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Abstract

Cancer is a large group of diseases and a second leading cause of death globally with an estimated 9.6 million death in 2018 according to World Health Organization (WHO). It is an abnormal and uncontrollably growth of cells that go beyond their boundaries and eventually invade and spread to the other part of the body. Acute myeloid leukemia (AML) is a type of blood cancer that is most common in adults. It is an abnormal differentiation and proliferation of myeloid precursor cells. Mutational complexity of AML is a challenge for proper diagnosis and a barrier for effective treatment strategy. Alternative drug strategies are continuously being tested and amongst them Metformin, the most prescribed and well tolerated anti-diabetic drug, have shown to have a potential as anti-cancer agent. Phenformin, a more potent derivative of Metformin is also interesting, but have shown to cause severe lactic acidosis in patients and has therefore been pulled-out as an alternative drug from the market. Numerous studies of metformin elucidating its effect point out that the metformin seems to be acting at multiple sites, however the import of the drug into cells is primarily facilitated through the Organic cation transporter 1 (OCT1) receptor. Thus, expression of the receptor would play an important role in the cell response to metformin or phenformin. This project had therefore the aim to assess the effect of metformin/phenformin treatment on two different AML cell lines, MOLM-13 and HL-60, and further examine the OCT1 expression of each cell line under the influence of metformin and phenformin treatment over time.

Our results showed that metformin and phenformin effects is dose- and time exposure-dependent and their potency and efficacy varied in the two cell lines. MOLM-13 was observed to be more sensitive compared to HL-60 in both drug treatments. OCT1 expression in MOLM-13 was upregulated by metformin and phenformin treatments after over time, while in HL-60 it was downregulated with both treatments, but more so in phenformin treatment.

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List of Abbreviations

ATP	Adenosine Triphosphate
NADPH	Nicotinamide adenine di nucleotide phosphate
mTOR	mammalian target of rapamycin
AMPK	AMP-activated protein kinase
LKB1	liver kinase B1
TSC2	tuberous sclerosis complex 2
OCT1	Organic cation transport 1
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
PI3K	Phosphoinositide 3-kinase
AKT	Protein kinase B
TKI	Tyrosine kinase inhibitor
mGPD	Mitochondrial glycerophosphate dehydrogenase
IRS	Insulin receptor substrate
DNA	Deoxyribonucleic acid
REDD1	Regulated in development and DNA damage responses 1
SLC22A	Solute carrier family 22
AML	Acute myeloid leukemia
DMSO	Dimethyl sulfoxide
FLT3	fms-like tyrosine kine 3
1°Ab	Primary antibody
2°Ab	Secondary antibody
IC50	Half maximal inhibitory concentration

1 Introduction

Cancer is a term used to a group of diseases caused by the abnormal cells that develop continual unregulated proliferation [1]. It is a second cause of death globally with an estimated 9.6 million death in 2018 [2]. Cancer cells grows and divide in an uncontrolled manner disregarding the signals that control the normal cell behavior and invading normal tissues and organs and eventually spreading throughout the body [1]. These abnormalities of cell mechanism were reviewed by Hanahan and Weinberg (2000) who introduced "the hallmarks of cancer" [3]. The six core hallmarks of cancer include self-sustained growth signal, growth suppressor aversion, resistance to apoptosis, limitless proliferative potential, sustained angiogenesis and metastasis. Two emerging hallmarks were also considered in addition to the existing core hallmarks of cancer: alteration of energy metabolism and evasion of immune destruction [4].

This thesis is about glucose metabolism in cancer cells and its relation to drugs used in the treatment of high blood sugar.

1.1 Glucose metabolism in cancer cells

Glycolysis normally occurs as a physiological response to hypoxia in normal somatic cells. It is a response mechanism of cells to metabolize glucose to supply energy (ATP) in the absent of oxygen, known as anaerobic glycolysis [5]. However, in 1920's, Otto Warburg observed that a cancer cells performed glycolysis and produce lactate even in the present of oxygen, and conceptualized that mitochondrial cellular respiration is partially damaged in cancer cells [6]. This phenomenon of excessive lactate production of glycolysis, referred as a "Warburg effect", is observed predominantly in most cancer [6,5]. The novel discovery of Warburg inclined interest of many researchers for further exploration in this area to disclose the mechanism behind it, and it also open possibilities for therapeutic target of cancer [8,7].

Contrary to the hypothesis of Warburg, it showed in subsequent work that mitochondrial function is not damaged in most cancer cells [9,5]. That high glycolytic flux and lactate production is cause from bioenergetic mutation of cancer cells which activate transcriptional genes responsible for lactate production. It also revealed that the high

rate of glucose metabolism of cancer was benefited by bioenergy and biosynthesis as the glucose degradation provides intermediates needed for biosynthetic pathways, including ribose sugar for nucleotides; glycerol and citrate for lipids; nonessential amino acids; and NADPH [10]. As proliferating cells requires extra nutrients, energy, and biosynthetic activity, all macromolecular components must be supplemented during each passage through the cell cycle for cells to be able to divide and duplicate [10]. in addition, it was observed that cancer cells prioritized biosynthesis that supports proliferation in mitochondria, rather than generating energy [9,11].

Studies of anti-diabetic drugs metformin and its derivative phenformin, was found to inhibits mitochondrial complex 1 of cancer cells, making it as a target for alternative treatment for cancer.

1.2 Metformin and phenformin as potential anti-cancer agents

Biguanides are a group of organic compounds that associated to a class of drugs that acts as antihyperglycemic drugs used to treat diabetes mellitus (type 2 diabetes) or prediabetes treatment. Guanidine, an active agent in lowering the glucose level in the blood was discovered in early 1920s in Galega officinalis plant extracts. The active ingredient of the plant was found to be galegine or isoamylene guanidine, a compound to have anti-hyperglycemia activity [13,12]. However, the hypoglycemic activity of guanidine was accompanied by adverse toxicity. Thus, several attempts were made to synthesize derivatives that are less toxic. The demonstration of biguanidines was proved to be useful and it exhibit a greater hypoglycemic effect than those monoguanidines [14]. Three biguanides, metformin, phenformin and buformin, became available for diabetes therapy in 1950s. Although in 1970s, phenformin and buformin were withdrawn from the market due to the emergence of lactic acidosis and increased cardiac mortality [12]. Metformin was proved safer and remained commercially-available biguanide drugs for treating diabetes mellitus [15,12].

1.2.1 Metformin

Metformin (dimethyl biguanide) is now a widely prescribed oral drug for type 2 diabetes, is also found to have therapeutic effect in various diseases and disorders including

cancers [15,17,16,12]. Type II diabetes has been associated to incidence of cancer. Patients with diabetes mellitus have higher risk to likely develop cancer, and those patients who have developed cancer have higher mortality compared to population of cancer patients without diabetes [18,19]. The used of metformin in diabetic patients with cancer was reported to have a low mortality compared to those patients that used other forms of therapy [20,21]. Results in numerous meta-analyses confirmed that metformin reduces cancer incidence by 30-50% [22].

1.2.1.1 Metformin mode of action

Though various studies of metformin have been conducted in many years, the actual mechanism and target of metformin are still not yet fully understood. Two of the proposed mechanism of metformin that potentially contributed to inhibiting the neoplastic activity are indirect effect by lowering insulin activity via liver and direct inhibition of mitochondrial complex I in cancer cells, where both of the mechanisms endpoint is the inhibition of the proliferative activity of mTOR pathway [15,23,22,24,25].

Activation of 5' adenosine monophosphate-activated protein kinase (AMPK), a central cellular energy sensor of the cell, by liver kinase B1 (LKB1), plays a major role in inhibition of mTOR pathway, by activating the mTOR inhibitor, tuberous sclerosis complex 2 (TSC2) [23]. By entering into the cancer cells via organic cation transport 1 (OCT1), metformin directly involve in activating the AMPK/LKB1/TSC2 pathway which leads to inhibition of mTOR downstream pathways (figure 1) [23,22]. While the indirect effect of metformin involves suppression of IGF-1/IGF-1R binding, which inhibit the signaling pathways of PI3K/AKT/mTOR and RAS/RAF/mitogen-activated protein kinase (MAPK), the inhibiting factors of TSC2 activity which inhibit the activation of mTOR pathways promoting cell growth and proliferation, and indirect activation of AMPK/LKB1/TSC2 pathway, by decreasing the circulating insulin (figure 2) [23,22,26,25].

On the other hand, a contrasting report demonstrated that AMPK and LKB1 are not required for the antiproliferative effect of metformin [27]. Metformin inhibits mitochondrial ATP production leading to induced cell death when ATP diminished in a limited glucose availability and it inhibits the biosynthetic capacity of mitochondria to generate macromolecules in cancer cells [27,17].

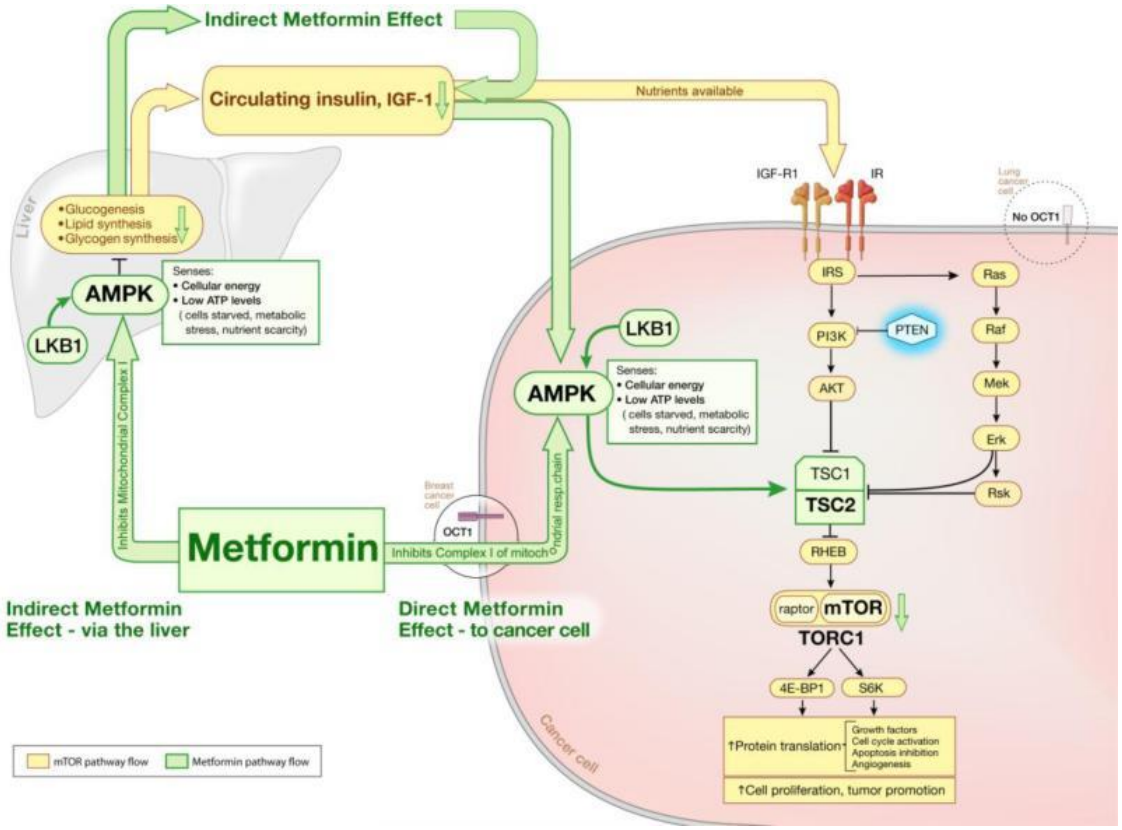

Figure 1. Pathway of direct and indirect effect of metformin in cancer cell [23].

1.2.2 Phenformin

Due to the increasing evidence of preventative and therapeutic effect of metformin in various types of cancer, the potential of phenformin as anti-cancer was also subjected to various research. Previous study demonstrated that phenformin and metformin have similar metabolic profile, with phenformin having increased potency [17]. Study in phenformin was found to eliminate resistance to angiogenic tyrosine kinase inhibitor (TKI), which prevent the uncontrolled metabolism of glucose in tumor cells [14]. Similarly, study in hedgehog-dependent tumor demonstrated that phenformin inhibits mitochondrial glycerophosphate dehydrogenase (mGPD), promoting association between corepressor CtBP2 and Gli1, thereby inhibiting Hh transcriptional output and tumor growth [28]. In a recent review of phenformin, it was proposed that it acts in three different ways: inhibition of complex I of mitochondrial respiratory chain, inhibition of insulin receptor substrate (IRS), and activation of regulated in

development and DNA damage responses 1 (REDD1) protein [14]. All three mode of action leads to inhibition of mTOR downstream pathway.

The import of metformin and phenformin into cells is primarily through the organic cation transporter 1 (OCT1) receptor, which is one of the biomarkers in measuring the effect of metformin and phenformin in cancer cells [29].

1.3 Organic cation transport 1 (OCT1)

Organic cation transporter 1 (OCT1) is a member of solute carrier family 22 (*SLC22A*) that encoded by the *SLC22A1* gene which mediates the facilitated transport of diverse organic cation solutes, endogenous compounds, toxins and drugs [30]. OCT1 expressed primarily on the sinusoidal membrane of hepatocytes and is also located in other type of cells in lower abundance like tumor cells. The level of expression and specific localization of the protein varies in different type of cells [31]. It is located, for example, in the lateral membrane of intestinal epithelial cells, apical membrane of ciliated cells in the lung and of tubule epithelial cells in the kidney [32]. OCT1 is known to be highly polymorphic and studies shows that the genetic variance of OCT1 modulated the subcellular localization and function of the transporter [33,34,32,35]. Also, study in mouse hepatocytes showed that deletion of OCT1 resulted to reduction in the effect of metformin on AMPK phosphorylation and gluconeogenesis [35]. Previous study also demonstrated that hOCT1 can be regulated by various intracellular signaling pathways, such as inhibition of hOCT1 by PKA activation and endogenous activation by the Ca^{2+}/CaM complex, the Ca^{2+}/CaM-dependent CaMKII and $p56^{lck}$ tyrosine kinase [36].

In cancer research, leukemia cells as a cell model are widely used to test new drugs and to study of signaling pathways/receptors. In this project, acute myeloid leukemia (AML) cell lines were used to study the relationship of OCT1 receptor to metformin and phenformin treatments.

1.4 Leukemia as cell model

Leukemia cell lines are a self-renewing and irreplaceable resources of living cells and are used as models for researching and developing new therapeutic targets and drugs [37]. With its advantages of unlimited supply and availability of identical cell materials

worldwide, indefinite storage in liquid nitrogen and recovery, while preserving its cellular features and viability, leukemia cell lines have become an important tool in research in several areas of biomedicine and biotechnology [37]. Well-characterized leukemia cell lines have provided fundamental information and insight into the biology of hematopoietic neoplasia [37]. Common characteristics of leukemia cell lines are: monoclonal origin, differentiation arrest at a discrete maturation stage, sustained proliferation in culture, genetic alterations, and stability of most features in long-term culture [37].

1.4.1 HL-60

HL-60 is a suspension cell line established from the peripheral blood of a patient with acute myeloid leukemia (AML FAB M2) in 1976 [38,39]. This cell line can be used as model to study the cellular and molecular events involved in proliferation and differentiation of leukemic cells, including the effect of physiologic, virologic and pharmacologic elements [41,40]. About 5 - 10% of HL-60 cell culture have properties of differentiated granulocytes, such as phagocytic ability and the ability to respond to chemotactic peptides. Differentiation of HL-60 can be inducted by DMSO, phorbol ester TPA and other reagents towards monocytic, eosinophilic and granulocytic myeloid cell lineages, depending on the environmental condition and the chemical inducers used [42,43]. Cytogenetic analysis shows that HL-60 cells possess many karyotypic abnormalities, including monosomy, trisomy and tetrasomy and a variety of chromosomal translocation [41]. On genetic alteration, for example, it has been found to carry amplified MYC gene, that the p53 gene has been largely deleted, and one allele of the GM-CSF gene is rearranged and partly deleted [41]. The cells used in this thesis were apparently tetraploid derivates of hypodiploid original where MYC was amplified in dmin (double minute chromosome) [44].

1.4.2 MOLM-13

MOLM-13 cell line is stablished from the peripheral blood of a patient with acute myeloid leukemia AML FAB M5a at relapse in 1995 after the myelodysplastic syndromes, along with sister cell line MOLM-14, with a growth pattern of single cells in suspension [45]. MOLM-13 carries an internal tandem duplication of FLT3 and CBL

deltaExon8 mutant, however the FLT3 protein is not expressed [46]. Cytogenic analysis shows hyperdiploid karyotype with 4% polypoidy, carrying occult chromosome insertion which affect the KMT2A-MLLT3 fusion [46,45]. Differentiation to macrophage-like morphology can be induced and stimulated with INF-*y*, alone or in combination with TNF-α, which treatments also upregulated the expression of certain myelomonocyte-associated antigens [45].

Numerous studies of metformin and phenformin on cancer cells annotates the relationship of OCT1 expression to the response to drug treatment, but little can be found about how drugs affect the expression of OCT1. This project was therefore designed with the following objectives.

1.5 Objectives

The objectives of the study are:

(1) to assess the effect of Metformin and Phenformin drugs in cancer cell lines of acute myeloid leukemia MOLM-13 and HL-60

(2) to examine OCT1 expression levels in MOLM-13 and HL-60

(3) to see how/if the expression of the receptor is influenced by Metformin or Phenformin treatment over time.

2 Materials and Methods

Materials and chemicals used in this thesis are listed in detailed in appendix, section 7.1. An overview of different methods used in the experiment are listed in table 1 below. Proliferation assay was conducted to assess the proliferation of different cell populations that would fit/or in the range for fluorescence reading for Alamarblue assay. Viability assay was performed to measure the dose response of HL-60 and MOLM-13 to metformin and phenformin treatment over 24- and 48-hours exposure. OCT1 determination and protein expression assessment were carried out using flow cytometry to determine the levels of OCT1 expression between cell lines and to evaluate the influenced of metformin and phenformin treatment over time to the expression of OCT1 receptor.

Table 1. Overview of the Experimental Outline.

Procedure of analysis	Parameters	
	HL-60	MOLM-13
	Drugs Incubation time	
1. Proliferation Assay	24 hours	24 hours
2. Viability Assay	Metformin 24 hours/48 hours	Metformin 24 hours/48 hours
	Phenformin 24 hours/48 hours	Phenformin 24 hours/48 hours
3. Viability Assay in lower range of concentration	Metformin 24 hours/48 hours	Metformin 24 hours/48 hours
	Phenformin 24 hours/48 hours	Phenformin 24 hours/48 hours
4. OCT1 determination and protein expression assessment	Metformin 24 hours/48 hours	Metformin 24 hours/48 hours
	Phenformin 24 hours/48 hours	Phenformin 24 hours/48 hours

2.1 Retrieval and maintenance of MOLM-13 and HL-60 cell lines.

2.1.1 Thawing and recovery of cells

HL-60 and MOLM-13 cells were recovered from a cryogenic state in a cryotank at -196°C to growth condition at 37°C. The cells were cryopreserved in a freezing medium containing Dimethyl sulfoxide (DMSO). DMSO is used as a protecting agent to minimized formation of ice crystals thereby preventing cell death in the freezing process. However, at a room temperature DMSO is toxic to the cells, thus, a rapid thawing process is crucial to avoid cell damage and to ensure rapid recovery of the cells.

Procedure:

1. Cryovials containing cells from cryotank were held in a water bath at 37°C until 80% of the vials were thawed.
2. The contents of the vials were immediately transferred into a prewarmed 5 ml culture medium in 15 ml centrifuged tubes and were mixed gently using sterile micropipette.
3. Cells were centrifuged at 900 rpm for 5 minutes in a compact tabletop refrigerated centrifuge (Kubota 2800, RS-240 rotor), discarded supernatant, and resuspended the cell pellet with prewarmed 10 ml culture media and transferred into T25 cell culture flasks.
4. After 24 to 48 hours, depending on the cell population growth, cells were transferred into T75 cell culture flask and maintain the growth and viability of the cells for the experiment. Cell cultures were incubated in a cell culture incubator with temperature at 37°C and 5% CO_2 concentration.

Cell culture medium RPMI 1640 supplemented with 10% FBS, 100 units/ml Penicillin and 100 µg/ml Streptomycin was used for cell growth throughout the experiments. T25 cell culture flasks were used to keep the cells close together and have a good cell-signaling from the neighboring cells for faster recovery. The procedures were carried out in a biosafety cabinet and done in an aseptic technique to avoid contamination in the cell culture.

2.1.2 Cell count determination using MUSE™ Count and viability kit

MUSE™ Count and viability kit was used to check the cell concentration and viability information of the cells in this experiment. It provides absolute cell count and viability data on cell suspensions by staining viable and non-viable cells based on their permeability to the two DNA binding dyes present in the reagent (Figure 2).

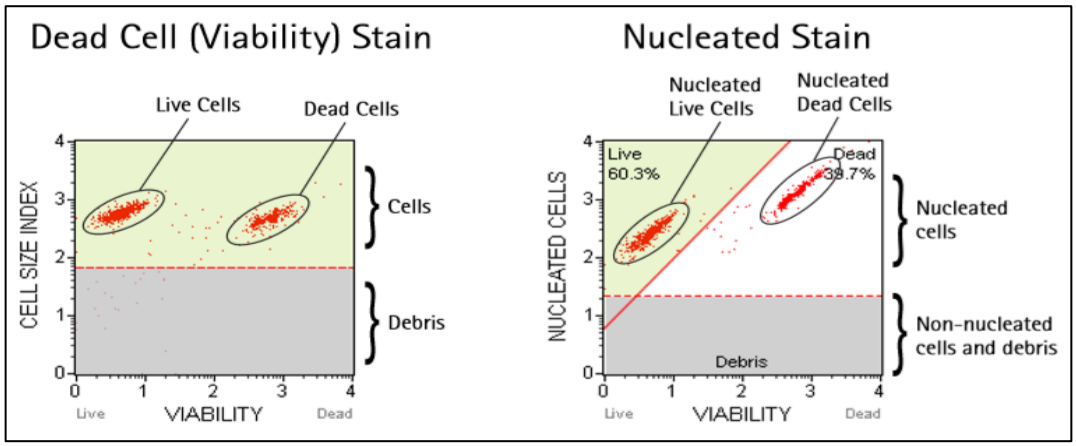

Figure 2. Staining of viable and non-viable cells bases on their permeability to the two DNA binding dyes present in the reagents. First plot has a moveable gate marker for adjustment to eliminate debris based on size. The second plot has a threshold marker to eliminate cells that do not have a nucleus and angled marker to separate live cells from the dead cells.

Protocol:

1. Prepared uniform cell suspension for counting. The cell suspension in the flask was mix thoroughly using sterile pipette and a small portion of cells were withdrawn for counting.
2. Prepared stained cell samples. Referred to recommended volumes for dilution that comes with the kit, cells were mixed with MUSE® Count and viability reagent in the in a sample tube and incubated for a minimum of 5 minutes to stain the cells.
3. Set up and acquisition on the MUSE® cell analyzer. MUSE® cell analyzer was set up by selecting the “Count and Viability” from the main menu then “run assay”. The stained cell sample was loaded by following the on-screen instructions. Optimization and verification of settings were asked and set up, entered the sample specific information, and then select the “Run” to run the sample. After the acquisition is complete, the results are displayed on the screen.

2.1.3 Cell passage

Cells growth and proliferation usually follow a growth pattern characteristic in four phases: lag, log, stationary and decline phase. Cells begins lag phase right after seeding where they are growing slowly while recovering from sub-culturing; then it undergo log phase when they grow and divide exponentially until the cell density exceeds the capacity of the medium; and eventually reach stationary phase where cells slowdown and stop to proliferate; then it enters into decline phase, decrease in cell viability, if medium is not changed and the cell number is not reduced.

To ensure the viability of suspension cells in culture, cells were kept in the log growth phase by passing them when they become 75-80% confluent. For the MOLM-13 and HL-60, culture media were changed every 2 to 3 days maintaining the ideal cell density of 0.5-1.5 x 10^6 cells/ml.

Procedure:

1. Cell density of the cultures were determined by running a cell count and viability check (refer to section 2.1.2).
2. Cell cultures were carefully mixed using sterile pipette and calculated volumes to be reseeded were withdrawn and transferred into a 15 ml centrifuge tubes.
3. Cells were centrifuged at 900 rpm for 5 minutes in a compact tabletop refrigerated centrifuge (Kubota 2800, RS-240 rotor). Discarded supernatant.
4. Cells were resuspended into a prewarmed fresh media back to the cell culture flask.

2.1.4 Cryopreservation of HL-60 cell line

Established cell line is a valuable resource and preserving it for long-term storage is important for an ongoing research project. As cell cultures are prone to microbial contamination or in any circumstance there is a need to put down the cell culture, there is always a use for new seeding stock.

Procedure:

1. HL-60 cells were counted using the MUSE® Count and viability kit (refer section 2.1.2).

2. Freezing medium containing 70% RPMI, 20%FBS and 10% DMSO was prepared. The volume of freezing medium was calculated based on the total cell concentration and recommended cell population of $5x10^6$ cells/ml per aliquot.
3. Cells were thoroughly mixed using sterile pipette and transferred into 15 ml centrifuge tubes and centrifuged at 900 rpm for 5 minutes in a compact tabletop refrigerated centrifuge (Kubota 2800, RS-240 rotor).
4. Supernatant was discarded and the cells were resuspended in a freezing medium to cell population of $5x10^6$ cells/ml.
5. Cell stock were aliquoted 1ml into labeled cryovials and were placed into a pre-chilled Mr. Frosty™ Freezing Container and stored for an hour at -20°C and at -80°C for an overnight storage.
6. The cryovials containing preserved HL-60 cells from an overnight storage were put away in the cryotank at -196°C for long-term storage until further use.

2.2 Cell proliferation assay

Proliferation of MOLM-13 and HL-60 cell in different seeding densities were evaluated using Microplate AlamarBlue assay. AlamarBlue is a non-toxic and reliable cell proliferation and viability reagent, containing resazurin as an active reagent which metabolized by cells into resorufin, a compound that is highly fluorescent. Black-walled 96-well microplates for fluorescence-based assay were used for optimal fluorescence reading. This microplate minimizes well-to-well crosstalk and autofluorescence which will affect the results.

Procedure:

1. Cell count were determined (refer section 2.1.2) and series of seeding densities 2500, 5000, 10000, 15000, 20000, 30000 and 40000 cells/ml were prepared.
2. 200 µl of cell culture with different seeding densities were loaded into the wells along with the control (blank and blank+Resazurin) in 6 technical replications. Blanks were only contained 200 µl of fresh media. Incubated for 20 hours.
3. Wells containing different concentration of cells and blank+Rez were added 20 µl of 484 µM AlamarBlue making it to a final concentration of 44 µM, and the wells containing blank were added 20 µl 1x PBS. Incubated for 4 more hours.

4. After 24 hours of total incubation time, fluorescence was measured with Excitation wavelength at 560 nm and Emission wavelength at 590 nm using the SpectraMax Paradigm Multi-Mode Microplate Reader.

2.3 Cell viability assay

2.3.1 Assessment of Metformin and Phenformin treatment on MOLM-13 and HL-60

Cell viability assay was conducted using Microplate AlamarBlue assay. From the acquired data on cell proliferation assay, 20000 cells/well was used as seeding density for both cell lines to test series treatment of metformin and phenformin over 24- and 48-hours incubation time. Metformin concentration used were 100, 250, 500, 1000, 2000, 4000, 8000, 12000 µM and phenformin concentration were 25, 50, 100, 250, 500, 1000, 2000, 4000 µM. 1M Metformin and 250 mM Phenformin stocks were used in the dilution series of treatment in culture medium. Concentration of cell cultures and treatments were adjusted to the loading volume of 100 µl, such that final concentrations were maintained in 200 µl total volume per well.

Procedure:

1. Series dilution of metformin and phenformin treatments were prepared.
2. Cell count were determined (refer section 2.1.2) and cell culture stocks for seeding were prepared.
3. Cell culture were loaded to 96-well microplate (black-walled) then treated with different concentration of metformin and phenformin. Each treatment was carried out in 4 replicates together with the control (no treatment), blank (culture media) and blank+resazurin. Two microplates were prepared for MOLM-13 and HL-60 and incubated for 24 and 48 hours.
4. After 20 hours for 24-hour treatment, wells containing treatment, control and blank+resazurin were added 20 µl of 484 µM AlamarBlue making it to a final concentration of 44 µM. Wells containing blank were added 20 µl 1x PBS. Incubated for 4 hour and measured the fluorescence with excitation wavelength at 560 nm and emission wavelength at 590 nm using the SpectraMax Paradigm Multi-Mode Microplate Reader.
5. After 44 hours for 48-hour treatment, the same procedure in number 4 were done.

2.3.2 Assessment of Metformin and Phenformin treatment on MOLM-13 and HL-60 in lower range of concentration

Based on the results from dose response assessment on MOLM-13 and HL-60, lower range of concentrations were further investigated using the IC50 as reference. The concentrations used for MOLM-13 were 100, 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 µM metformin and 5, 10, 20, 25, 30, 35, 40, 45 and 50 µM phenformin. As for HL-60, 100, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000 µM metformin and 10, 20, 40, 50, 60, 70, 80, 90, 100 µM phenformin were used.

Procedure:

Using the lower concentration treatment, the same procedure in section 2.3.1 were followed.

2.4 Determination of OCT1 by flow cytometry analysis

2.4.1 OCT1 determination together with titration of antibodies

The presence of OCT1 protein in MOLM-13 and HL-60 were determined using flow cytometer. OCT1 (SLC22A1) primary antibody (1°Ab) and CF®488A Llama Anti-mouse IgG (H+L) secondary antibody (2°Ab) were used to stain the cells. Titration of antibodies were determined for optimal dilutions/concentrations of 1°Ab and 2°Ab. Primary antibody titration carried out in 3 dilutions 1/200, 1/300 and 1/400 in two sets, along with 3 controls 1°Ab, 2°Ab, blank (no antibodies) ; and the secondary antibody carried out in 3 concentrations 1, 5, and 10 µg/10^6 cells in two sets with 3 controls 1°Ab, 2°Ab, blank.

Procedure:

2.4.1.1 Cell fixation

1. Cells were harvested from cell culture after determining the viable cell count (refer section 2.1.2).
2. Cells were carefully mixed and transferred to the centrifuged tubes, centrifuge at 900 rpm for 5 minutes using refrigerated centrifuge (Kubota 2800, RS-240 rotor). Removed supernatant.

3. mixed well to dissociate and prevent crosslinking of individual cells.
4. Fixed for 15 to 30 minutes at room temperature (20 - 25°C).
5. Washed with excess 1x PBS, centrifuged and discarded supernatant. Washed twice.
6. Resuspended cells in 1x PBS and stored at 4°C.

2.4.1.2 Immunostaining of antibodies

1. Fixed cell samples were aliquoted 5 x 10^5 cells per tube into 1.5 ml Eppendorf tubes. Centrifuged at 1000 x g for 5 min in Eppendorf refrigerated microcentrifuge 5702 R (F-45-24-11). Removed supernatant.
2. Prepared dilution series of 1°Ab. Antibody dilution buffer used was 0.5% BSA PBS buffer.
3. Cell samples 1/200, 1/300 1/400 and 1°Ab and were resuspended in 100 µl of diluted OCT1 (SLC22A1 antibody) 1°Ab and samples 2°Ab and blank were resuspended in 100 µl 0.5% BSA PBS buffer. Incubated overnight at 4°C.
4. Cell were washed with excess 1x PBS, centrifuged at 1000 x g for 5 min and removed supernatant. Washed twice.
5. Cell samples 1/200, 1/300 1/400 and 2°Ab and were resuspended in 100 µl of 5 µg Llama Anti-mouse IgG (H+L) 2°Ab and samples 1°Ab and blank were resuspended in 100 µl 0.5% BSA PBS buffer. Incubated away from the light for one hour at room temperature.
6. Washed twice with excess 1x PBS and finally resuspended into 500 µl for flow cytometer reading.

For the titration of secondary antibody, the same procedure was followed in immunostaining using 100 µl 1/400 1°Ab and 100 µl of 0.5µg, 2.5µg and 5µg 2°Ab.

2.4.1.3 Flow cytometry analysis

1. Cells for gating were loaded in S3e Cell Sorter and were analyzed using ProSoft™ Software.

2. Region of cells of interest were gated for sorting and gating of single cells to be analyzed for OCT1, voltage of FSC (forward scatter) was set to 250 and SSC (side scatter) to 270 to both cell lines (figure 3).

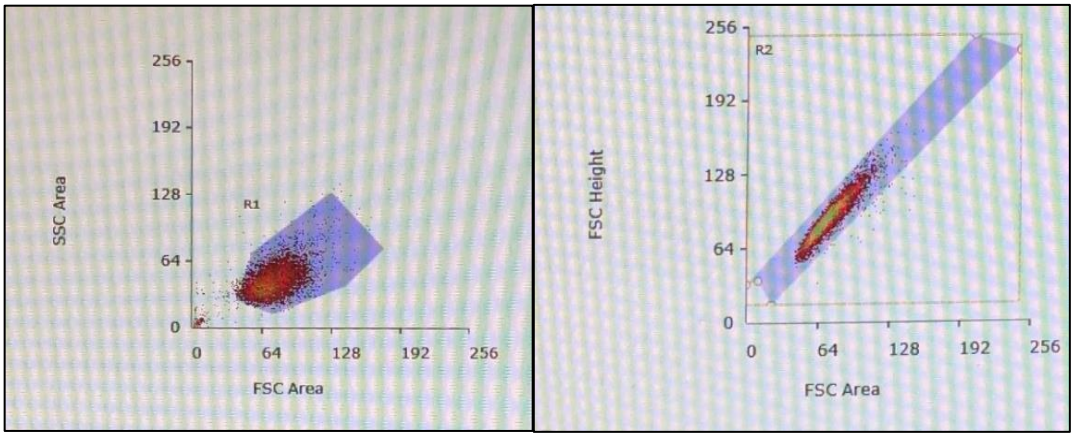

Figure 3. Example of gating the cells of interest. Left: gated region of cells of interest. Right: gated single cells to be analysed for OCT1.

3. After the gate were set up, samples were then analyzed for OCT1 and FL1 525/30 filter was used in data acquisition.

2.4.2 Determination of OCT1 protein expression in treated cells

The effect of Metformin and Phenformin treatments on OCT1 protein expression in different concentrations over 24 and 48 hours were further investigated. Three different concentrations of 200, 1000 and 2000 µM Metformin and 10, 25, and 50 µM Phenformin were tested for MOLM-13 along with the control. For HL-60, concentrations of 200, 2000 and 4000 µM Metformin and 10, 50, 100 µM Phenformin were used (Table 2). Each sample (cells with treatments) were grown in a 6-well plate for 24 and 48hours before harvesting. Cell concentration used for MOLM-13 was $2x10^5$ cells/ml and for HL-60 were $2x10^5$ cells/ml and $2.5x10^5$ cells/ml.

Procedure:

2.4.2.1 Cell treatment with Metformin and Phenformin

1. Different dilution of 1M Metformin and 250mM Phenformin treatment in culture medium were prepared. The concentrations of treatments were adjusted to the loading volume of 2 ml to achieve the final concentration of 4 ml.

Table 2. Experimental sample design.

MOLM-13				
24hours Treatments	Control	200 µM metformin	2000 µM metformin	4000 µM metformin
		10 µM Phenformin	50 µM Phenformin	100 µM Phenformin
48hours Treatments	Control	200 µM metformin	2000 µM metformin	4000 µM metformin
		10 µM Phenformin	50 µM Phenformin	100 µM Phenformin
HL-60				
24hours Treatments	Control	200 µM metformin	1000 µM metformin	2000 µM metformin
		10 µM Phenformin	25 µM Phenformin	50 µM Phenformin
48hours Treatments	Control	200 µM metformin	1000 µM metformin	2000 µM metformin
		10 µM Phenformin	25 µM Phenformin	50 µM Phenformin

2. Cell count were determined (refer section 2.1.2) and cell culture stocks for seeding were prepared. The concentrations of cells were adjusted to the loading volume of 2 ml to achieve the final concentration of 4 ml.
3. Cell culture were seeded into 6-well microplate then treated with different concentration of Metformin and Phenformin. Incubated for 24 and 48 hours.

2.4.2.2 Cell fixation

Refer to section 2.4.1.1

2.4.2.3 Immunostaining

1. Fixed cell samples were aliquoted 2.5 x 10^5 cells per tube into 1.5 ml Eppendorf tubes. Centrifuged at 1000 x g for 5 min in Eppendorf refrigerated microcentrifuge 5702 R (F-45-24-11). Removed supernatant.
2. Prepared 1/200 OCT1 (SLC22A1) 1°Ab in an antibody dilution buffer 0.5% BSA PBS buffer.
3. Cell samples were resuspended in 100 µl of and samples 2°Ab and blank were resuspended in 100 µl 0.5% BSA PBS buffer. Incubated overnight at 4°C.

4. Cell were washed with excess 1x PBS, centrifuged at 1000 x g for 5 min and removed supernatant. Washed twice.
5. Cell samples were resuspended in 100 µl of 2.5 µg Llama Anti-mouse IgG (H+L) 2°Ab and samples 1°Ab and blank were resuspended in 100 µl 0.5% BSA PBS buffer. Incubated away from the light for one hour at room temperature.
6. Washed twice with excess 1x PBS and finally resuspended into 250 µl for flow cytometer reading.

2.4.2.4 Flow cytometry analysis

Refer to section 2.4.1 c.

3 Results

3.1 Cell proliferation of MOLM-13 and HL-60 cell lines

To evaluate the ideal cell population of HL-60 and MOLM-13 for Alamarblue assay and to assess the health and growth behavior of the cells prior to further experiments with metformin and phenformin treatments, proliferation of different cell population was tested. The linear trend of cell proliferation showed that the seeding densities used in the experiment were in the range of Alamarblue assay (figure 4), with the ideal seeding densities of 10000 to 40000 cells/well for both cell lines. The linear increase of cell proliferation in an increasing cell seeding densities showed that both cell lines were in good condition.

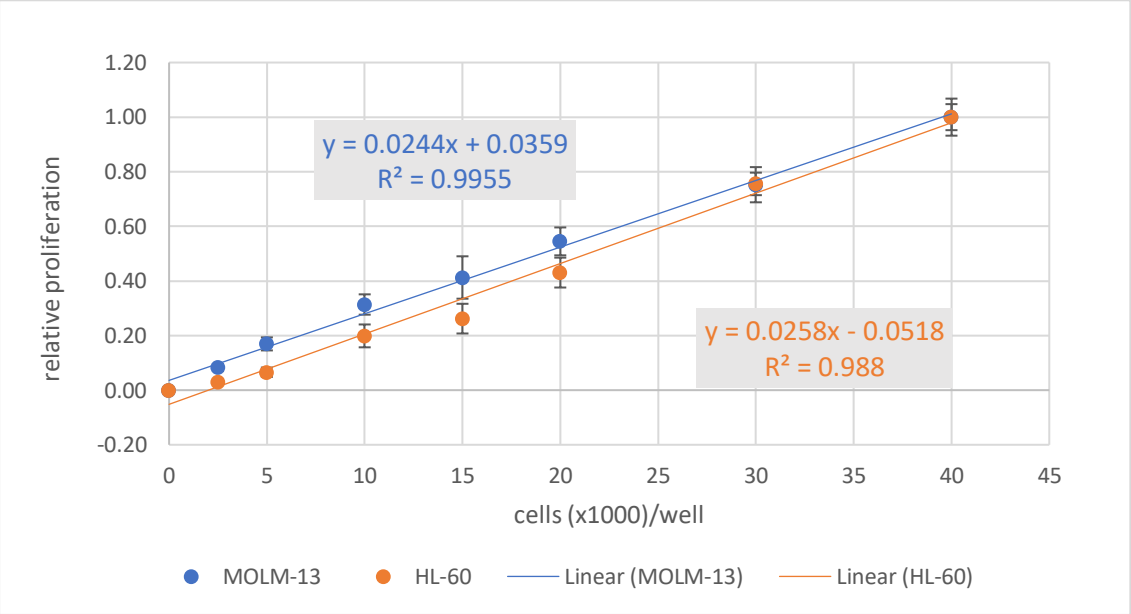

Figure 4. Cell proliferation of different cell populations of MOLM-13 and HL-60 over 24 hours incubation time. The data acquired as fluorescence intensity at 590 nm and each cell lines were normalized to own highest value and standard deviation were calculated from 6 biological replicates in one experiment.

3.2 Dose response of MOLM-13 and HL-60 to metformin and phenformin treatment

In evaluation to the effect of metformin and phenformin treatment on MOLM-13 and HL-60, viability assay were performed by subjecting the cells to a range of concentration of metformin (100 – 12000 µM) and phenformin (25 - 4000 µM) over 24 and 48 hours incubation and assessed by using Alamarblue assay. The acquired data in fluorescence intensity at 590 nm was calculated into cell viability as percentage of control and the

percent inhibition was calculated by subtracting the percent viability from 100. The calculated values of percent inhibition were plotted as dose response curves over drug concentration in logarithmic form and thereby calculated the approximate half maximal inhibitory concentration (IC50) of metformin and phenformin in MOLM-13 and HL-60 cells using the log concentration value in excel.

The results in MOLM-13 showed to have a positive response to both drugs after 24- and 48-hours treatments (figure 5). Inhibition of cell activity in 50 % was reached after 24 hours treatment of metformin at 2210 µM and 2750 µM after 48 hours (figure 5A). As for phenformin, the IC50 were 40 µM and 50 µM after 24 and 48 hours, respectively (figure 5B).

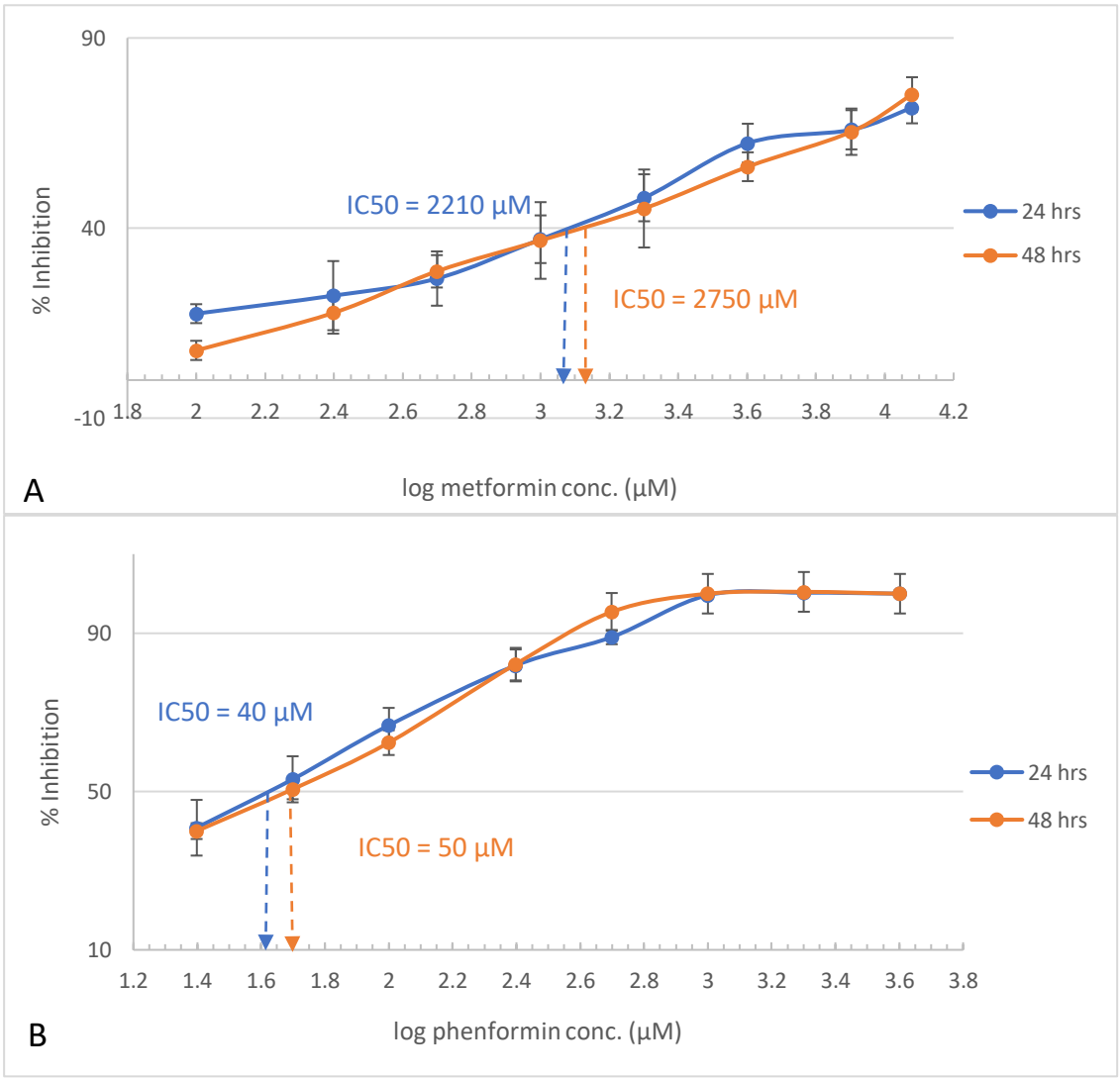

Figure 5. Dose response of MOLM-13 treated by (A) metformin and (B) phenformin after 24 and 48 hours. Acquired data as florescence intensity at 590 nm were calculated as % inhibition from cell viability % of control and standard deviation were taken from 3 biological replicates in one experiment.

On the other hand, HL-60 was observed to be less responsive to both drug treatments, as the observed IC50 of both drugs are higher compared to MOLM-13 (figure 6). And the effect of both drug treatments to HL-60 showed to be more evident in 48 hours compare to 24 hours exposure. The result showed that metformin treatment failed to inhibit the 50 % activity of the cells at the given range of concentration after 24 hours exposure, but in longer in longer time exposure, the IC50 was observed at 3020 µM (figure 6A). Phenformin treatment on the other hand, showed lower IC50 in 48 hours treatments at 110 µM compared to 24 hours treatment at 158 µM (figure 6B).

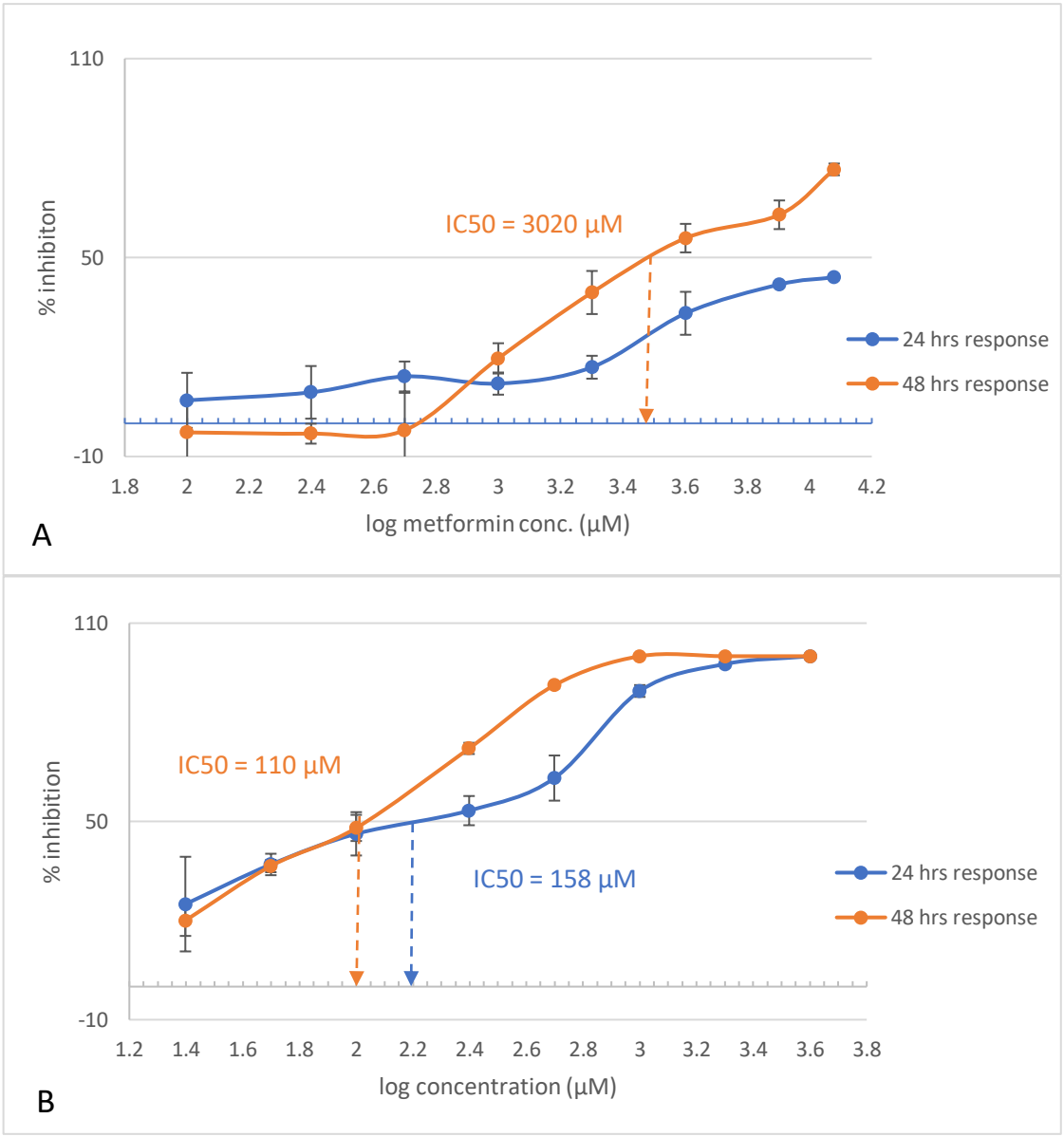

Figure 6. Dose response of HL-60 treated by (A) metformin and (B) phenformin after 24 and 48. hours. Data as florescence intensity at 590 nm were calculated as % inhibition from cell viability % of control and standard deviation were taken from 3 biological replicates in one experiment.

The Dose response of MOLM-13 and HL-60 to metformin and phenformin over time revealed that MOLM-13 is more responsive to drug treatments than HL-60. MOLM-13 showed a response in the first 24 hours exposure to both drugs, while HL-60 showed a lower response to drug treatments and had best response when treatment was continued for 48 hours for both drugs. The IC50 results demonstrated that the potency of different drugs varied in each cell line and the efficacy of each drug varied between cell lines (table 3). Phenformin showed 55 folds more potent than metformin in MOLM-13, while in HL-60 lower difference in potency was observed with phenformin 27 folds stronger than metformin. In comparison of the efficacy of each drug between cell lines showed that metformin is 1.1 folds more effective in MOLM-13, and similar pattern was observed in phenformin with 2.2 folds.

Table 3. IC50 of metformin and phenformin in MOLM-13 and HL-60 after 48 hours treatments and the corresponding fold change between cell lines and drugs.

	Metformin µM	**Phenformin** µM	**Fold change of phenformin/metformin**
MOLM-13	2750	50	**55**
HL-60	3020	110	**27**
Fold change of HL-60/MOLM-13	**1.1**	**2.2**	

3.3 Dose response of MOLM-13 and HL-60 to metformin and phenformin treatment at lower range of concentration

To further evaluate the effect of metformin and phenformin on the proliferation of MOLM-13 and HL-60, a range of lower concentration of metformin from 100 to 2000 µM and phenformin from 5 to 50 µM were used to treat MOLM-13, and from 100 to 4000 µM metformin and 10 to 100 µM phenformin were used to treat HL-60 over 24 and 48 hours incubation time using the same Alamarblue assay.

The response of MOLM-13 to both drug treatments showed an increased inhibition to increasing drug concentration in 24 and 48 incubation time (figure 7). Metformin

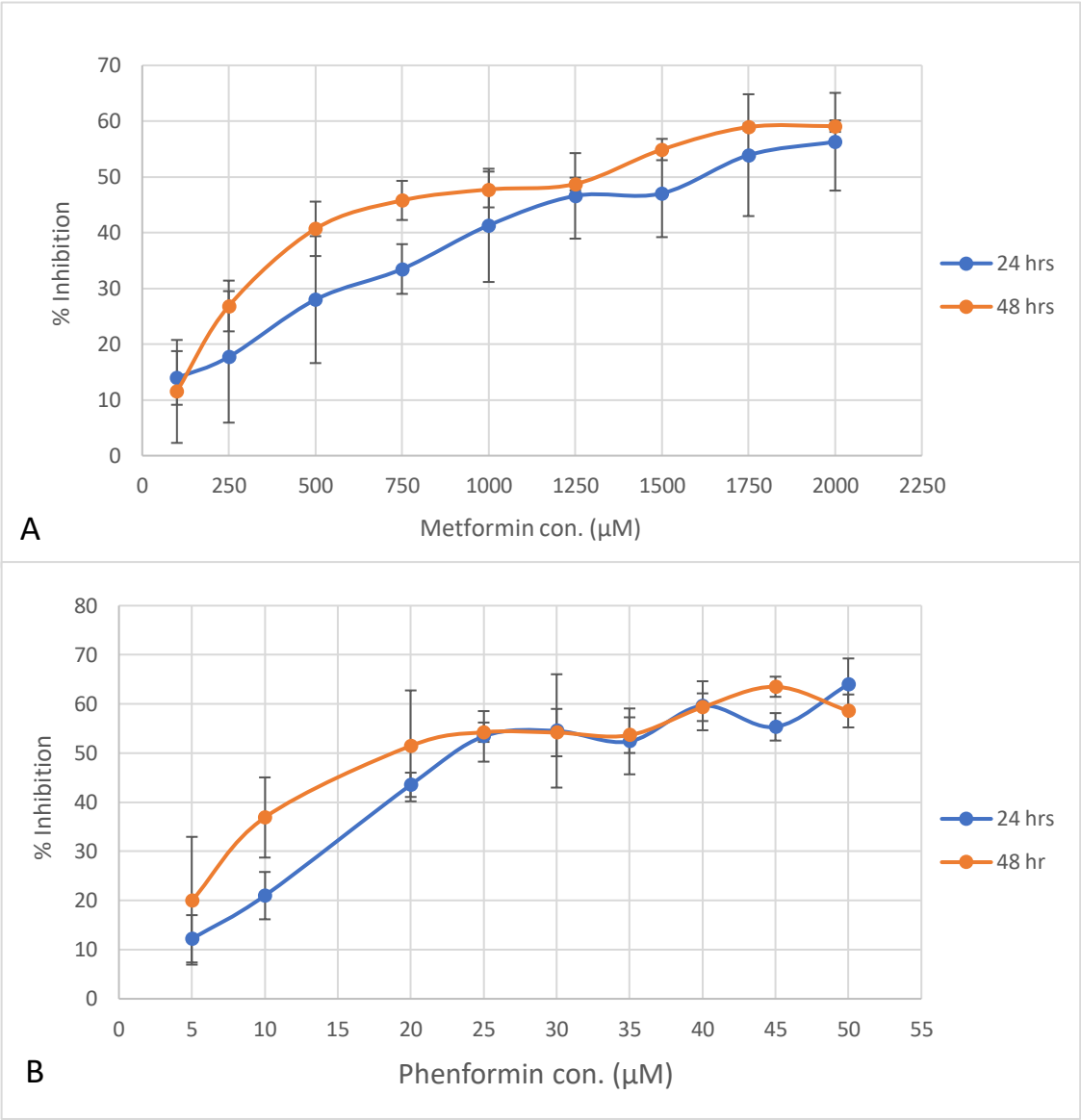

Figure 7. Dose response of MOLM-13 to A) metformin and B) phenformin over 24- and 48-hours treatments in lower range concentration. Florescence intensity were acquired at 590 nm and calculated as % inhibition from cell viability % of control and standard deviation were taken from 3 biological

treatment after 24 hours showed an inhibition of 42% from 100 µM to 1250 µM, proceeding with only 9 % increase from 1250 µM to 2000 µM (figure 7A). In 48 hours treatment, a curve increase in 36% inhibition observed from 100 µM to 1000 and gradual increase of 11% from 1000 µM to 2000 µM. While phenformin treatment after 24 hours observed a sharp increase with 41% inhibition from 5 µM to 25 µM and from 25 µM to 50 µM only 11 % increase in inhibition was observed (figure 7B). In 48 hours treatment, a curve increase in response was observed from 5 µM to 25 µM with 20% to 54% inhibition, and with only 9% increase in inhibition from 25 µM to 50 µM.

In HL-60, longer exposure of cells to metformin and phenformin showed notably increase in response compared to 24 hours exposure to the drugs (figure 8). Metformin effect after 24 hours of exposure showed that only 24% inhibition was reached in the highest given concentration (figure 8A). While in 48 hours exposure, a notably increase in 28% inhibition from 1000 µM to 2500 µM was observed, and from 2500 µM to 4000 µM showed almost no difference in response. Concentration from 500 µM and below showed no inhibitory effect, rather an increase in viability was observed with 16% in the lowest concentration. In phenformin, the inhibitory effect was observed in 48 hours treatments with a relative increase of 50 % (6% to 56%) inhibition from 20 µM to 70 µM (figure 8B). Concentration below 20 µM showed no inhibitory effect. In 24 hours treatments, 32% inhibition was observed in the highest concentration.

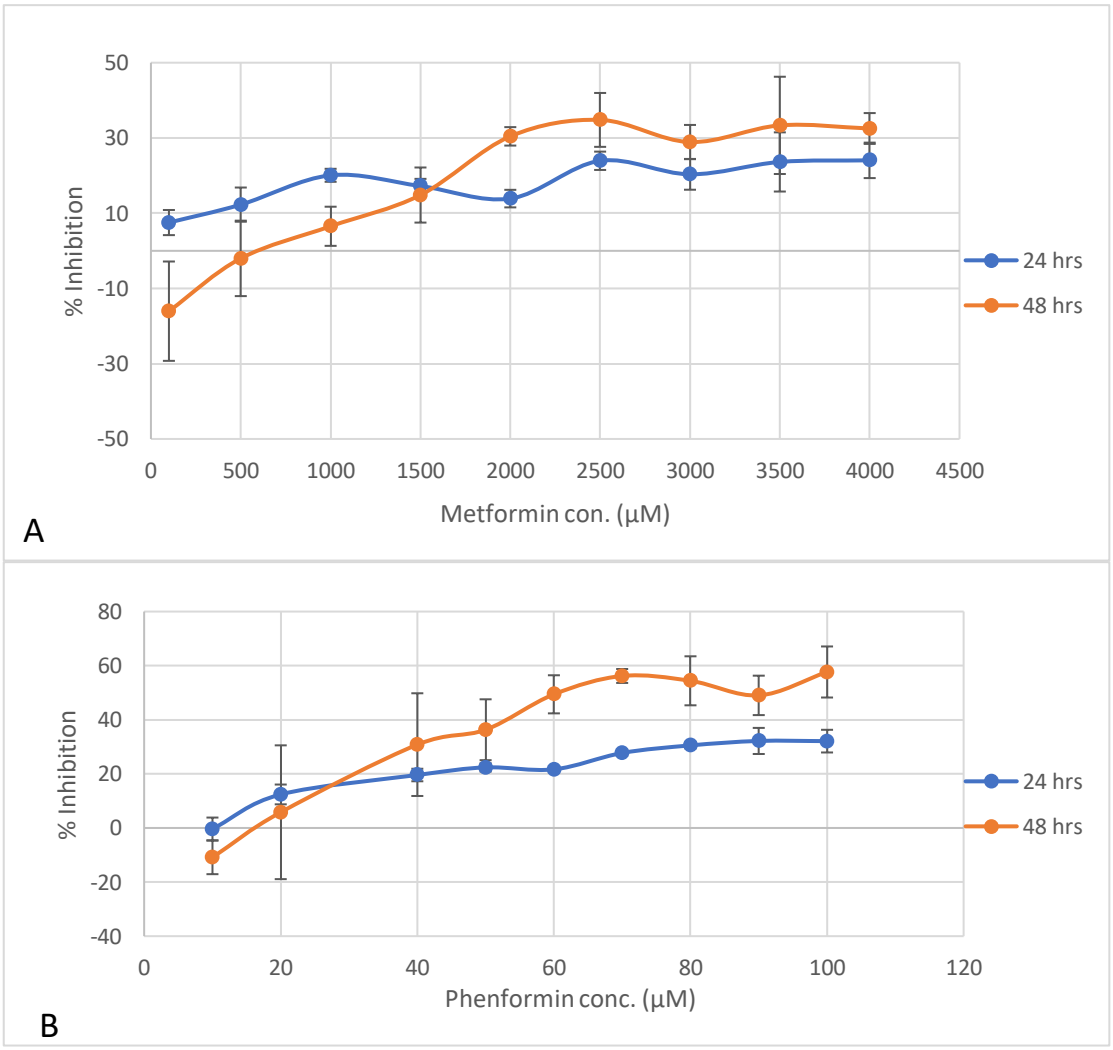

Figure 8. Dose response of HL-60 to A) metformin and B) phenformin over 24- and 48-hours treatments in lower range concentration. florescence intensity collected at 590 nm were calculated as % inhibition from cell viability % of control and standard deviation were taken from 3 biological replicates in one experiment.

In general observation, the results showed that the longer incubation time of metformin and phenformin treatment in both cell lines was more reliable in measuring the inhibitory effect of the drugs.

3.4 Effect of metformin and phenformin on cell viability of MOLM-13 and HL-60 using MUSE™ Count and viability assay.

The concentration-dependent effect of metformin and phenformin were further investigated using MUSE™ Count and viability kit assay. Cells were subjected into 3 different concentrations of metformin and phenformin and incubated for 24 and 48 hours. Metformin concentrations used were: 200, 1000 and 2000 µM for MOLM-13; and 200, 2000 and 4000 µM for HL-60. Phenformin concentrations used were: 10, 50 and 100 µM for MOLM-13; and 10, 50 and 100 µM for HL-60.

In MOLM-13, addition of metformin lowered the viability of cells after 24- and 48-hours incubation time aside from 200 µM which seems to be recovered after 48 hours (Figure 9A), while the addition of phenformin was more evident to lowered cell viability after 48 hours (Figure 9B).

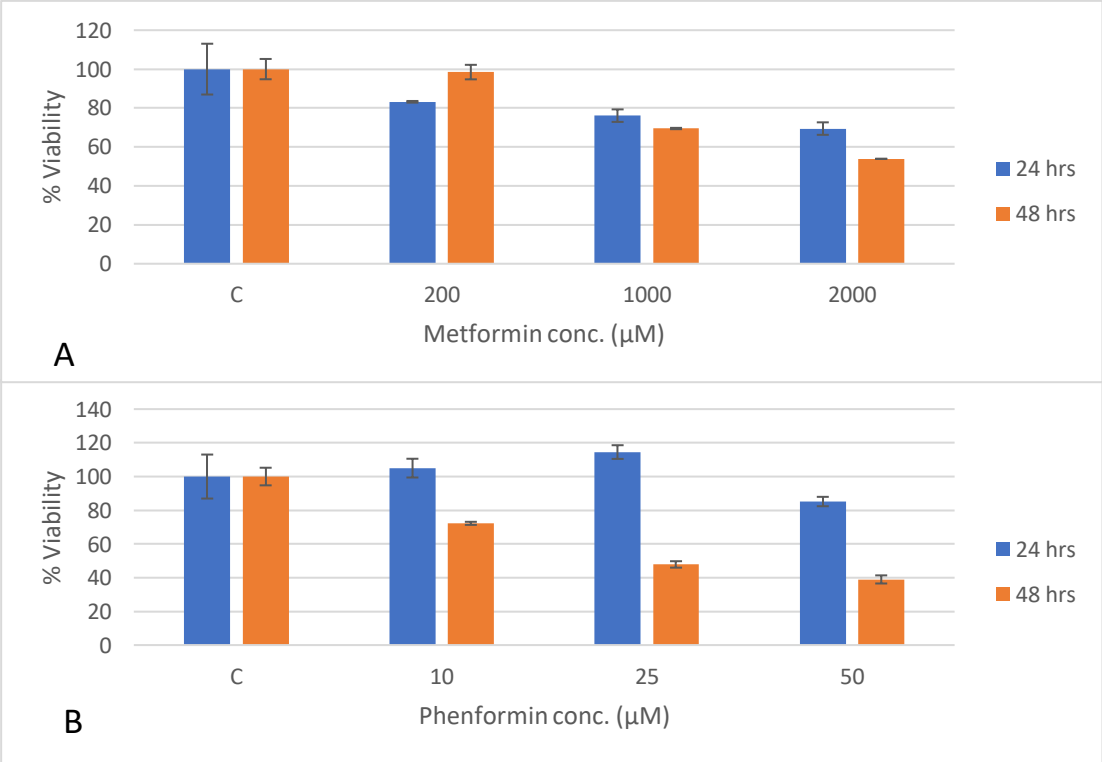

Figure 9. Cell viability of MOLM-13 treated by A) metformin and B) phenformin. The cell count of each treatment was calculated to % viability of control and standard deviation were obtained from two biological replications in one experiment.

In HL-60, metformin treatment showed to lowered cell viability in 2000 and 4000 µM with more evident after 48 hours exposure (figure 10A). While phenformin treatment showed to lowered cell viability in all treatment but more evident in 24 hours (figure 10B).

The inhibitory effect of metformin and phenformin showed to be consistent with the previous experiments in both cell lines as a concentration and time dependent drugs. MOLM-13 showed to be more responsive than HL-60 in both treatment and phenformin was more effective in inhibiting growth of both cell lines than metformin.

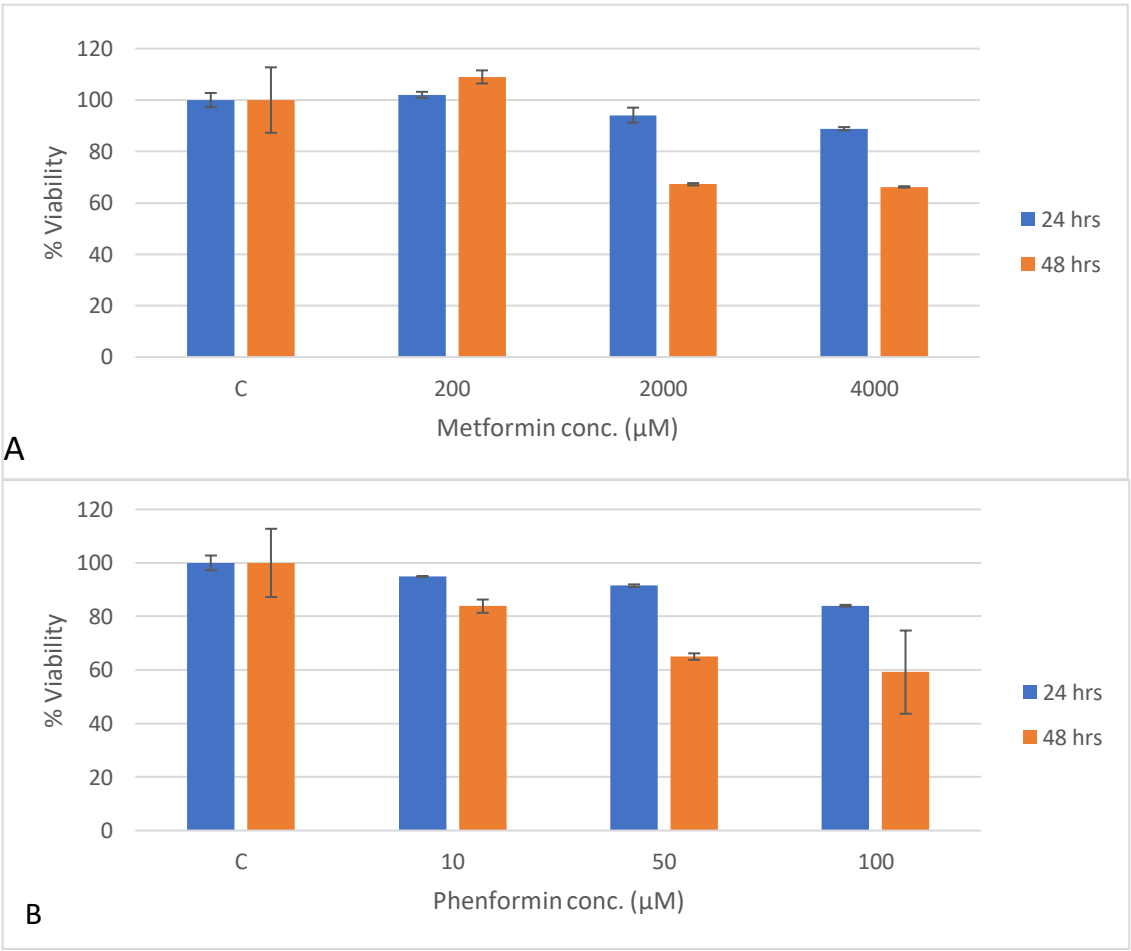

Figure 10. Cell viability of HL-60 treated by A) metformin and B) phenformin. The cell count of each treatment was converted to %viability of control and standard deviation were obtained from two biological replications in one experiment.

3.5 OCT1 expression levels of MOLM-13 and HL-60

OCT1 expression analysis were carried out using protein expression and flow cytometry.

Using the negative control of no anti-body for OCT1 (primary), the expression of the OCT1 levels seem to be similar, but HL-60 seems to have slightly higher expression (figure 11).

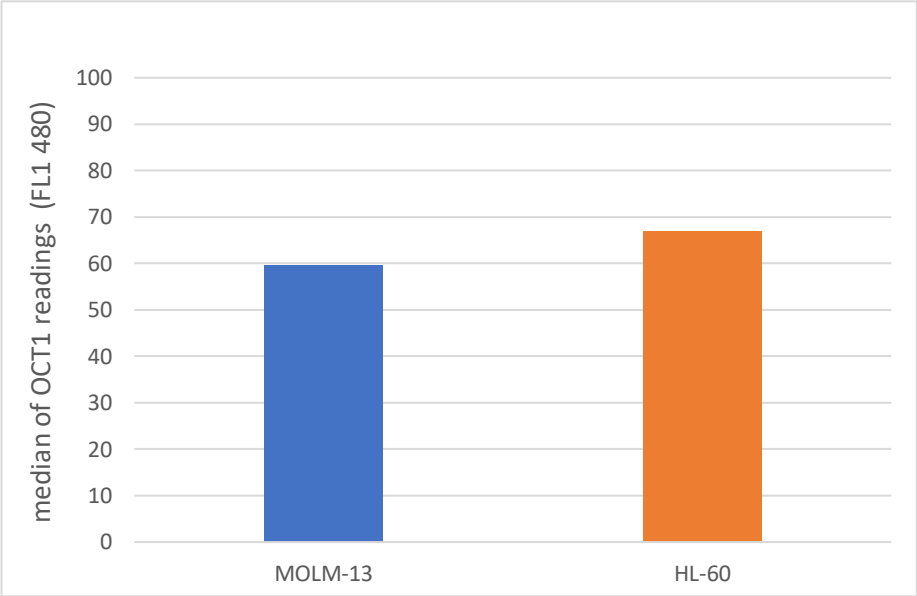

Figure 11. Oct1 expression levels of MOLM-13 and HL-60.

3.6 Metformin and phenformin influence on expression of OCT1 in MOLM-13 and HL-60

Results revealed that both drug treatments upregulated the expression of OCT1 in MOLM-13 and downregulated in HL-60 (figure 12). In metformin treatment, it showed that OCT1 expression lowered in MOLM-13 in the first 24 hours and increased up to 1.55 times more than control after 48 hours, while HL-60 were downregulated with almost the same rate after 24 and 48 hours of treatments (figure 12A). Similar pattern of response were observed in phenformin treatment to MOLM-13, the expression of OCT1 were lowered in the first 24 hours and increased after 48 hours up to 1.59 times more than control. While in HL-60, down regulation of OCT1 were observed only after 48 hours exposure (figure 12B).

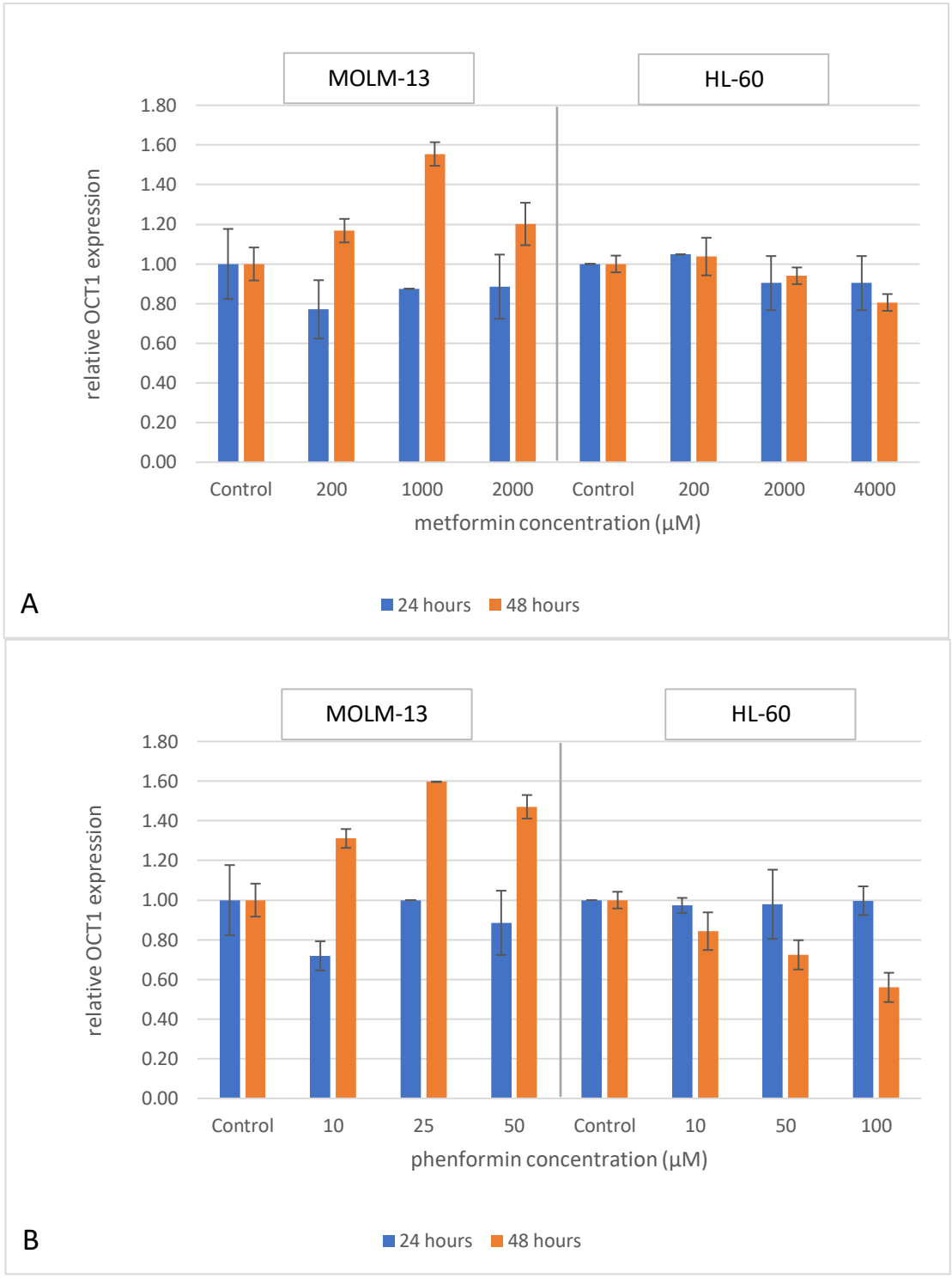

Figure 12. OCT1 expression in MOLM-13 and HL-60 in response to A) metformin and B) phenformin. Data acquired as median of florescence at FL1 (525/30), and the mean of two replicates were normalized to its own control. Standard deviations were calculated from 2 biological replicates of one experiment.

4 Discussion

Metformin and phenformin are drugs used for treating diabetes 2 but have recently been associated with a beneficial effect in cancer patients [23,22]. However, the drugs need to reach their target cells to have an effect and here we show that even between cancer cells of similar origin there is a differential effect from both drugs, both in concentration used and treatment time. We found that phenformin is 27 to 55 folds stronger drug than metformin and MOLM-13 is 1.10 folds more responsive to metformin and 2.2 folds to phenformin treatment than of HL-60. We also found that the longer exposure time (48 hours) is more reliable in measuring the inhibitory effect of the drugs in this experiment. MOLM-13 shows to readily took up the drug and showed a response in the first 24 hours, however after 48 hours, it showed that higher concentration is needed to achieve the 50% inhibition in the cell activity. In contrary, it needs longer time exposure for the drugs to show an effect in HL-60, the 50% inhibitory concentration showed in 24 hours is much higher than in 48 hours. It shows that the drugs are not completely taken up by the cells and it seems that HL-60 tried to repel the toxic chemical from entering the cells. The differential response of cell lines to metformin and phenformin treatment maybe dependent to the expression of OCT1 [35,16]. However in previous studies, the mRNA levels of OCT1 were three-folds higher in MOLM-13 compared to HL-60 [47], while our data in expression levels of OCT1 receptor shows that MOLM-13 is slightly lower than HL-60. The reduced in expression of OCT1 may account to posttranscriptional modification of mRNA [35]. The fact that not all mRNA can be translated into protein due different kinds of modification in transcription and translation, we could assume that functional OCT1 expression would be more relevant indicator to measure the effect of drugs.

Various studies demonstrated that metformin mode of action is reliant to OCT1 expression and that genetic variation and polymorphisms in *OCT1* may contribute to variation in drug disposition and response [30,35]. Previous study of hepatocytes, demonstrated that deletion of *Oct1* resulted in a reduction in the effects of metformin on AMPK phosphorylation and gluconeogenesis [35]. Here we investigated the influenced of drug treatment, metformin and phenformin, to the expression of OCT1 receptor overtime. We show that metformin and phenformin have influenced the regulation in expression of OCT1 receptors. Interestingly, it shows that MOLM-13

increased expression of OCT1 over time during treatment, while the same is not true to for HL-60. The differential regulation pattern of OCT1 expression in MOLM-13 and HL-60 shows to corresponds to the response to treatment. The higher response of MOLM-13 to treatment was apparently due to the increased in OCT1 expression, while the withstanding effects of HL-60 to treatment was because of the decreased in OCT1 expression.

Previous study demonstrated that expression of OCT1 is regulated by various intracellular pathways, such as inhibition of OCT1 by PKA activation [36]. And from another study it was demonstrated that induction of c-MYC can cause an increase in PKA catalytic activity independent of cAMP-mediated signaling [48] and the activation of the cAMP-PKA pathway leads to metformin resistance [49]. The over expression of MYC in HL-60 must be a contributing factor to the downregulation in expression of OCT1. And the different mutations between cell line play an important role in the up and down regulation of the OCT1 expression in MOLM-13 and HL-60.

Our data suggest that regulation of OCT1 expression could be an important biomarker for determining response of metformin and phenformin treatment.

From the point of view of our results, we can say that it can be an important for cancer patients who want to be treated by metformin (phenformin) to test the OCT1 expression of the cancer cells before using and for the doctors to better understand the regulation of OCT1 expression before administering the metformin (phenformin) to the cancer patients.

4.1 Future perspectives

In the experiment where the analysis in expression levels of OCT1 between cell lines, using negative control as cell line with no expression of OCT1 would be ideal to this analysis, however due to limited resources, the analysis were carried out using negative control of no antibody for OCT1 (primary), if given longer time it could nice to confirmed the results using different methods. Also, the experiment of OCT1 expression using flow cytometry was performed only once, thus repetition of the experiment, also with using different methods of analysis, would interesting to see if the result will be the same and if so, to further validate and fortify our findings. And finally, it would be interesting to test

a relevant cell lines where one confirms high/low OCT1 expression and see if they are more or less responsive to metformin or phenformin treatment.

5 Conclusions

Our results showed that metformin and phenformin effects is dose- and time exposure-dependent and their potency and efficacy varied in the two cell lines. Phenformin is more potent than metformin, and MOLM-13 is more responsive to drug treatments, while HL-60 appears to develop resistance to drug treatments. The expression levels of OCT1 is slightly higher in HL-60 compared to MOLM-13. And metformin and phenformin treatment over time influences the expression of OCT1 receptors in MOLM-13 and HL-60. OCT1 expression in MOLM-13 is upregulated by metformin and phenformin treatments overtime, while in HL-60 it is downregulated in both treatments, but more so in phenformin treatment.

From the perspective of the results we conclude that the regulation OCT1 expression could be an important biomarker for determining the response of cancer cells to drug (metformin and phenformin) treatments. And also, it can be of importance to better understand the regulation of OCT1 expression in different types of cancer before treating patients with metformin.

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7 Appendix

7.1 List of materials used in the experiment

Table 4. Information of products and instruments used in the experiment.

Product name	Company	Reference	Notes
Cell Culture			
MOLM-13 cell line	DSMZ	ACC 554	Biological material-mammalian cell line
HL-60	DMZ	ACC 3	
RPMI 1640 Medium	Life Technologies AS (Invitrogen Dynal AS)	21875034	
Fetal bovine serum	Biowest SAS	-	
Penicillin Streptomycin, liquid	Life Technologies AS (Invitrogen Dynal AS)	15140122	
Compact Tabletop Refrigerated Centrifuge	Kubota	2800	
T25 Nunclon™ cell culture flask	Thermo Scientific	174951	
T75 Nunclon™ cell culture flask	Thermo Scientific	174952	
Muse™ Count & Viability Kit (600 tests)	Merck Life Science AS	637365	
Muse™ Cell Analyzer	Merck Millipore		
Proliferation and Viability Assay			
96-well black-walled microplates	VWR®		
PBS Tablets	Life Technologies Europe BV	18912014	1x concentration, 1 tablet dissolved in 500 ml dH_2O
AlamarBlue	Invitrogen	DAL1100	484 µM in 1x PBS
SpectraMax Paradigm Multi-Mode Microplate	Molecular Devices		
SoftMax Pro Microplate Data Acquisition and Analysis Software	Molecular Devices		
Metformin hydrochloride	Merck Life Science AS	M0605000	
Phenformin hydrochloride	Sigma-Aldrich Norway AS	P7045	
OCT1 determination (fixation, immunostaining, and flow cytometry)			
6-well plates, flat bottom	VWR®	734-2323	
Formaldehyde 37%	VWR international AS	81033	Diluted into 4% in 1x PBS
Centrifuge 5702 R (F-45-24-11)	Eppendorf		Fixed-angled Rotor F-45-24-11

Bovine Serum Albumin	Merck Life Science As	A9418	0.5 g dissolved in 100 ml 1x PBS
OCT1 Antibody	Nordic Biosite	GTX80400	Host: Mouse
CF®488A Llama Anti-mouse IgG (H+L)	Biotium	20454-500µL	Ex/Em: 490/515 nm
S3e Cell Sorter	Bio-Rad		
ProSoft™ Software, Version 1.6			
Proline™ Universal Calibration Beads		1451086	
Proflow™ Sort Grade 8x Sheath fluid		1451082	
Milli-Q Water			
Cell freezing			
Dimethylsulfoxide (DMSO)	Life Technologies Europe BV	L34951 COMPONENTC	Used 10% DMSO
Cryovials			
Mr. Frosty™ Freezing Container	ThermoFisher Scientific	5100-0001	

7.2 Supplemental data for flow cytometry analysis

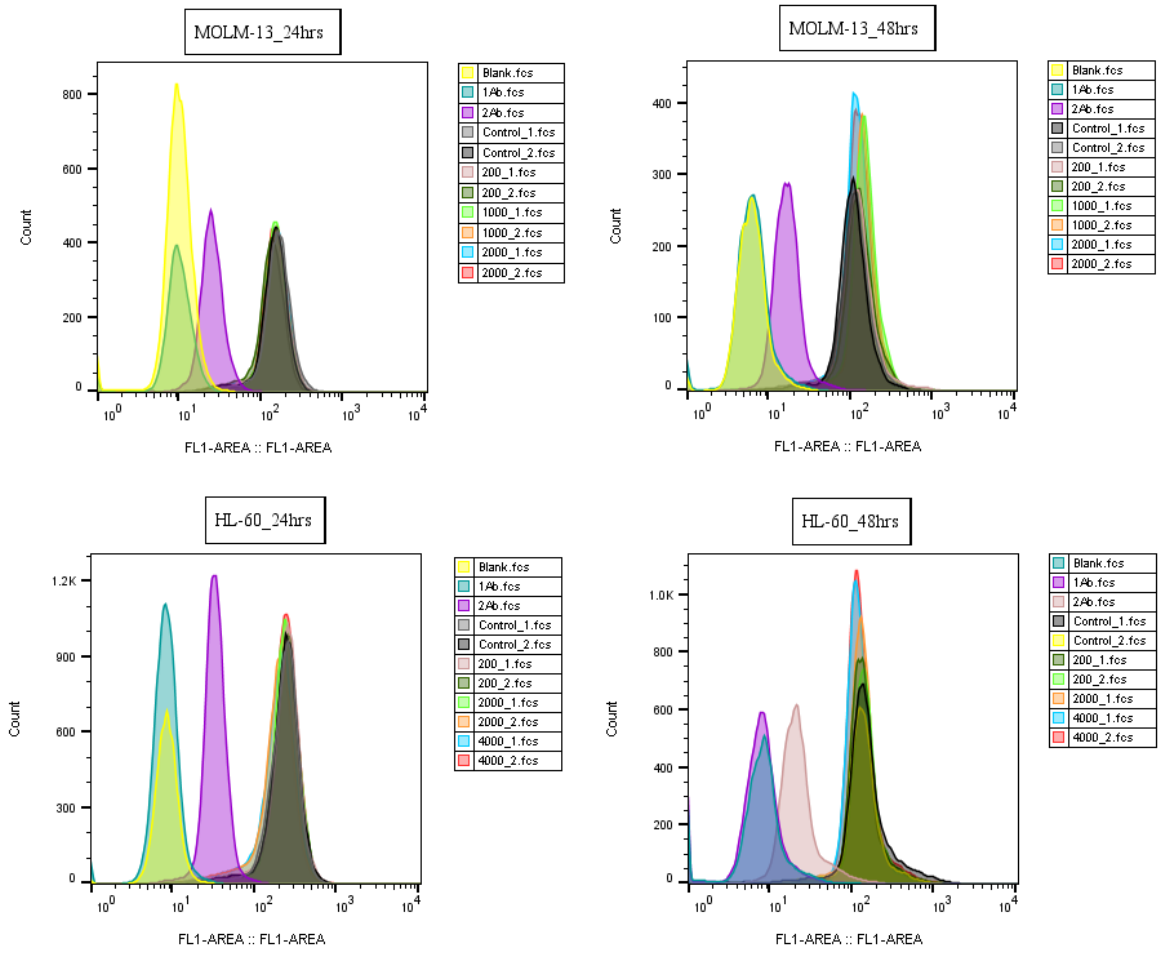

Figure 13. Overlays histograms of OCT1 expression in MOLM-13 and HL-60 with Metformin treatment over 24- and 48-hours exposure.

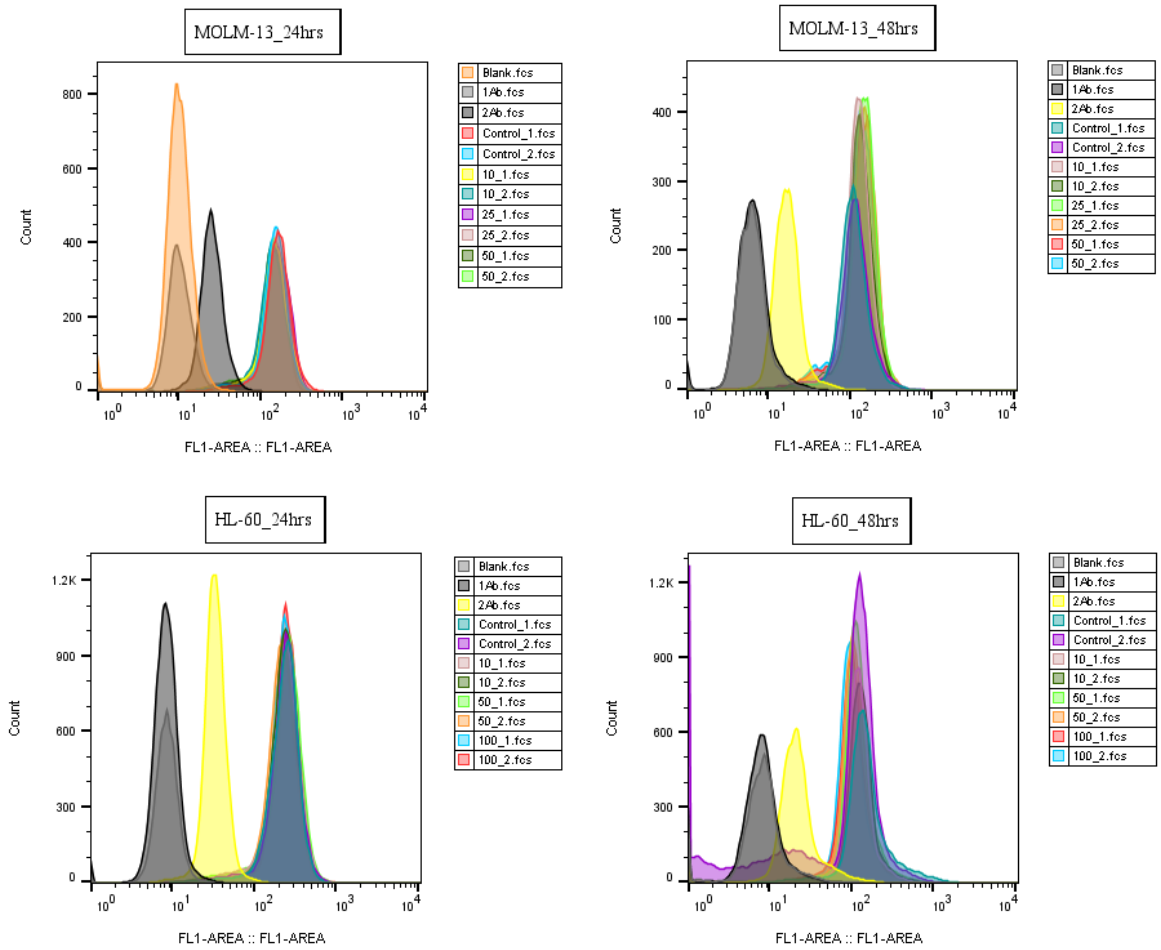

Figure 14. Overlays histograms of OCT1 expression in MOLM-13 and HL-60 with Phenformin treatment over 24- and 48-hours exposure.

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