Metformin Inhibits Growth of Breast Cancer Cell T47 through Decreasing Expression of Protein P53, BCL2 and Cyclin D1

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Abstract
Breast cancer is a disease that afflicts women only 0.5 to 1 % are male breast cancers. Breast cancer has several variants and requires a different therapeutic approach, and until now the therapy has not been satisfactory due to the emergence of resistance. Metformin as the main choice drug type 2 diabetes mellitus which is known to have a cytotoxic effect for breast cancer. This study aimed to analyze metformin cytotoxic mechanisms covering the cell cycle, apoptosis, expression of p53, bcl-2 and cyclin D1 T47D cells which exposed to metformin HCl. The study was conducted invitro on T47D breast cancer cells which exposed to metformin concentrations of 1738.2 µg / mL and 3476.4 µg / mL and doxorubicin concentrations of 0.1µg / mL and 0.2µg / mL for 24 hours. Cell cycle testing and apoptosis using the flowsitometry method and expression test of p53 protein, bcl-2 dancycline D1 in T47D cells with immunocytochemistry. Data was analyzed by one way Anova with Bonferroni's advanced test. The results showed that metformin inhibited the G0-G1 phase of the T47D cell cycle, triggered T47D cell apoptosis, significantly reduced p53, bcl-2 and cyclin D1 protein expression (p <0.05). Conclusion of the study, metformin inhibits T47D cells through inhibition of the cell cycle G0-G1 phase, reducing protein expression p53, bcl-2 and cyclin D1.

Keyword: Metformin; T47D Cell; Cytotoxic; Cell Cycle; Apoptosis;P53; bcl-2; Cyclin D1
Introduction

Cancer is one of the serious world health problems and in 2008 there were 12.7 million new cancer cases and 7.6 million died of cancer. The most diagnosed cancers worldwide are lung cancer (1.6 million, 12.7%), breast cancer (1.38 million, 10.9%) and colorectal cancer (1.23 million, 9.7%) \(^1\). The 2012 Globocan statistics showed that around 1.7 million women were diagnosed with breast cancer with a mortality rate of around 522 thousand\(^2\). Clinical breast cancer is classified according to morphological characteristics including infiltrating ductal carcinoma, infiltrating lobular carcinoma, tubular, mucinous, medullary, and adenoid cystic carcinoma. Classification based on the expression of estrogen (ER) receptors and progesterone (PR) and Her2 oncogene\(^3\).

The types of breast cancer are diverse according to various therapeutic approaches. It reported that many kinds of therapy have been carried out so far; some of them had problems including therapeutic effectiveness that varies among individuals or chemotherapy resistance made new problems\(^3\). This study focused on the Metformin, 1, 1-dimethyl biguanide is derived from galegine alkaloid or isoamyl guanidine, the active substance of Gallegaofficinalaes, also known as Goat'es Rue. It has been used since 1958 as a type 2 diabetes mellitus drug in the United Kingdom, and 1995 at United States and recommended as first line therapy for all patients newly diagnosed with type 2 DM by the 2014 American Diabetes Association\(^4\). Previous studies showed a reduced risk of cancer in type 2 DM patients by using metformin compared to patients who did not use metformin such as insulin, sulfonylureas, sitagliptin and pioglitazon\(^5, 6\). In practice, metformin works by inhibiting complex I respiratory chains in mitochondria \(^5, 7\) where the mitochondrial respiratory chain functions to produce ATP\(^8\). Complex I barriers in the mitochondrial respiratory chain resulted in the decreased cell ATP concentrations and increased AMP, then stimulated AMP-Kinase\(^9\). Set the activated AMP-Kinase would phosphorylate various substrates that played a role in cell life \(^10\).

It found that the research in invitro has been done including among several types of cancer cells such as WiDr cells \(^11\), Hep G-2 cells \(^12\), MCF-7 cells \(^9\), MDA-MB-231\(^13\) and MDA-MB-435 all showing suppression effects of cancer cell growth. This study used T47D cancer cells, ductal type breast cancer cells that express p53 proteins that might be mutated (missence mutation) at residue 194 (in the zinc-binding domain, L2) \(^14\). p53, a protein encoded in the TP53 gene, a tumour suppressor gene (tumour suppressor gene) which acts to block cell changes into cancer cells through three inhibiting mechanisms, namely quiescence, induction of permanent cessation of cells (senescence) or spurring programmed cell death (apoptosis) and barriers to angiogenesis \(^15\). Thus, p53 causes anti-apoptotic effects occurs through its bond with bcl-2, a family anti-apoptotic protein from Bcl-2. Then p53 activates the proapoptotic bcl-2 protein family, as a result there is an increase in permeability of the mitochondrial membrane, cytochrome c is released and finally apoptosis occurs through activation of the caspase\(^16\). The p53 also has an effect on the cell cycle,
where p53 acts as a negative regulator protein and cyclin kinase groups *Cyclin Dependent Kinase* (CDK) as a positive regulator of cell cycle\(^{16}\).

It found that p53 mutations in breast cancer cells contribute to tumour formation, because it increases the breast cancer cell defence through the effects of cell apoptosis \(^{14,18,19}\) and also increases resistance to chemotherapy \(^{18,19}\) genomic instability, disruption of spindle checkpoint control\(^{18}\) and mutant p53 protein that have shown to be activators a number of genes, including c-Myc, topoisomerase I and MDR-1\(^{20}\).

Early research conducted by Rangkuti et al., 2018 showed that metformin had a cytotoxic effect on T47D breast cancer cells with IC\(_{50}\) 1738.2 µg / mL, much smaller than the control of doxorubicin with IC\(_{50}\) 0.1 µg / mL\(^{21}\). The mechanism of inhibition of T47D cell growth is due to exposure to metformin. It requires further investigation of the cells cycle and apoptosis along with the proteins that play a role in it.

**Materials and Method:**

*Cell line and reagent*

This research used the T47D breast cancer cell line which was provided by the Faculty of Medicine, GadjahMada University of Indonesia. Cells are cultured in RPMI 1640 media (Sigma, USA) with 10% fetal bovine serum (Thermo, Chile), under standard culture conditions (37 oC, 95% humidified water, and 5% CO2). Metformin was produced by PT. DexaMedica Palembang (Indonesia) and Oxoxorubicin of PT. Ferron Parr Pharmaceuticals of Indonesia. This study used the anti-p-53 antibodies; anti bcl-2 and anti-cyclin D1 (Sigma, America). The serial dilution solution of the sample is prepared immediately before the experiment was carried out.

*Cell cycle testing and apoptosis*

Apoptosis and T47D cell cycle testing were using the method of flow cytometry. The number of cells needed 5 x 10\(^5\) cells / well on the 6-well-plate, incubated for 24 hours, the next day the cell plus the metformin solution, was re-incubated for 24 hours. Then the media of each well is inserted in each 15 mL cone tube, washed with PBS 1 time re-enter the same cone. Added 250 µL trypsin to each well then incubated for 3 minutes. After that, plus 1 mL of culture media; then the media was accommodated in the same cone tube. Centrifuging at a speed of 600 rpm for 5 minutes, the supernatant was discarded. Added 1 mL PBS, the media was transferred to a 1.5 mL microtube; it was centrifuged again at a speed of 2000 rpm for 3 minutes, after which the supernatant was removed. Cell cycle testing plus iodiapropidium was done while apoptosis testing plus Annexin V and iodiapropidium were made. Then it was measured by means of the FACS Calibur (Beckton Dickinson) flowsitometer\(^{22}\) The cell pellets were suspended in 50 µg / ml propidium iodide in the presence of 100µg / ml RNase and triton-x solution and incubated for 10 min at 37ºC in the dark. Cell cycle distribution was analyzed by using the Cell Quest program.
Immunochemistry

Analysis of protein expression of p-53, Bcl-2 and cyclin D1 was using T47D cells grown Immunochemistry. Before T47D cell is being planted, first put each coverslip in a 24-well pitting plate. The cell planted at a density of 5 x 10^4 cells / wells, incubated for 24 last hours then the media discarded. Cell that being incubated then added with metformin test solution, incubated back for 24 hours. After incubation, the metformin test solution and the culture media is removed, washed with PBS one time. Cells were fixed with cold methanol (Merck) for 10 minutes and discarded. After that, coverslip on each of the wells is removed and placed on a glass slide, washed with PBS 2 times, washed with distilled water and then got dried; add the prediluted blocking serum at room temperature for 10 minutes, then discarded. After incubation, the monoclonal antibodies p-53 / Bcl-2 / cyclin D1 was added to the cells and then incubated for 1 hour at room temperature. Coverslip then washed with PBS and etched secondary antibody (Biotinylated universal secondary antibody) (Star Trek Universal HRP Detection Kit, Ref. STUHRP 700L10- KIT, Biocare Medical) and incubated for 10 minutes. The cells washed again using PBS and etched with streptavidin enzyme horse radish peroxidase (HRP Star Trek Universal Detection Kit, Ref. STUHRP 700L10-KIT, Biocare Medical) and incubated for 10 minutes. Cells were washed and then added 3,3'-diaminobenzidin (DAB) (Star Trek Universal HRP Detection Kit, Ref. STUHRP 700L10-KIT, Biocare Medical), incubation for 2 minutes (until the brown color). Cells were washed again with PBS and distilled water then with a solution Mayer-Haematoksilin (Dako) and incubated for 5 minutes, then cells were washed again using distilled water to clean, add 70% ethanol incubation for 2 minutes, wipe, then dropped into a solution of xylol and dry. After drying, coverslip placed on a glass slide and a few drops of mounting media and covered with a cover glass. Cells observation was performed using Olympus brand light microscope with 400 x.

Results: Metformin inhibits the G0-G1 Phase of the T47D Cell Cycle

Previous research showed that the metformin has a cytotoxic effect against cancer cells T47D. To find its effect, then a test to determine the cell cycle bottleneck effect of metformin on cell cycle was administered. It was made by the exposure to metformin concentrations of 1738.2 mg / mL and 3476.4 g / mL for 24 hours which showed the accumulation of cells in G0-G1 phase; that is dose dependent, whereas exposure to doxorubicin concentration of 0.1 mg / mL and 0.2 mg / mL for 24 hours showed barriers G0 phase of the cell cycle at the G2-M-G1d and (Figure 1 and Table 1).
Fig 1. T47D Cell Cycle Histogram in (A). Control Cell (B). Exposure to Metformin concentration of 1738.2 µG / mL (C). Exposure to Metformin concentration 3476.4 µG / mL (D). Exposure to Doxorubicin concentration 0.1 µG / mL (E). Exposure to Doxorubicin concentration of 0.2 µG / mL.
TABLE 1. Percentage of Accumulation in each phase in the T47D Cell Cycle

| Kinds of Treatment | Cell Cycle Phase (%) |
|--------------------|----------------------|
|                    | G0-G1    | S       | G2-M    |
| Control Cell       | 49.20    | 20.93   | 30.15   |
| Metformin 1738.2 µg/mL | 52.99    | 22.03   | 25.36   |
| Metformin 3476.4 µg/mL | 54.48    | 22.07   | 23.79   |
| Doksortabin 0.1 µg/mL    | 27.90    | 24.75   | 47.31   |
| Doksortabin 0.2 µg/mL    | 39.97    | 28.22   | 31.34   |

Metformin decreases cyclin D1 Protein expression in T47D Breast Cancer Cell

Cell accumulation in the G0-G1 phase due to exposure to metformin is then required to trace the protein that plays a role, for that the cyclin D1 expression is examined. G1.

Exposure to metformin concentrations of 1738.2 µg / mL and 3476.4 µg / mL for 24 hours to T47D cells showed a decrease in cyclin D1 expression compared to controls (Figures 2, 3, 4, Table 2).

Fig 2: The Expression of Cyclin D1 in Control Cells T47D
Description: The black arrow indicates cells expressing cyclin D1

Fig 3: The Expression of Cyclin D1 in Control Cells T47D Cells Described Metformin Concentration 1738,2mG / mL
Description: The black arrow indicates a cell that expresses Cyclin D1
Fig 4: Cyclin expression D1 in T47D Cell Described Metformin Concentrate 3476.4 mG/mL
Description: The black arrow indicates a cell that expresses Cyclin D1

TABLE 2 Analysis of Cyclin D1 Protein Expression in T47D cancer Cell Using ANOVA Test

| No. | Treatment                  | Expression of cyclin D1 in T47D cells | p Value |
|-----|----------------------------|----------------------------------------|---------|
| 1.  | Control Cell               | 14.61 ± 2.49 %                        |         |
| 2.  | Metformin 1738.2 µg/mL     | 7.76 ± 2.13 %                         | 0.014   |
| 3.  | Metformin 3476.4 µg/mL     | 4.40 ± 3.89 % *                       |         |

Description: Data was analyzed by one way Anova with Bonferroni’s advanced test
* p <0.05 vs. Control

Based on the ANOVA test obtained p = 0.014 (p <0.05), there were significant differences in the expression of cyclin D1 in all three groups. The results according to Table 4.5 show a significant difference in the expression of cyclin D1 in the cell group which was exposed to metformin 3476.4 µg/mL (4.40 ± 3.89%) with control cells (14.61 ± 2.49%) with p values < 0.05.

Metformin has Apoptotic ability for T47D Breast Cancer Cells
The results of apoptotic testing showed that the ability of apoptosis metformin was smaller than doxorubicin (Figure 5 and Table 3).

(A)
(B)
Fig 5: T47D Cell Apoptosis test in (A). Control Cells (B). Exposure to Metformin Concentration of 1738.2 µG / mL (C). Exposure to Metformin Concentration 3476.4 µG / mL (D). Exposure to Doxorubicin Concentration 0.1 µG / mL (E). Exposure to Doxorubicin 0.2 µG / mL Concentration.
Description: R1 = living cell; R2 = cells undergo initial apoptosis; R3 = cells undergo final apoptosis; R4 = cells undergo necrosis.

**TABLE 3: RESULTS OF T47D CELL APOPTOSIS TESTS**

| Treatment                      | R1 (%) | R2 (%) | R3 (%) | R4 (%) |
|--------------------------------|--------|--------|--------|--------|
| Control Cell                   | 96.35  | 0.64   | 1.43   | 1.48   |
| Metformin 1738.2 µg/mL         | 94.67  | 1.38   | 2.44   | 1.42   |
| Metformin 3476.4 µg/mL         | 91.31  | 1.86   | 3.92   | 2.80   |
| Doxorubicin 0.1 µg/mL          | 35.61  | 7.99   | 28.41  | 10.14  |
| Doxorubicin 0.2 µg/mL          | 31.71  | 11.19  | 31.25  | 26.20  |

Description: R1 = living cell; R2 = cells undergo initial apoptosis; R3 = cells undergo final apoptosis; R4 = cells undergo necrosis.

**Metformin Decreases P53 Protein Expression T47D Breast Cancer Cells**

In this step, p53 protein expression testing was performed on control cell T47D cells, T47D cells were exposed to Metformin 1738.2 µg / mL and 3476.4 µg / mL for 24 hours and T47D cells were exposed to doxorubicin 0.1 µg / mL and 0.2 µg / mL for 24 hours. The expression of p53 protein in cells exposed to doxorubicin 0.2 µg / mL is difficult to assess because of lysis cells.

Cells that positively express p53 visually appear to have a brown / dark cytoplasm, whereas cells that do not express p53 are bluish transparent.

![Fig 6: Expression of P53 Proteins in T47D Control Cells](image)

Description: Black arrows indicate cells expressing p53.
Fig 7. Expression of P53 in T47D Cells Exposed to Metformin Concentration of 1738.2 µG / mL
Description: Black arrows indicate cells expressing p53

Fig 8: Expression of P53 in T47D Cells Exposed to Metformin Concentration of 3476.4 µG / mL
Description: Black arrows indicate cells expressing p53
Fig 9: Expression of P53 in T47D Cells Exposed to Doxorubicin Concentration 0.1 µG / mL
Description: Black arrows indicate cells expressing p53

TABLE 4. Analysis of P53 Protein Expression in T47D Cancer Cells using ANOVA Test

| No | Treatment         | Expression of p53 to T47D Cell (mean±SD %) | p Value |
|----|-------------------|------------------------------------------|---------|
| 1. | Control Cell      | 14,54 ±1,60                              |         |
| 2. | Metformin 1738,2 µg/mL | 11,69 ± 3,28                       | 0,028   |
| 3. | Metformin 3476,4 µg/mL | 6,98 ± 2,74*                     |         |
| 4. | Doxorubicin 0,1 µg/mL | 12,33 ± 1,59                      |         |

Description: Data was analyzed by *one way Anova* with Bonferroni's advanced test

* p <0.05 vs. control

Based on the ANOVA test obtained p = 0.028 (p <0.05), there were significant differences in the expression of p53 in the four groups. The results according to Table 4 show a significant difference in the expression of p53 in the cell group which was exposed to metformin 3476.4 µg / mL (6.98 ± 2.74%) with the control cell group (14.54 ± 1.60%) with a p value < 0.05.

**Metformin Decreases BCL-2 T47D Breast Cancer Protein Expression**

Testing of bcl-2 expression was performed on T47D cell control cells, T47D cells which were exposed to metformin 1738.2 µg / mL and 3476.4 µg / mL for 24 hours and T47D cells exposed to doxorubicin 0.1 µg / mL and 0.2 µg / mL for 24 hours. Expression of bcl-2 in cells exposed to doxorubicin 0.2 µg / mL is difficult to assess because of lysis cells.

Visually positive cells expressing bcl-2 appear to have a brown / dark cytoplasm, while cells that do not express bcl-2 are colored bluish transparent.
Fig 10: Expression of BCL-2 Proteins in T47D Control Cells
Description: Black arrows indicate cells expressing bcl-2

Fig 11: Expression of BCL-2 in T47D Cells Exposed to Metformin Concentration of 1738.2 µG / mL
Description: Black arrows indicate cells expressing bcl-2
**Fig 12:** Expression of BCL-2 in T47D Cells Exposed to Metformin Concentration of 3476.4 µG / mL

Description: Black arrows indicate cells expressing bcl-2

**Fig 13:** Expression of BCL-2 in T47D Cells exposed to Doxorubicin Concentration of 0.1 µG / mL

Description: Black arrows indicate cells expressing bcl-2

**TABLE 5: Analysis of BCL-2 Protein Expression in T47D Cells using ANOVA Test**

| No. | Treatment            | bcl-2 Expression to T47D Cell | p Value |
|-----|----------------------|--------------------------------|---------|
| 1.  | Control Cell         | 14,04 ± 1,32%                 | 0.000   |
| 2.  | Metformin 1738,2 µg/mL | 5,46 ± 1,19% **               |         |
| 3.  | Metformin 3476,4 µg/mL | 2,84 ± 1,16% ***              |         |
| 4.  | Doxorubicin 0,1 µg/mL  | 7,4 ± 2,00% **                |         |

Description: Data was analyzed by one way Anova with Bonferroni’s advanced test

* p < 0.05 vs. Control
** p < 0.01 vs. Control
*** p < 0.001 vs. Control
Based on the ANOVA test, it obtained $p = 0.000$ ($p < 0.05$), there were significant differences in the expression of bcl-2 in the four groups. The corresponding results in Table 5 show a significant difference in the expression of bcl-2 in the cell group exposed to metformin $1738.2 \mu g / mL$ $(5.46 \pm 1.19\%)$ and doxorubicin $0.1 \mu g / mL$ $(7.4 \pm 2.00 \%)$ with the control cell group $(14.54 \pm 1.60\%)$ with a $p$ value $< 0.01$, and in the metformin group $3476.4 \mu g / mL$ $(2.84 \pm 1.16\%)$ significantly different from the control cell $(14.54 \pm 1.60\%)$ with a value of $p < 0.001$.

**Discussion**

It was found that in the previous studies showed that metformin had cytotoxic activity against T47D cells with IC$_{50}$ $1738,1875 \pm 141.63 \mu g / mL$. The toxicity of metformin to cells T47D was based on the mechanism of metformin inhibiting complex I respiratory chains in mitochondria which ended up stimulating various substrates which caused various effects in the form of activation of glycolysis, and inhibition of anabolic pathways in inhibiting fatty acid synthesis, cholesterol, synthesis of ribosomal RNA, resistance to cycles cells through AMPK phosphorylation to p-53 which causes an increase in transcription of p-21 a CDK inhibitor that plays a role in the cell cycle, and has an effect on the occurrence of apoptosis $^{5,7,8,10}$.

The mechanism of cytotoxic metformin had been tested through the cell cycle and apoptosis. It was a cell cycle of proliferation process that mediated the growth and development of living things, had two main phases, namely the S phase (synthesis) and the M phase (mitosis). Phase S was the phase of chromosomal DNA replication in cells while in phase M there is a separation of 2 sets of chromosomal DNA into 2 cells $^{24}$. In addition, there were phases that limit the two main phases called Gap.G1 (Gap-1) before the phase S and after the S phase are called G2 (Gap-2). In the G1 phase, the cell prepares for DNA synthesis. That phase was the initial phase of cell cycle progression regulated by extracellular factors such as mitogens and adhesion molecules. That phase marker was the expression and synthesis of protein as preparation for entering the S phase. In the G2 phase, the cell carried out further synthesis which sufficient for the cleavage process, so that the cell was ready to do division in the M phase $^{25}$.

This study displayed metformin leading to cell cycle arrest in the G0-G1 phase, that was in accordance with the results of the Qu and Cai study $^{12,26}$. G1 (Gap-1) is a cell preparation phase for DNA synthesis, and this phase would be exceeded if cell requirements such as nutrition, cell size and growth factors have been fulfilled. Metformin working by inhibiting the mitochondrial respiratory chain I complex with the consequent inhibition of ATP formation resulting in AMPK activation which caused barriers to protein synthesis, lipids and ribosomal RNA which were needed by cells to live and develop $^{12,27}$. That explained the occurrence of cell growth barriers through cell cycle arrest in the G0-G1 phase. The extension of the G0-G1 phase also occurred in MCF-7 cells exposed to a concentration of 2.5; 5; 10 and 20 mM, and increasingly elongated in the next two and three days of metformin exposure $^9$. Different from doxorubicin which also worked in the G2-M phase,
Doxorubicin cytotoxic activity through topoisomerase II inhibition, DNA intercalation, cell membrane binding and the formation of semiquinone free radicals and oxygen free radicals.

Doxorubicin is one of the anthracycline anti-breast cancer drugs currently used in combination regimens and for other types of cancer such as leukemia. Doxorubicin was used as a positive control in this study, other than because the drug was still used, but also because doxorubicin showed an anti-cancer effect on T47D cells. Doxorubicin working specifically in the S phase, that was related to its mechanism of action to inhibit DNA and RNA synthesis, intercalation between base pairs and interact with topoisomerase II.

Metformin exposure slightly increased T47D cell apoptosis compared to the effect of doxorubicin apoptosis. Previous metformin research on T47D cells indicated that the metformin inhibits T47D cell growth through activation of AMPK and histone H2B inhibition monoubiquitination and metformin caused apoptosis in T47D cells and increased expression of caspase 8 and 9. The study used the serial concentrations of 5, 10 and 50 µM and evaluated for 24, 48 and 72 hours, the maximum effect occurred after exposure to metformin 50 µM for 72 hours.

Other studies on MDA-MB-231 and MDA-MB-435 cell types of breast cancer cells using serial concentration of 1.25; 2.5; 5; 10 and 20 mmol/l for 24, 48 and 72 hours. Cells undergoing apoptosis increased from 7% with exposure to 5% metformin to 39.6% with exposure to 20 mM metformin for 24 hours, cytotoxic effects and apoptosis of metformin through the intrinsic mitochondria-mediated apoptosis pathway.

It noted that p53 was a tumour suppressor that maintained gene stability, played a role in the cell cycle, apoptosis, senescence and differentiation. The mutation of p53 will cause a loss of the function of maintaining genome stability, even triggering cancer. Decreasing expression of p53 protein that undergoes mutations in T47D cells will reduce the growth of cancer cells, this is in line with the effect on the extension of the G0-G1 phase in the cell cycle there are barriers to cell growth. P53 acts as a mediator for cell cycle arrest. This is consistent with research on T47D cells and MDA-MB-468 cells by silencing endogenous p53 via lentiviral shRNA which shows massive cell death through apoptosis with nuclear blebbing, PARP-1 cleavage, but does not occur in MCF-7 or MCF-10A with normal p53 conditions.

It is also stated that mutant p53 is an activator of a number of genes such as c-Myc, topoisomerase I and MDR-1 which cause genome instability and the occurrence of spindle checkpoint control interference. This study observed three protein expressions, namely p53, bcl-2 and cyclin D1. The results showed a decrease in expression of p53, bcl-2 and cyclin D1 proteins. Decreasing the expression of p53 in T47D cells which in a state of mutation explains the effects of metformin and doxorubicin inhibiting T47D cell growth. Lim et al (2009) in their study stated that in cells T47D and MDA-MB-468 (both cells experienced
a p53 mutation), after p53 was silenced with lentiviral shRNA, there was no change in the expression levels of pro-apoptotic proteins that were directly regulated p53 such as PUMA and NOXA. The connection of p53 with bcl2 could be seen from the following figure, which explained that the regulation of p53 could occur directly by binding to bcl2, or through PUMA, NOXA and bim.

![Connection of P53 with BCL2](image)

**Fig: 14 Connection of P53 with BCL2 (Hemann and Lowe, 2006 With Modification)**

Decreasing bcl2 expression in T47D cells exposed to metformin and doxorubicin could explain the increase in cells undergoing apoptosis in this study. It would be stated that a decrease in bcl2 expression is one of the mechanisms of apoptosis that occurred in T47D cells exposed to metformin and doxorubicin.

Replication and division of cell DNA occurs through the cell cycle, where the cell cycle was a sequence of sequences which included G1 (pre synthesis), S (DNA synthesis), G2 (premitotic) and M (mitotic) phases. Each phase of the cell cycle will occur if the previous cell cycle is complete and will stop if there is an essential deficiency of gene function. Each phase of the cell cycle will be regulated by the cyclin and the type of cyclin that regulates differently for each phase.

In the cell cycle occurs when critical in the form of checking (checkpoint) in several places including the phase G1-S and G2-M. For checkpoints in phase G1-S are regulated by cyclin and cyclindependent kinase inhibitors (CDKI) including cyclin D1, cyclin E, p21 and p27. Over expression of cyclin D1 and cyclin E supports the development of cancer. There was a decrease in the expression of D1 cyclin between control T47D cells and T47D cells exposed to metformin. Decreasing cyclin D1 expression can explain the occurrence of arrest in the phase cell cycle G0-G1 and in accordance with the study of Cai et al (2013) on HepG2 cells.
Conclusions

Conclusions of this experimental research are followings;

(1). The results of research conducted on the metformin in T47D cells; it found the effect of metformin on small cell cycle barriers, inhibits G0-G phase. Metformin has a smaller effect in stimulating T47D breast cancer cell apoptosis than that of doxorubicin.

(2) Metformin was able to reduce the expression of p53, bcl-2 and cyclin D1 in T47D breast cancer cells compared to control cells.

(3). As a single drug, metformin could effectively destroyed breast cancer because it only makes a small quantity to halt the cancer growth. It needs to combine metformin with other drugs.

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