Anti-Tumor Activity of Metformin in Human Epidermal Growth Factor Receptor 2 Positive Breast Cancer Cells

(Aktiviti Anti-Tumor Metformin pada Reseptor Faktor Pertumbuhan Epidermis Manusia 2 Positif Sel Kanser Payudara)

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ABSTRACT

Breast Cancer (BC) is the leading cause of cancer death in women. One BC subtype is very aggressive with amplification of human epidermal growth factor receptor 2 (HER2) protein. Although specific HER2+ targeting agents are available, most of HER2+ BC patients develop resistant to these agents. Recent studies show that metformin, is able to become anti-tumor in various cancer cells. This research aims to evaluate anti-tumor activities of metformin to HER2+ BC cells in both sensitive and resistant to trastuzumab. A series of assays were performed to evaluate metformin anti-tumor activities in HCC-1954 and SKBR-3 HER2+ BC cells. MTT assay was performed to evaluate cell death, and inhibitory concentration (IC_{50}), while scratch assay was performed to assess inhibition of cell migration and clonogenic assay to assess cell proliferation. $p<0.05$ was considered to be significant. Metformin could suppress the number of HER2+ BC cells. Viability assay showed suppression of viable cells after metformin incubation of 60 and 600 μM compared to control, 30 and 90%, respectively. Surprisingly, IC_{50} of metformin was smaller in HER2+ BC HCC-1954 cells that resistant to trastuzumab compare to the sensitive one (SKBR-3). Both were below 1 μM, with R^2 more than 0.95. Additionally, clonogenic assay showed less colony number and colony area with at least $p < 0.05$ in colony number and $p < 0.01$ in the area. In addition, metformin inhibited cell migration of HER2+ BC cells. Metformin shows a potency as anti-tumor by inducing cell death, inhibiting cell proliferation and cell migration of HER2+ BC cells.

Keywords: Breast Cancer; HER2+; metformin; Trastuzumab resistant

ABSTRAK

Kanser Payudara (BC) adalah penyebab utama kematian akibat barah pada wanita. Satu subjenis BC sangat agresif dengan penguatan protein reseptor 2 faktor pertumbuhan epidermis manusia (HER2). Walaupun agen sasaran HER2 + khusus ada, kebanyakan pesakit HER2 + BC mengalami ketahanan terhadap agen ini. Kajian terbaru menunjukkan bahawa metformin mampu menjadi anti-tumor pada pelbagai sel barah. Penyelidikan ini bertujuan untuk menilai aktiviti anti-tumor metformin kepada sel HER2 + BC dengan sensitif dan tahan terhadap trastuzumab. Satu siri ujian dilakukan untuk menilai aktiviti anti-tumor metformin pada sel HCC-1954 dan SKBR-3 HER2 + BC. Ujian MTT dilakukan untuk menilai kematian sel dan kepekatan penghambatan (IC_{50}), sementara ujian awal dilakukan untuk menilai penghambatan penghijrahan sel dan pengujian klonogenik untuk menilai percambahan sel. $p <0.05$ dianggap signifikan. Metformin dapat menekan bilangan sel HER2 + BC. Ujian daya maju menunjukkan penekanan sel yang berdaya maju setelah inkubasi metformin 60 dan 600 μM berbanding kawalan, masing-masing 30 dan 90%. Anehnya, IC50 metformin lebih kecil pada sel HER2 + BC HCC-1954 yang tahan terhadap trastuzumab dibandingkan dengan sel sensitif (SKBR-3). Kedua-duanya berada di bawah 1 μM, dengan R2 lebih daripada 0.95. Selain itu, ujian klonogenik menunjukkan bilangan koloni dan kawasan koloni yang kurang dengan sekurang-kurangnya $p <0.05$ pada bilangan koloni dan $p < 0.01$ di kawasan koloni. Sebagai tambahan, metformin menghalang penghijrahan sel HER2 + BC sel. Metformin menunjukkan potensi sebagai anti-tumor dengan mendorong kematian sel, menghambat percambahan sel dan penghijrahan sel HER2+ sel BC.

Kata kunci: HER2 +; kanser payudara (BC); metformin, rintangan Trastuzumab

INTRODUCTION

Breast cancer (BC) is becoming one of the main causes of death in the world, in 2012 it causes around 8.2 million deaths (Ferlay et al. 2015). According to the data from Population-based-cancer registration in Indonesia, BC ranks first as the most common death causing cancer in women with an incidence rate of 18.6%, and fifth in all death due to cancer cases (Wahidin et al. 2012).

BC is a malignancy of the breast tissues that may come from either the ductal epithelium or the lobules. Risk factor for breast cancer varies greatly, from inherited genetic factor (most common), hormones, lifestyle, to environment. In several tumors and cancers, mutation and pathological over-expression of normal growth factor receptor can be found. One of such cases is the Human Epidermal Growth Factor Receptor 2 (HER2), that controls growth, proliferation, and healing of breast cells. HER2 gene synthesizes HER2 protein, which is a receptor in breast cells (Hacioglu et al. 2015). However, in around 25% of BC patient, HER2 is provoking multiplication or amplification and called HER2 enrich or HER2+ (Cobleigh et al. 1999).

HCC-1954 cell line was generated from BC patient and has been proven to be resistant intrinsically to trastuzumab. While SKBR-3 cell line is a trastuzumab sensitive HER2+ BC cells, with large epithelial types and often found in human breast glands, metastatic tissues, and also cells in pleural effusion (Holliday & Speirs 2011).

The main strategies of BC management are still centered on surgeries and systemic therapy. Surgery is the earlier treatment of choice in BC. In systemic therapy, arrays of choices are available, starting from hormonal therapy estrogen hormones, chemotherapy, to targeted therapy, such as trastuzumab for HER2+. Trastuzumab is standard therapy for HER2+ BC patients. However, treatment with trastuzumab is costly and possess the risk of cardiotoxicity that increases the possibility of heart failure. Furthermore, some patients develop resistance in trastuzumab therapy, thus, this problem makes it harder to treat BC HER2+ patient (Sharma et al. 2010).

In a study by Kasznicki et al. (2014), beside as an anti-diabetes mellitus type 2 therapeutic agent, metformin can also inhibit tumor cell proliferation as observed in prostate, liver, and pancreas tumor cells. The mechanism of action of metformin in cancer is still under investigation. It's mechanisms in inhibiting tumor cell proliferation were thought to occur by: AMP kinase pathway activity, sensory cellular energy pathway; and reducing insulin resistance, that can reduce cancer growth factor (Viollet et al. 2012). A preclinical study suggests a direct antineoplastic activity (Cazzaniga et al. 2009).

In this study, we want to investigate the anti-tumor activity of metformin towards HER2+ BC cell in trastuzumab resistant cells, HCC-1954, and trastuzumab sensitive cells, SKBR-3, using a series of assays e.g. MTT assay, migration assay, and clonogenic assay.

MATERIALS AND METHODS

MATERIALS

Dulbecco's Modified Eagle's Medium was purchased from Gibco, USA. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), and trypan blue were purchased from Sigma-Aldrich, USA. Metformin was a gift from PT Kimia Farma. All the other chemicals were of analytical grade purchased from Merck, USA.

CELL CULTURE AND CONDITIONS

Different human BC cells were used in this study. HCC-1954 and SKBR-3 cells (both HER2+) were used. HCC-1954 cells were given by Prof. Stefan Wiemann (DKFZ, Germany) and SKBR-3 cell were given by Prof. Andreas Trumpp (DKFZ, Germany). In addition, Luminal A BC cells, MCF-7 and triple negative BC (TNBC) cells, MDA-MB 231 were also used. MDA-MB 231 cells were given by Prof. Stefan Wiemann (DKFZ, Germany). All cells were cultured using Dulbecco's Modified Eagle's Medium with supplementation of 10% FBS and 1% streptomycin penicillin. Cells were incubated under a controlled temperature of 37 °C and 5% CO_2. All cells were between passage range 2 and 20. A summary of breast cancer cell line characteristics that were used are listed in Table 1 (Subik et al. 2010).

TABLE 1. Characteristic of receptors in various breast cancer cell lines (Subik et al. 2010)

	ER	PR	HER2	subtype	Trastuzumab
MCF-7	6	6	0–1+	Luminal A	*-unrelated*
MDA MB-231	0	0	0–1+	Basal	*-unrelated*
HCC-1954	0	0	3+	HER2	Resistant
SKBR-3	0	0	3+	HER2	Sensitive

TRYPAN BLUE EXCLUSION ASSAY

A standard protocol was used. Briefly, cells were seeded into 6-well plate and incubated for 24 h before treated with indicated concentrations of metformin. Cells then were harvested, stained with trypan blue and counted under a microscope at 0 and 72 h after treatment (Strober 2015).

CYTOTOXICITY ASSAY

The cytotoxic effect of metformin was evaluated in BC cells using MTT assay, modified from our previous study (Bashari et al. 2016; Rezano et al. 2013). Briefly, cells were seeded into 96-well plate, and then treated with indicated metformin concentrations (1% DMSO as control) on the next day, followed by incubation for 72 h (in 5% CO_2 at 37 °C). MTT solution was then added and incubated for 4 h, followed by addition of DMSO to dissolve the formazan crystal. Absorbance was measured at the wavelength of 450 nm for finding percentages of viable cells on treated cells compared to the control cells. All tests were carried out at least 3 times in triplicate.

CLONOGENIC ASSAY

To evaluate anti-proliferative effect of metformin, we analyze plating efficiency (PE) and area per seed according to the previous study using clonogenic assay or colony formation assay (Franken et al. 2006). Briefly, 100 BC cells were seeded on 6-well plate. After attachment, they were treated or untreated (control) with metformin or DMSO 1% in fresh complete medium, respectively, and incubated for 2 weeks. On the last day, after medium removal, cells were fixed using methanol for 5 min followed by staining with crystal violet for 3 min then rinsing with tap water (Franken et al. 2006).

The number of the colonies formed were counted manually by measuring each of colony using a ruler. A colony was counted if the diameter measured more than 1 mm. The formula for calculating PE is as follow

$$PE\ (\%) = \frac{\text{number of colony formed}}{\text{number of cells seeded}} \times 100\%$$

The area of colonies were calculated semi-automatically by scanning the plate, then measuring the area using Colony Area plugin in ImageJ software (NIH, USA) (Guzmán et al. 2014). The formula for calculating the area per cell seeded is as follow

$$\text{Area per seed (mm}^2) = \frac{\text{total area of colony formed}}{\text{number of cells seeded}}$$

SCRATCH ASSAY

Consistent equal scratch with perpendicular angle and equal scratch pressure was performed (at 80% confluence, in plate 24 well) using 200 µL pipette tip. Plate then was washed slowly with phosphate buffer solution (PBS) twice to remove dirt and detached cells. Fresh medium was added afterwards. For control group, a complete medium were given without metformin, while for the experiment group were give a complete medium with metformin (2, 60, or 600 µM). Cells were incubated in 37 °C and CO_2 5% for 48 h.

GAP AREA MEASUREMENT

Serial observations were done under a microscope. The distance between gap and marking line were the observation point. Image of the gap was captured using a camera connected to microscope and a computer. Gap distances were evaluated quantitatively with ImageJ software (ImageJ 1.50i, National Institutes of Health, USA). Gap areas between borders were determined using image analyzing software that can calculate the distance reached in each time points determined.

COMBINATION INDEX

Combination index was analyzed using compusyn software (ComboSyn Inc, Paramus, NJ), Two doses (500 and 1000 µM) of metformin were combined with two doses (1 and 4 µM) of docrubicine or paclitaxel. The synergistic effects on cell viability were analyzed.

STATISTICAL ANALYSIS

Normalization was done to determine absorbance of cell death in each plate using Microsoft Excel program (Microsoft, USA). Drug curves, as well as IC_{50} was created and predicted using four parametric logistic regression by Sigmaplot ver.12 (SYSTAT Software Inc., USA). All other data were analyzed using Statistical Product and Service Solutions version 22 (SPSS; IBM, USA). To determine the association of inhibition of colony formation by each treatment group, we performed one-way Analysis of Variance (ANOVA) test with post-hoc Least Significance Difference (LSD). The statistical analysis was considered significant if p value < 0.05.

RESULTS AND DISCUSSION

VIABILITY TEST OF SKBR-3 CELL LINE

First, we evaluated cytotoxic activity of metformin using viability test in HER2+ BC SKBR-3 cells. We incubated

SKBR-3 cells with metformin for 72 h. Our finding showed that the higher the metformin concentration the higher the cytotoxicity. There were a statistically significant of non-viable cells after metformin incubation of 60 and 600 µM compared to control, 30 and 90%, respectively (Figure 1).
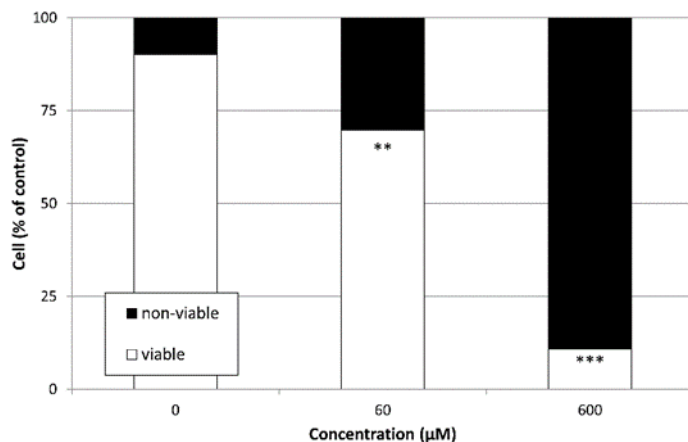

FIGURE 1. Trypan blue exclusion assay 72 h after incubation. Viable and non-viable cells were calculated. Statistical analysis was done using one-way ANOVA, post-hoc Tukey HSD. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$ against control

METFORMIN CAUSES CELL DEATH IN BOTH TRASTUZUMAB RESISTANT AND SENSITIVE BC HER2+ CELLS

Then, we evaluated cytotoxic activity of metformin using MTT assay of metformin in HER2+ BC cells using MTT assay. We used trastuzumab sensitive HER2+, SKBR-3 and trastuzumab resistant HER2+ HCC-1954 cells. Our data showed metformin causes cell death in both trastuzumab resistant and sensitive BC HER2+ cells in a dose dependent manner. We then performed four parameter logistics analysis to predict inhibition concentration 50% (IC_{50}). The IC_{50} of metformin in tested BC cell lines were less than 1mM. IC_{50} of metformin in HCC-1954, and SKBR-3 cells were 600 and 980 µM, respectively. A very significant R^2 value was obtained, for each dose curves are 0.9791 and 0.9723, respectively (Figure 2). Our data showed that metformin was more sensitive to the trastuzumab resistant HER2+ BC cells than to the trastuzumab sensitive HER2+ BC cells. Indicating that metformin may overcome the resistance to trastuzumab.
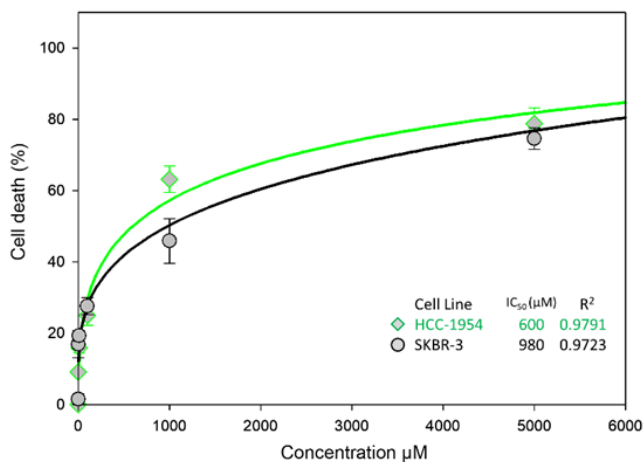

FIGURE 2. Dose response curves of metformin in HER2+ BC cells. Various concentration of metformin were tested. Data was presented as mean, SEM, of triplicate and three individual experiment. HCC-1954, trastuzumab resistant HER2+ (green). SKBR-3, trastuzumab sensitive HER2+ (black). IC_{50} were calculated using four parameter logistics regression in Sigmaplot ver. 12

METFORMIN SUPPRESSES CELL PROLIFERATION OF HER2+ BC CELLS

We analyzed the data of cytotoxic activity of metformin in HER2+ BC cells, and then wondered whether metformin has anti proliferative effect to HER2+ BC cells. Therefore, we used clonogenic assay, to assess cell proliferation and colony formation in HCC-1954 as well as SKBR-3 cells.

Data showed that metformin 600 µM decreases colony number in HCC-1954 into quarter of control while in SKBR-3 it was one-eight compared to control. The area of colony in 600 µM was a quarter of the control in HCC-1954 cells and one-fifth of control in SKBR-3 cells (Figure 3). This data was statistically significant as $p < 0.01$ in colony number and $p < 0.05$ in the area of colony between treatment and control group.
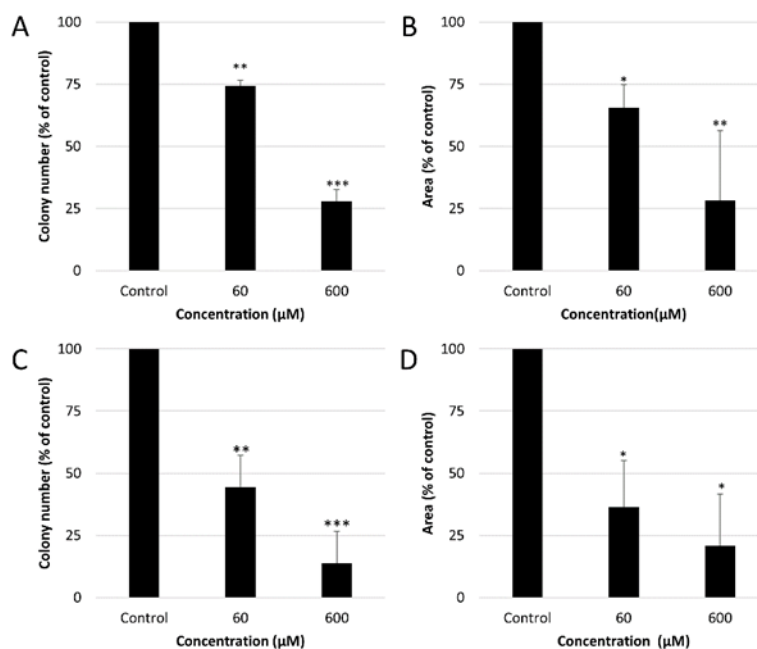

FIGURE 3. Metformin inhibits colony formation of HER2+ breast cancer cells. Metformin concentration were 0, 60, 600 µM and cell were incubated for 3 days, then replace with complete medium and incubate for 14 days. Colonies were fixed and stained with crystal violet. A and B for HCC-1954, C and D for SKBR, A and C for colony number, B and D for area. ANOVA one-way post hoc LSD was performed. *, $p < 0.01$, **, $p < 0.05$, ***, $p < 0.001$

METFORMIN INHIBITS HER2+ BC CELLS MIGRATION

To further evaluate anti-tumor activity of metformin, we then evaluated migration inhibition properties of metformin in HCC-1954 and SKBR-3 cells. Our data showed that metformin inhibits BC cell migration in dose dependent manner. After 48 h of intervention, 55% of gap area remains to the initial gap (0 h) of control group, while in the metformin 2, 60, and 600 µM, the gap area that remains were 72, 76, and 81% (respectively) in HCC-1954 cells. In SKBR-3, after 48 h of intervention, control shows 53% of gap area remains, but bigger in 2, 60, and 600 µM, which were 63, 69, and 76% (respectively). Statistical analysis was done using one way ANOVA followed by post hoc LSD, data of gap areas were statistically significant between any metformin treated groups compared to control (Figure 4).
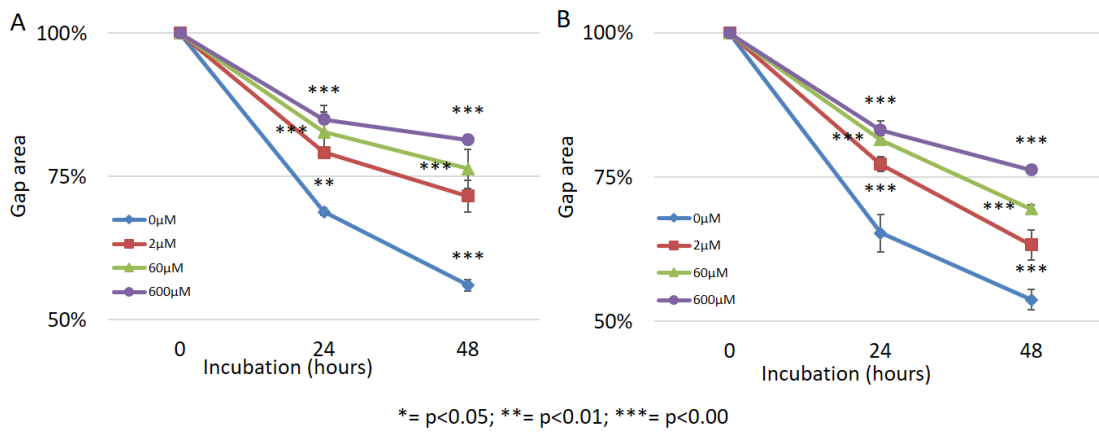
*= p<0.05; **= p<0.01; ***= p<0.00

FIGURE 4. Metformin inhibits cell migration of HER2+ BC cells. Metformin concentration were 0, 2, 60, 600 μM and cell were incubated for 0, 24, and 48 h. A. HCC-1954, B. SKBR-3. Gaps were capture and measure using ImageJ software. Data was presented as mean ± SD from duplicate of three individual experiments and analyzed by ANOVA one way, post hoc LSD. *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$ against control

COMBINATION THERAPY WITH PACLITAXEL AND DOXORUBICIN

We then evaluate metformin activity combined with other chemotherapeutic agents (paclitaxel and doxorubicin). We used 500 and 1000 μM metformin with combination of two concentration (1 and 4 μM) of doxorubicin and paclitaxel. Compusyn software was used to analyze the result. Combination index were all less than 1, means these combinations show a synergistic effect. Indicating that metformin is potentially be used in combination to enhance the effect of paclitaxel or doxorubicin (Figure 5).
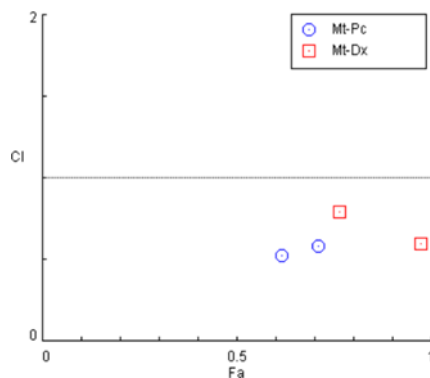

FIGURE 5. Combination index (CI) of Metformin with conventional chemotherapy agent. Metformin (Mt) with Paclitaxel (Pt) or Doxorubicin (Dx); Fraction affected (Fa)

Metformin is a well-known and foremost as diabetes mellitus type 2 drug. It is a biguanide compound which has the potential to downregulate insulin *via* IGF-I signaling pathway, and AMPK pathway (Kasznicki et al. 2014). AMPK activation leads to lower liver and cell glucose production and high insulin sensitivity leads to increase glucose uptake in peripheral tissue. Metformin increases glucose tolerant, avoid hypoglycemia, prevents hyperinsulenemia (Collier et al. 2006). Metformin also considered as a safe drug, abundance in production and stock, good tolerability profile, and low in cost.

The available evidence from basic science, clinical, and population-based research supports the anticancer effect of metformin (Kasznicki et al. 2014). Recent epidemiological and observational studies have shown association between metformin and cancer-related mortality, reduction in cancer incidence, compared with other antidiabetic treatment in diabetic patients (Bowker et al. 2006; Cazzaniga et al. 2013). Metformin is potential as a chemopreventive agent by lowering 31% overall relative risk from cancer incidence and mortality associated with cancer in type 2 diabetes treated with metformin, compared to diabetes with other treatments (Hadad et al. 2014).

Mechanisms of metformin in cancer prevention are suggested *via* activation of AMPK/LKB1 pathway, cell cycle arrest, apoptosis induction, protein synthesis inhibition, reduction in insulin level, immune system activation, cancer stem cell eradication, reduced IGF1/ insulin/HER-2 mediated signaling and angiogenesis inhibition (Malek et al. 2013; Pernicova & Korbonits 2014; Viollet et al. 2012). Activity in AMP kinase pathway for sensory cellular energy which is a cancer growth pathway via an inhibition in respiratory chain complex I and reducing circulating glucose (Gallagher & LeRoith 2011; Viollet et al. 2012). Insulin resistance reduction, in which insulin itself is a growth reduction factor for cancer, changes insulin-like growth factor level, sex hormones, and adipokinase that contributes in tumorigenesis (Viollet et al. 2012). Some studies have found that the anti-tumor ability of metformin is mediated *via* p53 dependent pathway (Buzzai et al. 2007). Since p53 communicates with other tumor suppressors (such as AMPK) to coordinate the cell growth during stress as in oxygen and nutrient deprivation, metformin triggers tumor regression In p53 deficient tumor, presumably *via* autophagy (Buzzai et al. 2007).

In trials of BC patient with diabetes mellitus showed that combination of metformin with neoadjuvant therapy resulting in increased pathological complete response compared to neoadjuvant alone (Jiralerspong et al. 2009). Anti-tumor effects of metformin and its potential use as a therapeutic agent for breast cancer are started to be shown (Jiralerspong et al. 2009). Another trial showed dual effects of metformin on BC growth related to insulin status (Cazzaniga et al. 2013). Previous studies have been conducted to evaluate cytotoxic effect of metformin in different cancer entities *in vitro* (Table 2).

TABLE 2. Study of metformin usage as anti-tumor agent, in other various cancer cell lines (excluding breast cancer)

No	Cancer cell lines	Cell source	Metformin Conc IC_{50} (micromolar)	Reference
1	143B; MG63; U2OS	Osteosarcoma	7,290 8,720 9,130	(Quattrini et al. 2014)
2	Calu-3	Human non-small cell lung carcinoma. K-RAS G13D TP53 CDKN2A	1,000	(Della Corte et al. 2016)
3	H1299	human non-small cell lung carcinoma. Mutation NRAS Q61K	1,500	(Della Corte et al. 2016)
4	H1975	human non-small cell lung carcinoma. Mutation EGFR	2,500	(Della Corte et al. 2016)
5	H358	human non-small cell lung carcinoma. Mutation KRAS G12C	1,000	(Della Corte et al. 2016)
6	ASPC-1; BxPc-3; PANC-1; SW1990	Pancreatic cancer (single dose)	<5,000	(Wang et al. 2008)
7	JB	Feline injection site sarcoma cell line	8,000	(Pierro et al. 2017)

Previous studies showed that metformin in micromolar concentration decreases BC cell viability using MTT assay was well as inhibits cell proliferation and cell migration in MDA-MB 231, MCF-7, MDA-MB-231, and MDA-MB-435 (Davies et al. 2017; Sharma et al. 2019). Metformin inhibits the development, and promotes the resensitization, of treatment-resistant breast cancer (Davies et al. 2017; Gao et al. 2016; Marinello et al. 2019). Metformin regulates multiple pathways of cancer cells including downregulating multiple proliferation controlling genes, activating mitochondrial pathway of apoptosis *via* inducing anti-apoptotic genes BCL_2 and Bcl-xL, suppressing cell migration regulating proteins such as N-Cadherin and MMPs (Lord et al. 2018; Sharma et al. 2019). Indeed, metformin regulates BC cells metabolism one of which by reducing cholesterol intracellular content that led to an inhibition of cell growth, cell survival and cell movement (Sharma et al. 2019).

Our study showed antitumor potential of metformin in BC. We treated HER2+ BC SKBR-3 and HCC-1954 cells with metformin, and our finding showed that metformin 60 and 600 µM trigger more cell death compared to control (Figure 1). Based on the drug curves, IC_{50} is predicted below 1 mM, with good R^2 (Figure 2). Colony number and area of colonies also showed that metformin suppressed cell proliferation in HER2+ BC cells (Figure 3). Moreover, HER2+ cell migration was more prevalent in metformin treated cells using migration assay (Figure 4). Interestingly, the cytotoxic effect, anti-proliferative as well as anti-cell migration of metformin is not only in SKBR-3 cells but also in HCC-1954 cells. HCC-1954 cells are trastuzumab resistant HER2+ BC cell line (Subik et al. 2010). Our data indicate the potency of metformin in overcoming resistance of trastuzumab therapy in trastuzumab resistance HER2+ BC cells.

HER2+ BC subtype is one of the most aggressive and poor prognostic BC subtype. Although there are available HER2 targeted agents, some BC patients develop resistant to these HER2 inhibitors. The relapse free survival probability of HER2+ BC patients is approximately 50% while the less aggressive luminal A BC patients is about 80% (Parker et al. 2009). Amplification of HER2+ gene in BC leads to over activity of HER2 receptor causing uncontrolled cell growth and survival, eventually lead to cancer (Iqbal & Iqbal 2014). Therefore, our data will open new frontier in treatment of HER2+ BC, that in present time dependent heavily on targeted therapy, shifting to a more basic metabolic drug treatment (Guo et al. 2017; Marcotte et al. 2016).

There are some well-known mechanisms of trastuzumab resistance including mutation of HER2, auto activation of downstream cascade of HER2 and downregulation of HER2. These all mechanisms keep the aggressive profile of HER2 BC cells including fast cell growth and tend to migrate which need a lot of energy. Metformin proposes to overcome indirectly the resistance of trastuzumab by activates AMPK pathways that lead to suppress gluconeogenesis, inhibit cell cycle, and inhibit protein fatty acid synthesis (Deng et al. 2019).

Analysis of genomic and proteomics in HER2-positive breast cancer that resistant to trastuzumab showed a deregulation of TRAIL - tumor necrosis factor-related apoptosis-inducing ligand (Diaz-Rodriguez et al. 2019). This is in the future promising as a possibility as treatment target.

A HER2+ breast cancer cell predicts a poorer prognosis, and higher sensitivity to chemotherapy. HER2 is a receptor of transmembrane tyrosine kinase included in human epidermal growth factor receptor family which function in epithelial cell proliferation and survival. The downstream pathways of HER2 including MAPK and PI3K/Akt (Deng et al. 2019).

Trastuzumab is considered a standard of care for BC. It is a humanized recombinant monoclonal antibody. A resistant in BC to trastuzumab may include HER3 or EGFR IGF receptor, activation of PI3K/AKT/mTOR, cMET, PTEN, SRC, MUC4, VEGF, p95 (Lavaud & Andre 2014). Metformin has anti-proliferative effect on trastuzumab-resistant breast cancer cells *via* inhibition of erbB2/IGF receptor interaction (Liu et al. 2011).

In a recent finding metformin may serve as adjuvant to enhance trastuzumab emtansine (T-DM1) to improve clinical efficacy of T-DM1 via caveolin-1 mediated endocytosis (Chung et al. 2018). This open a new hope for tumor chemotherapy. Whether it will work on trastuzumab resistant BC is another research subject.

Vazquez-Martin et al. (2011) using SKBR3 model that has acquired auto-resistance to trastuzumab to assess the effect of metformin. Synergistically metformin interacts with trastuzumab to suppresses self-renewal and proliferation (mammo-sphere size) of BC in both acquired and de novo HER2+ cell. The metformin concentration used is in 1 to hundreds of mmol^{-1}. However, this group just able to obtain proof of principle and still could not elucidate the mechanism behind the findings (Martin-Castillo et al. 2010).

In order to avoid overestimation of the potential benefit of experimental anti-BC therapy such as metformin in unselected populations, carrying out neo-adjuvant translational research may identify BC patients likely to benefit from metformin-based regimens. Metformin is a 'hybrid' anticancer compound that physically combines

the long-lasting effects of antibodies (by persistently lowering levels of blood insulin and glucose) and the immediate potency of a cancer cell-targeting molecular agent (by suppressing the AMPK/mTOR/p70S6K1 axis and crucial BC-related protein kinases such as HER2) (Martin-Castillo et al. 2010).

Metformin inhibits tumor cell migration *via* down-regulation of MMP2, MMP9 breast cancer cells *via* the AMPK/mTOR/autophagy pathway (Jang et al. 2014; Li et al. 2017). Metformin-inhibited cell viability, migration, colony, and sphere formations were reversed back by cholesterol treatment. Similarly, cholesterol treatment inverted metformin-reduced several gene expressions. Metformin decreased cell viability, migration and stemness in metastatic MDA-MB-231 cells. Similarly, metformin treatment suppressed expressions of anti-apoptotic genes BCL_2 and Bcl-xL, and mesenchymal genes vimentin, N-cadherin, Zeb1, and Zeb2 with simultaneous enhancement of apoptotic caspase 3 and Bax, and epithelial genes E-cadherin and keratin 19 expressions, confirming an inhibitory effect of metformin in tumorigenesis (Sharma et al. 2019).

Having promising data of metformin in HER2+ BC cells, another question arised. We wondered whether metformin has also cytotoxic effect in other BC subtype cells including luminal A and triple negative BC (TNBC) cells. We then conducted experiments in luminal A MCF-7 cells and TNBC MDA MB-231 cells. Cytotoxic assay showed metformin causes cell death in those cell lines. The IC_{50} of metformin in both BC cell lines are 900 and 775 μM in MCF-7, and MDA MB-231 cells, respectively. A very significant R^2 value was obtained, for each dose curves are 0.9304 and 0.9393, respectively (Figure 6).
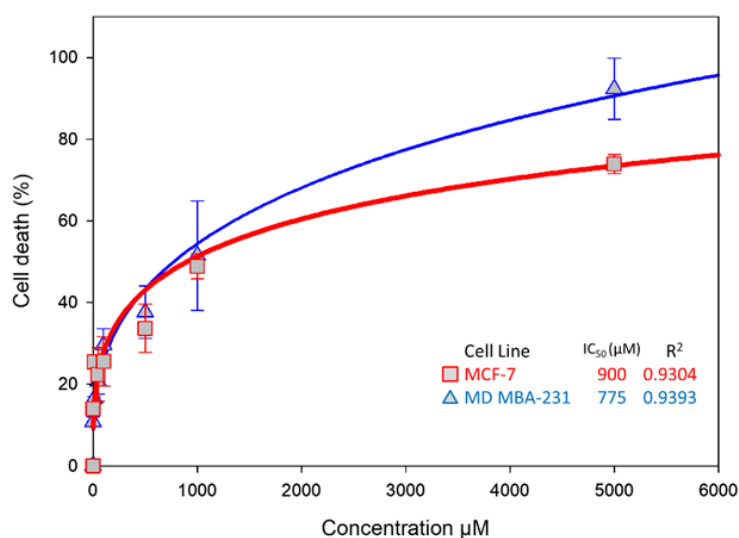

FIGURE 6. Dose response curves of metformin in luminal-A and triple negative BC cells. Various concentration of metformin were tested. Data was presented as mean, SEM, of triplicate and three individual experiments. MCF-7, Luminal A breast cancer cell (red). MDA MB-231, a triple negative breast cancer cells (blue). IC_{50} were calculated using four parameter logistics regression in Sigmaplot ver. 12

Metformin has been studied for its anti-tumor potency alone or with a combination with other conventional anti-tumor agent. Other studies showed different results on cytotoxicity of metformin in BC cells. All the IC_{50} result in BC cell line between 100 and 10 mM are shown in Table 3.

TABLE 3. Study of metformin usage as anti-tumor agent in breast cancer cell lines

No	Breast Cancer Cell Lines	Breast Cancer Cell Type	Metformin Conc IC$_{50}$ (microMolar)	Reference
1	BT-474;	Luminal B	3,000	(Zhu et al. 2014)
	SKBR-3;	HER2	3,000	
	MCF10A	Luminal-A	5,000	
2	BT474;	Luminal B	10,000	(Liu et al. 2011)
	SKBR-3;	HER2	5,600	
3	MCF-7;	Luminal A	4,500	(Guo et al. 2017)
	MDA MB-231	TNBC-basal	20,000	
4	MCF-7	Luminal A	10,000	(Scherbakov et al. 2016)

This study focused upon HER2+ BC cell. Using HCC-1954 and SKBR-3 we evaluated the dose curve. The IC$_{50}$ were below 1 mM. To the best of our knowledge, this is the first study that evaluate metformin antitumor activity only on HER2+ BC cell. Furthermore, Our IC$_{50}$ is lower in concentration compared to other previous studies. Other study using HER2+ cell (SKBR-3) has IC$_{50}$ at least 3 mM (Liu et al. 2011; Silvestri et al. 2015; Zhu et al. 2014).

In other type of BC cells, we evaluate metformin antitumor activity to luminal A (MCF-7) and TNBC cell (MDA MB-231). The IC$_{50}$ were below 1,000 μM. Previous studies using luminal A cell (MCF-7) has IC$_{50}$ at least 2,500 μM (Guo et al. 2017; Scherbakov et al. 2016; Silvestri et al. 2015; Zhu et al. 2014). In addition, research using TNBC cell (MDA MB-231) has IC$_{50}$ at least 10,000 μM, far much higher than our finding (Guo et al. 2017; Silvestri et al. 2015). Other research using luminal B (BT-474) has IC$_{50}$ at least 3,000 μM (Liu et al. 2011; Zhu et al. 2014).

CONCLUSION

From previously mentioned data, metformin has been proven to have cytotoxic effects and able to inhibit migratory growth of HER2+ BC cells sensitive and resistant types in regards to trastuzumab therapy. However, we realized several limitations of this study, which are length of study and data replication that was conducted in a very close time range, thus more research and reviews are warranted to be able to obtain a more comprehensive and accurate results.

Further studies need to be gathered before a clinical trial of metformin either as a single agent or in combination with chemotherapeutic agents or with HER2 targeted therapy. A clinical epidemiology intervention study with a strong relationship need to be carried carefully. Then at that time, as it is already easy to obtain and relatively in low price, metformin usage as an anti-tumor agent will be considered a breakthrough in cancer therapy, while at present standard treatment of BC centered on chemotherapy and other invasive therapy such as tissue removal.

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