saturation from cancer cell's receptor. Cancer cell's receptors showed no response of Celecoxib with concentration of 125 µm. The saturation point where the cell is the cell can no longer receive the higher concentrations. Higher doses are no more effective in inhibiting the growth of cancer cells.

The mean values of cancer cells' growth inhibition by the time showed time factor affected the occurrence of inhibition. The longer the time, the lower the cell growth rate showed diminished number of cancer cells. In the hour - 0 cells were almost the same growth rate. The barriers occurred in the 24 to 72 hours. The most significant decline was seen in 72 hours. This condition occurred due to longer contact time between Celecoxib and the cancer cells, so that the drug action became more effective.

Molecular mechanisms that lead to the cessation of the cell cycle by Celecoxib are still not known for sure. Based on several studies, there are molecular targets that could lead to anti-proliferative Celecoxib, namely the inhibition of AKT signaling and NF-κB. Akt plays an important role in the regulation of cell cycles and proliferation defenses to cancer cells by affecting the phosphorylation status of Akt excessively. Akt signaling blockade lead to the cessation of growth inhibition and induction of cancer cells cycles'

apoptosis.[18,19,20] NF-κB is a transcription factor involved in broad cellular functions, including apoptosis and cell cycle control. NF-κB regulates the excretion of several gene products associated with carcinogenesis. Among the genes, anti-apoptosis gene, COX-2, and cell cycle regulatory genes NF-κB is maintained in the cytoplasm by the inhibitor protein IκB. Mediators in the transduction pathway can activate Akt through phosphorylation of NF-κB from IκB. NF-κB is activated by translocation to the nucleus, leading to transcription of several genes (eg,COX-2). As a result, the production of prostaglandins will further activate NF-κB. NF-κB activation can lead to excessive and permanent effects, through activation of COX-2 pathway. In addition to inhibiting the production of prostaglandins Celecoxib inhibits Akt by the inhibition of the Akt cannot activate NF-κB resulting in cancer cell proliferation resistance. [18,19,20]The results of this study indicated Celecoxib could inhibit SP-C1 cancer cell proliferation and the most effective concentration was 100 μm at 72 hours.

4. Conclusion

The results of this study showed the greater concentration of Celecoxib administered will provide a greater effect on cancer cell invasion barriers from tongue cancer cell SP-C1. On proliferation barriers, Celecoxib may inhibit tongue cancer cell SP-C1's proliferation. The interaction between time and concentration factor obtained a linear relationship, with the increasing time and concentration showed the more effective inhibition of Celecoxib. The most effective concentration and maximum inhibition the concentration of 100 μm at 72 hours.

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