Antiproliferation and Apoptosis Effect of Cisplatin and Nanocurcumin on Ovarian Cancer SKOV3 Cell

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ABSTRACT

Background: Ovarian cancer is one of the leading cancers in women. Seventy percent were founded in the advanced stage, with a 5-year survival rate of only 46%. The current treatment modality is cytoreduction with platinum-based adjuvant chemotherapy as the first line. The effectiveness of chemotherapy is only 60% in the advanced stage, later developing into recurrent. Therefore, additional types of therapy are required based on agents that work specifically in cancer cells and synergize with current standard treatments. This study aimed to know the anti-proliferation effect and apoptosis effect of combination cisplatin with nanocurcumin on SKOV3 cells.

Methods: This experimental study was conducted in vitro using the biological cell line SKOV3 to determine the anti-proliferation effect (expression Ki67) and the apoptosis effect (caspase 3 and 8) of combination cisplatin with combination with cisplatin nanocurcumin on the cell. The data were analyzed with unpaired T when regular distribution / Mann Whitney test when the distribution is abnormal and using Graph Pad Prism.

Results: Based on this result, 50cc of nanocurcumin is 67 µm, and 50cc of cisplatin is 54 µm, using the MTT Assay method. The viability of the cells in this study decreased according to the dose-dependent, whereas the combined dose of 134 µm nanocurcumin with 108 µm cisplatin found the lowest life cell, 24.3% (p <0.001). Ki67 expression was low in the SKOV3 cells post-exposure to cisplatin, but increased post-exposure to nanocurcumin, suspected nanocurcumin at certain doses of pro-oxidant properties that triggered proliferation. Caspase 3 and 8 cannot be detected in this cell by the ELISA method.

Conclusions: Nanocurcumin has a potential effect on chemosensitive cells. However, our study shows no healing effect in chemoresistance cells, particularly SKOV3.

INTRODUCTION

Ovarian cancer is one of the leading causes of death due to cancer in women, with 295,414 new cases and 184,799 deaths worldwide based on Globocan 2018.[1] In Indonesia, most patients came in advanced stage (54-67%),[2,3] and contributed to 58.9% mortality rate.[4] Standard management of ovarian cancer is a combination of cytoreductive surgery and/or chemotherapy with a platinum and taxane-based regimen.[5,6]

A 5-year overall survival rate of ovarian cancer is not satisfying, 79-90% for early-stage and 24-25% for the advanced stage. With current standard treatment modalities, extensive cytoreductive surgery (no macroscopic residual disease), and chemotherapy, the effectivity is 60 -89%.[7,8] Thus, we need to develop a new agent to increase chemotherapy's potential action, targeting several pathways of carcinogenesis and synergistically acting with standard chemotherapy. The agent should have potential cancer cell killing while maintaining the integrity and function of a healthy cell. One of potential agents is curcumin *1,7-bis(4-hydroxy-3-methoxyphenyl-1,6-hepta-dien-3,5-Dion),* extracted from Curcuma Longa (turmeric). Curcumin was known for its anti-cancer and anti-inflammation effects.[9] Several studies reported the effect of curcumin to induce apoptosis in vitro, but no report in the ovarian cancer cell. This study analyzed curcumin's apoptosis and anti-proliferation effect on ovarian cancer cell SKOV3.

METHODS

Cell culture, Sample Formation, Co-culture Experiment

This is an experimental study using ovarian cancer SKOV3 cells to analyze the effect of curcumin (nanocurcumin), cisplatin, and a combination of nanocurcumin and cisplatin on cancer viability, Ki67 expression, caspase 3 and 8. SKOV3 cell was obtained from ATCC (Catalog no HTB-77), Manassas, USA. Nanocurcumin was obtained from Plamed Green Science Ltd, China, with 331 µm particle size. Human CASP3 and CASP8 were obtained from Elabscience (E-EL-H0017 and E-EL-H0659, respectively). Ki67

expression was analyzed with Tali Image-Based Cytometer, Invitrogen.

We used 4 repetitions for each treatment to sample. The total sample is 24, consisting of 4 pieces of SKOV3 with solvent (DMSO); 4 samples of SKOV3 with five µmolar, ten µmolar, 20 µmolar, 40 µmolar, and 80 µmolar nanocurcumin; 4 pieces of SKOV3 with 2,5 µmolar, five µmolar, ten µmolar, 20 µmolar, and 40 µmolar cisplatin; 4 samples of SKOV3 with cc50 nanocurcumin and cc50 cisplatin; 4 samples with cell line treated with cc50 cisplatin and cc50µg/mL (-1 level); 4 samples with cell line treated with cc50 cisplatin and cc50µg/mL (+1 level). The outcome of each sample is the number of live cells, Ki67 expression level, caspase 3, and caspase 8 levels.

Nanocurcumin solution was prepared by diluting 37 mg nanocurcumin into 100 µL dimethyl sulphoxide. SKOV3 culture media was prepared by diluting blank media into a 370°C water bath, mixing 44 mL of it with 5 mL FBS, 500 µl *L-glutamine*, 500 µl *Penstrep* the mix with a filtering syringe into a tube. SKOV3 and medium were served in a 3 mL tube. Cryotube was melted in the water bath and mixed with DMEM medium. The solution was mixed by dripping it into the tube until it became homogeneous. The tube was centrifuged 1200 rpm for 5 minutes at 4°C; then the medium was placed into a small flask. The medium was added with 4 mL DMEM and shook until the cell was well-spread on the flask. The flask was incubated at 37°C for 2-3 days. The cell was ready to harvest if cell growth was confluent and the medium turned yellow.

Cytotoxicity of Nanocurcumin and Cisplatin on SKOV3

Cytotoxicity test on SKOV3 was tested by using MTT [3-(4,5-dimethylthiazol- 2-il)-2,5 diphenyltetrazolium bromide]. SKOV3 cell was distributed into 96 well plates for nanocurcumin and 96 well plates for cisplatin, repeated 3 times and incubated for 24 hours. Nanocurcumin was added with 5 different concentration 5 mM, 10 mM, 20 mM ,40 mM, 80 mM. Cisplatin was added with 5 different concentration 2.5 mM, 5 mM, 10 mM ,20 mM, 40 mM. DMSO 0.025% was used for control with similar concentration (5 mM, 10 mM, 20

mM ,40 mM for nanocurcumin control and 2.5 mM, 5 mM, 10 mM, 20 mM, 40 mM for cisplatin control) in each treatment. MTT test absorbance data was

read using an ELISA reader with a 560 µm wavelength. Data were analyzed after 3 times repetitions by using the formula:

$$\% \text{ live clls} = \frac{\text{experiment cell absorbance-media absorbance}}{\text{control cell absorbance-media absorbance}} \times 100\%$$

Figures 1 and 2 show nanocurcumin and cisplatin's toxicity levels on cell viability. Nanocurcumin and cisplatin dosages with 50% cell viability were 67 mm and 53.6 mm, respectively.

Ki67 Expression, Caspase 3 and Caspase 8 Analysis

The cell was observed with an inverted microscope. Confluent cell growth was indicated when media turned yellow and the cell was ready to be harvested. Culture cell was introduced with different doses of nanocurcumin and cisplatin: 67mm nanocurcumin, 54mm cisplatin, 33.5 mm nanocurcumin + 27 mm cisplatin, 67 mm nanocurcumin + 54 mm cisplatin, 134 mm nanocurcumin + 108 cisplatin. Ki67 expression was analyzed with Tali

Image-Based Cytometer, Invitrogen, while Caspase 3 and Caspase 8 were analyzed using the ELISA method. We observed the cell viability after 48 hours of treatment.

RESULTS

After 48 hours of treatment, a live SKOV3 cell was observed and calculated. The highest mean live cell (95.3%) was observed in SKOV3 with solvent treatment, and the lowest mean live cell (24.3%) was observed in SKOV3 with a combination of 134 µM nanocurcumin and 108 µM cisplatin.

An increased dose of a combination of nanocurcumin and cisplatin decreased the live-cell mean. Compared to 67 µM nanocurcumin alone, the combination of 67 µM nanocurcumin and 54 µM cisplatin has more live-cell mean.
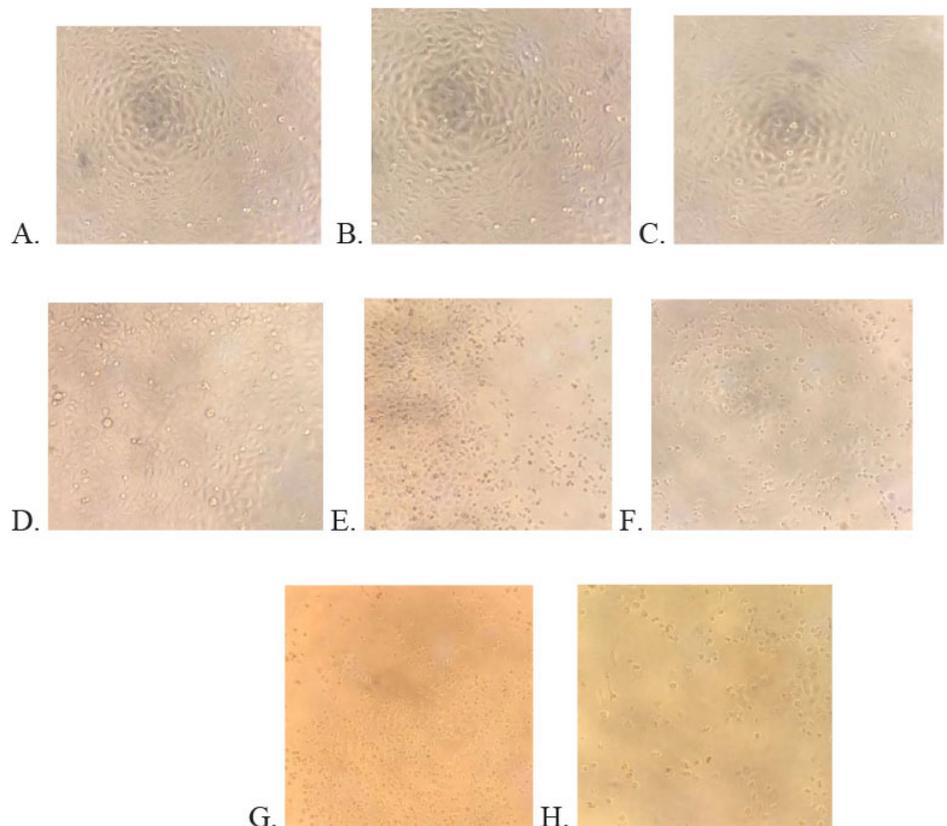

Figure 1. SKOV3 cell in 10x10 magnification.

(A). SKOV3 cell was confluent at >80% in 4 days post platting; (B). SKOV control cell before treatment; (C). SKOV3 cell in a solvent; (D). SKOV3 cell after treatment of 54 mm cisplatin; (E) SKOV3 cell after treatment of 67 mm nanocurcumin; (F). SKOV3 cell after treatment of 33.5 mm nanocurcumin and 27 mm cisplatin; (G). SKOV3 cell after treatment of 67 mm nanocurcumin and 54 mm cisplatin; (H). SKOV3 cell after treatment of 134 mm nanocurcumin and 108 mm cisplatin.

Expression of Ki67 level was low in control cell and solvent control but high in treatment cell. Even though the difference between control and treatment was high, it is not statistically different. The highest level of Ki67 expression was observed on 67 μm nanocurcumin + 54 μm cisplatin (96%), and the lowest was solvent control

(34%). The caspase 3 and Caspase 8 level test was started by testing different Caspase 3 and Caspase 8 optical density (OD) concentrations. R2 of Caspase 3 was 0.9893, and R2 of caspase 8 was 0.9405. This result means the curve is valid because the R2 value was closed to 1.

We observed a deficient absorbance level after treatment on the SKOV3 specimen. Cell control absorbance level of caspase 3 was 0.24 and had a similar absorbance level with the treatment of 33.5 μM Nanocurcumin + 27 μM Cisplatin and 67 μM Nanocurcumin + 54 μM Cisplatin. The highest absorbance level of caspase 3 (0.29) was observed on 134 μM Nanocurcumin + 108 μM Cisplatin. Caspase 8 absorbance level of cell control was 0.22. This result was similar to other treatments, such as 67 μM Nanocurcumin, 54 μM Cisplatin, 33.5 μM Nanocurcumin + 27 μM Cisplatin. The highest caspase 8 absorbance level was observed on 134 μM Nanocurcumin + 108 μM Cisplatin.

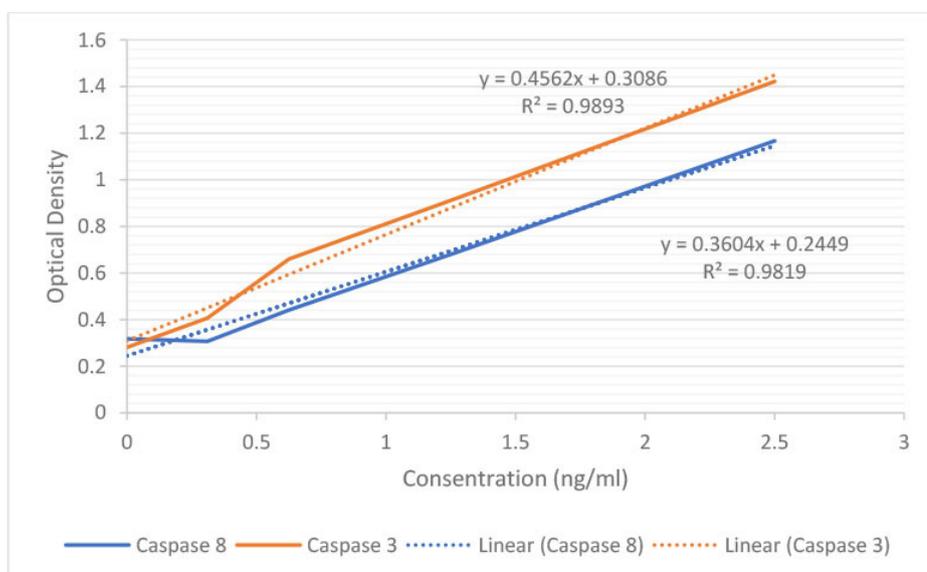**Table 1.** Mean of live SKOV3 Cell After 48 of Different Treatment.

Concentration	Live-cell mean (%)	SD	P-value
Solvent	95.3	1.5	
Nanocurcumin 67 μM	26.4	3.7	<0.001*
Cisplatin 54 μM	92.6	3.8	Reference
Nanocurcumin 33,5 μM + Cisplatin 27 μM	52.4	12.7	0.006*
Nanocurcumin 67 μM + Cisplatin 54 μM	29.6	0.9	<0.001*
Nanocurcumin 134 μM + Cisplatin 108 μM	24.3	2.5	<0.001*

*Independent t-test; significant if the p value<0.05

Table 2. Expression of Ki67 after 48 hours of Treatment on SKOV3 Cell.

Condition	Mean of Percentage	SD	P*
Cell Control	37.75	33.41	
Solvent Control	34	16.70	
Nanocurcumin 67 μM	92	6.55	0.083
Cisplatin 54 μM	58	25	Reference
Nanocurcumin 33.5 μM + Cisplatin 27 μM	71.67	23.02	0.523
Nanocurcumin 67 μM + Cisplatin 54 μM	96	6.08	0.062
Nanocurcumin 134 μM + Cisplatin 108 μM	94	3	0.131

*Mann Whitney Test; significant if the p value<0.05

Statistical Analysis

We analyze the data using SPSS 25. We calculate the mean difference using the T-test to analyze the different effects if the data is a normal distribution. In abnormal distribution, we used the Mann-Whitney test.

DISCUSSION

Ovarian cancer has an overall poor prognosis, despite advanced progress on operation techniques and chemotherapy. Overall survival was poor, especially in an advanced stage. Platinum-based chemotherapy is a first-line chemotherapy regimen for ovarian cancer. Ovarian cancer's resistance to platinum-based chemotherapy contributes to poor outcomes. Several mechanisms were proposed to explain the phenomenon, including mutation of p53, mutation of BAMBI, miR-141, miR-200c, epigenetic changes, and dysfunctional DNA repair.[10–12] Therefore, a new agent as an anticancer was intensively explored to improve the prognosis. Curcumin is well-known for its anti-cancer effect. Its effect on apoptosis knew the anticancer effect of curcumin, inhibiting cell proliferation, inducing cell cycle arrest via modulation of transcription factors, such as NF-κB,

Figure 2. Curve of Relationship of Optical Density and Caspase 3 and Caspase 8 concentration (ng/mL).

Table 3. Optical Density Mean of Caspase 3 and Caspase 8 Based on Different Treatment.

Treatment	Caspase 3	Caspase8
Cell Control	0,24	0,22
Solvent Control	0,25	0,22
Nanocurcumin 67 μM	0,25	0,22
Cisplatin 54 μM	0,25	0,22
Nanocurcumin 33.5 μM + Cisplatin 27 μM	0,24	0,22
Nanocurcumin 67 μM + Cisplatin 54 μM	0,24	0,23
Nanocurcumin 134 μM + Cisplatin 108 μM	0,29	0,24

STAT3, AP-1, Erg-1, p53, β-catenin, Notch-1, Hif-1, and PPAR-α.[13] Curcumin was expected to increase apoptosis measured by the level of caspase 3 and caspase 8, and the anti-proliferation effect measured by Ki67.

Because the main problem of ovarian cancer is always about chemoresistance, both primary and secondary cases, the primary case will respond directly to treatment, and the secondary is the recurrence case. We had not found any study for the chemoresistance cell. Therefore, in this study, we evaluate whether nanocurcumin can be used for platinum chemoresistance cells or not. The evaluation included the cell cycle arrest, which can be measured by Ki67 levels, and apoptosis using caspase 3 and caspase 8.

Our finding showed that nanocurcumin has a more cytotoxic effect than cisplatin on SKOV3 cells because of SKOV3 resistance to platinum-based chemotherapy.[14] SKOV 3 is a chemoresistance cell from clear cell-type carcinoma. A study by Kurman and Scully about clear cell shows that it has very low sensitivity to chemotherapy, it may be less on SKOV3.[15] Nanocurcumin with 67 μM dose alone has more cytotoxic effect than 67 μM nanocurcumin and 54 μM cisplatin. After the dose was doubled, the cytotoxic effect was not doubled. This condition showed nanocurcumin alone has a higher cytotoxic effect, and it seemed SKOV3 cell was resistant to SKOV3 cell.

The proliferation effect was measured by detecting Ki67 expression as cell proliferation antigen. The lower level Ki67 indicates that it is responding to therapy.[16] This study found no statistical difference in Ki67 level among treatments. Nanocurcumin seemed induced proliferation compared to cell control and cisplatin. When 67 μM Nanocurcumin and 54 μM Cisplatin were combined, proliferation was higher than nanocurcumin alone and cisplatin alone. Curcumin was known for its anti-proliferation effect on different cell lines.[9,17–19] This finding failed to show curcumin's effect on cell proliferation and could be a dose-related effect of curcumin on the NF-κB signaling pathway like the previous study. NF-κB signaling induced by TNF can induce pro-inflammatory genes, which lead to tumor progression.[20] Curcumin has a potential effect on lowering TNF concentration,[21] we did not measure TNF levels in this study. This finding suggested curcumin failed to suppress TNF in a given dosage.

This study found low caspase 3 and caspase 8 absorbance levels, without significant differences in different treatments, and failed to show the effect of curcumin and cisplatin to induce apoptosis on SKOV3 cells. It could be related to the resistance characteristic of SKOV3 on the apoptosis signaling pathway. Therefore, we need other studies in the future to discover the new agent that will increase chemotherapy's potential action and synergistically act with standard chemotherapy.

CONCLUSION

In the previous study, nanocurcumin might affect the chemo-sensitive cell. However, our study does not show any effect or activity on the chemoresistance cells. There is no additional benefit from adding nanocurcumin to the standard chemotherapy because it offers no healing activity in the chemoresistance cell or the SKOV 3 cell particularly.

AUTHOR CONTRIBUTION

Sigit Purbadi, Muhammad Yusuf, Hariyono Winarto, Andi Darma Putra: conceptualization, methodology, resources, supervision. Sigit Purbadi, Muhammad Yusuf, Wawaimuli Arozal, Aroem Naroeni: experimental studies, data acquisition, data analysis. Sigit Purbadi, Muhammad Yusuf, Gilbert Elia Sotarduga: literature search, writing-original draft preparation, writing-review and editing.

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