香港城市大學
City University of Hong Kong

Department of Biomedical Sciences

Bachelor of Biomedical Sciences

BMS4006 Medical Laboratory Research

FINAL YEAR PROJECT 2023-24

Investigation of the Apoptotic Effects of Metformin: Potential for Anti-cancer Drug Development

Student Name: HUANG Zechao

Student ID: 56642581

Supervisor: Prof. Jackie Y. L. PON

ABSTRACT

Metformin is the most widely used antidiabetic drug globally, with increasing evidence suggesting its anti-cancer potential in recent years. Conventional therapies for colorectal cancer (CRC), like 5-FU, while effective, are often accompanied by side effects and drug resistance. This project intends to investigate the apoptotic effects of metformin on CRC cells and aims to explore its potential as a novel anti-cancer drug.

This project used a comparison of the cancer cell line, HT-29, and the normal cell line, CAL-1, together with a comparison of metformin and 5-FU for investigation. Techniques, including cell culture, MTS assay, flow cytometry, etc., are utilized in the research. After investigating metformin's effect on HT-29 cell viability, it suggests that the effect is dose-dependent and time-dependent. As for the detailed effect on HT-29 cells, by applying effective dosages and times, the findings indicate that metformin causes morphological changes and induces cell death, which predominantly necrosis over apoptosis. Besides, the discovery demonstrates the excellent synergetic anti-cancer effect in the combination of metformin and 5-FU.

All these findings illustrate that although no significant apoptotic effect is presented, metformin still possesses a substantial anti-cancer effect in inhibiting cancer cell

viability, causing cancer cell morphological alterations, and inducing cancer cell death. Possible underlying mechanisms include its ability to activate the AMPK pathway and suppress the mTOR pathway. Alteration of these pathways results in the anti-cancer effects observed in the project.

This work is significant in both CRC research and patient care, offering strong practicability. Further research may focus on underlying molecular mechanisms, in vivo investigation, and clinical trials of metformin on cancer.

ACKNOWLEDGEMENT

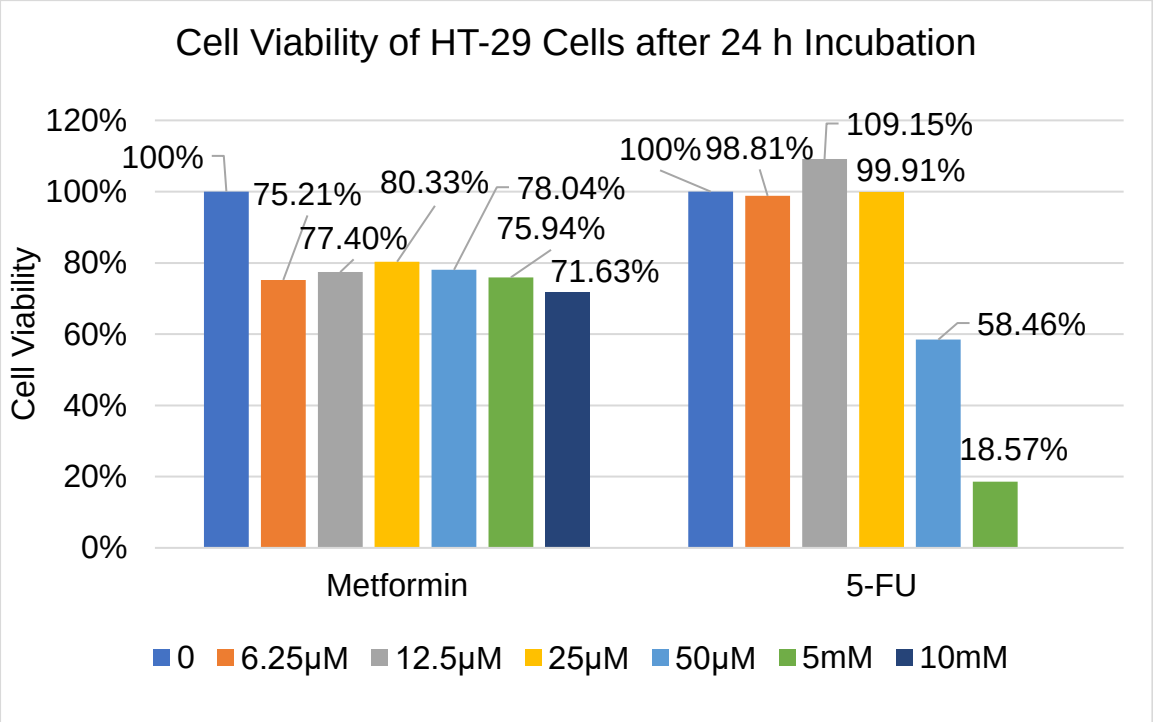
First of all, praise to Almighty GOD, who granted me countless blessings. Many individuals and groups assisted me a lot in this project, and I want to thank them all. I would like to express my gratitude to my supervisor, Prof. Jackie Y. L. Pon, for her valuable guidance, support, and reminders all along the way. As well, I am very thankful to Ms. Fannie Tsang and the whole technical team, for all their material purchase and technical support. Also, thank you to my classmates, for providing me advice and offering a helping hand. Furthermore, I would like to thank my beloved family, for their unconditional love and trust. Last but not least, I would like to convey my appreciation to City University of Hong Kong for all my four-year studies.

TABLE OF CONTENT

LIST OF ABBREVIATIONS

Abbreviations	*Definitions*
ATCC	American Type Culture Collection
AMPK	AMP-activated Protein Kinase
CRC	Colorectal Cancer
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene Diamine Tetraacetic Acid
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
mTOR	Mammalian Target of Rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PBS	Phosphate-Buffered Saline
PI	Propidium Iodide
ROS	Reactive Oxygen Species
RPMI 1640 Medium	Roswell Park Memorial Institute 1640 Medium
T2DM	Type 2 Diabetes Mellitus
5-FU	5-Fluorouracil

LIST OF FIGURES



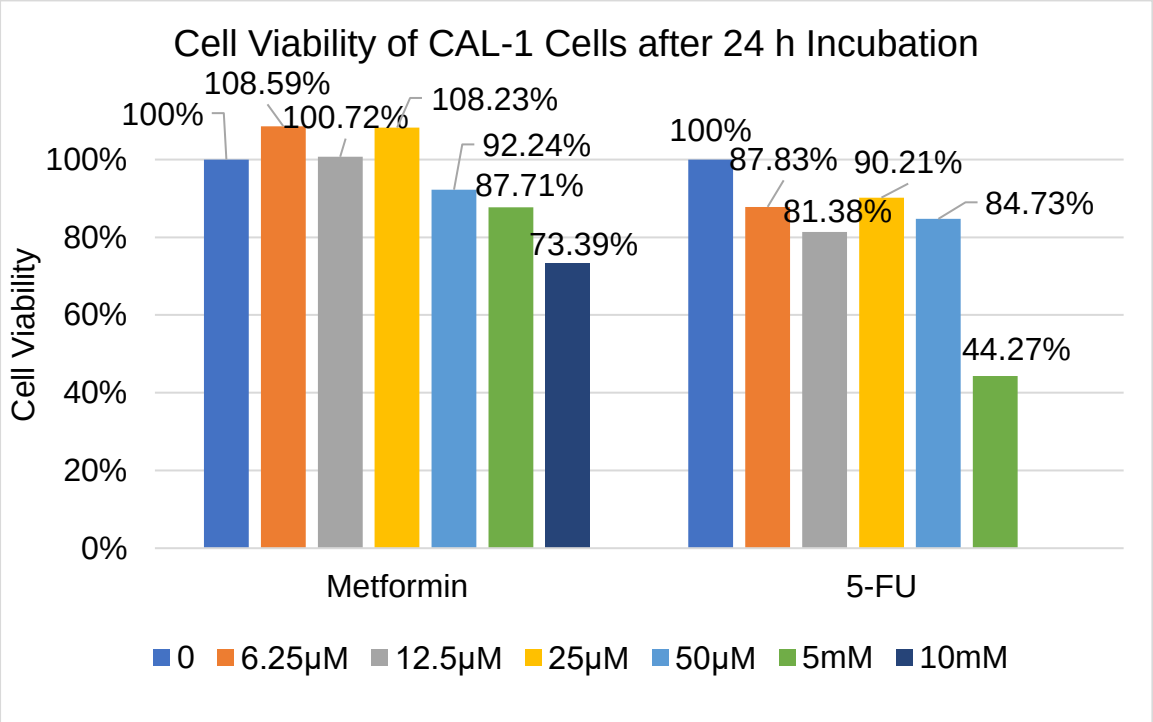
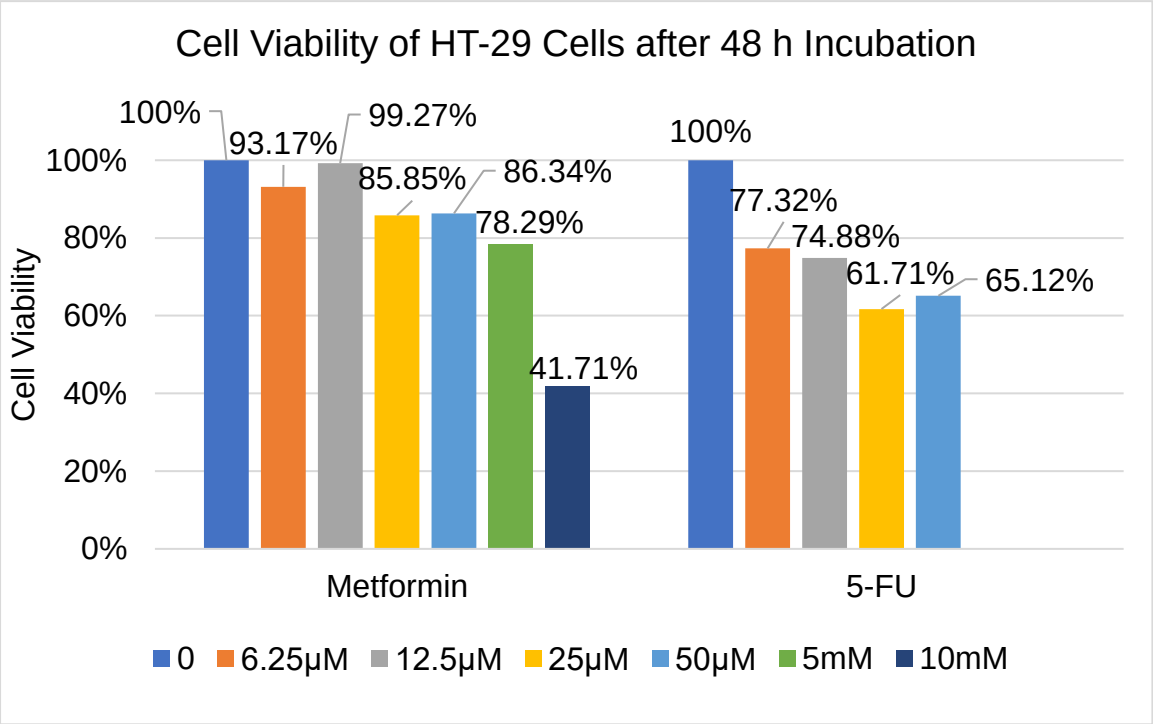


Figure 1A. *Cell Viability of Metformin or 5-FU Treatment in HT-29 and CAL-1 Cells after 24 h Incubation*



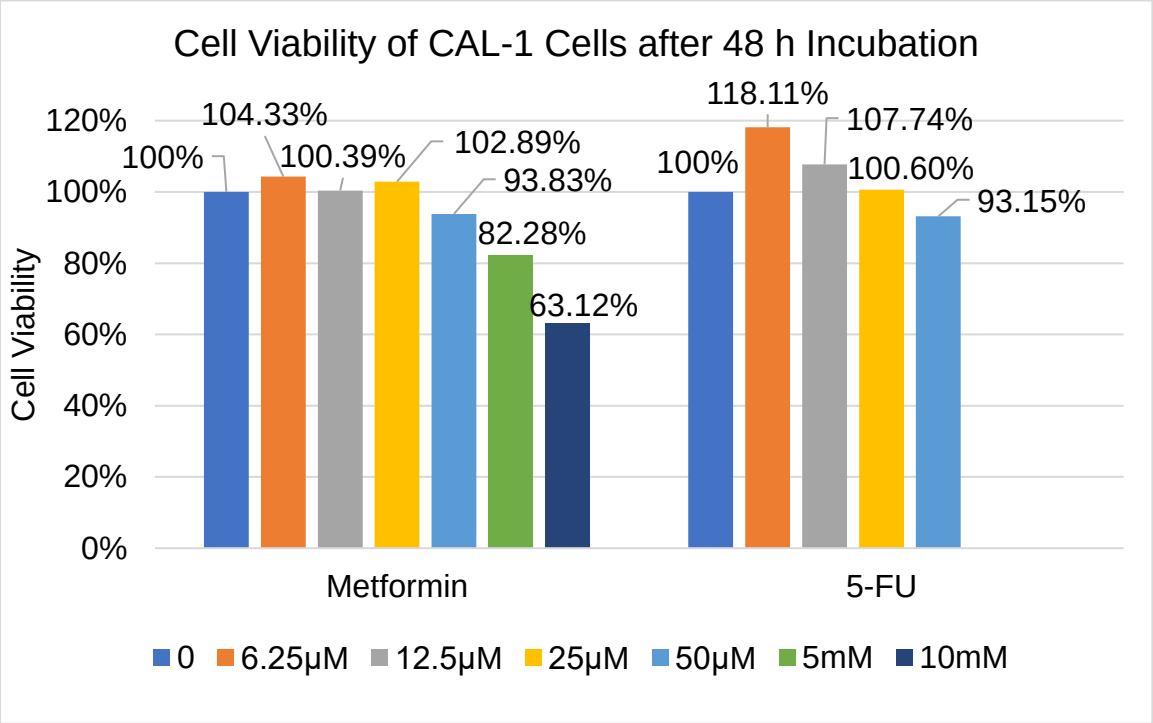


Figure 1B. *Cell Viability of Metformin or 5-FU Treatment in HT-29 and CAL-1 Cells after 48 h Incubation*

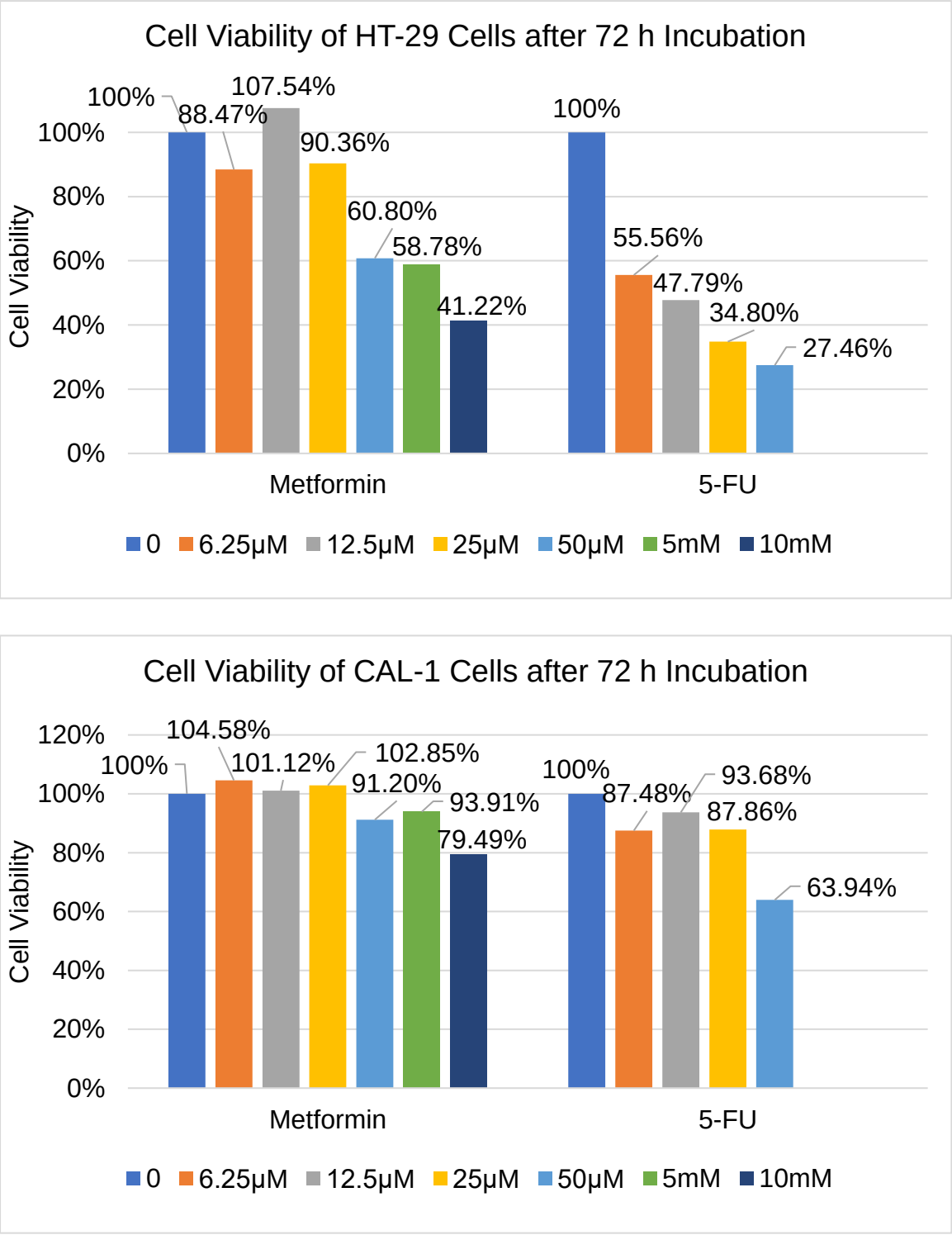
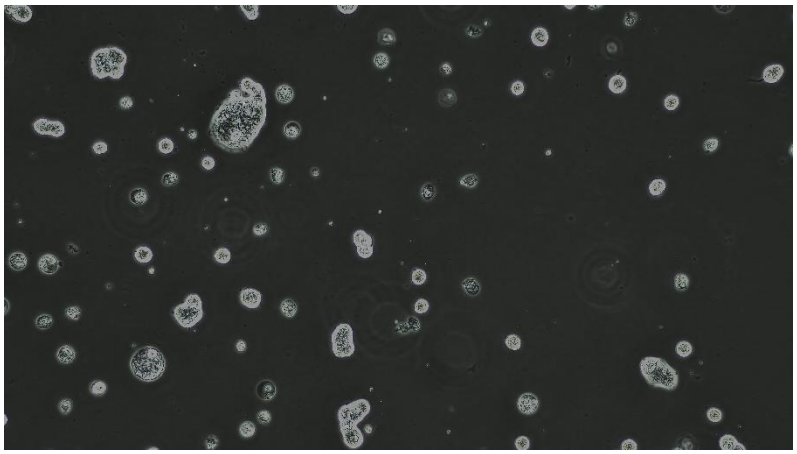
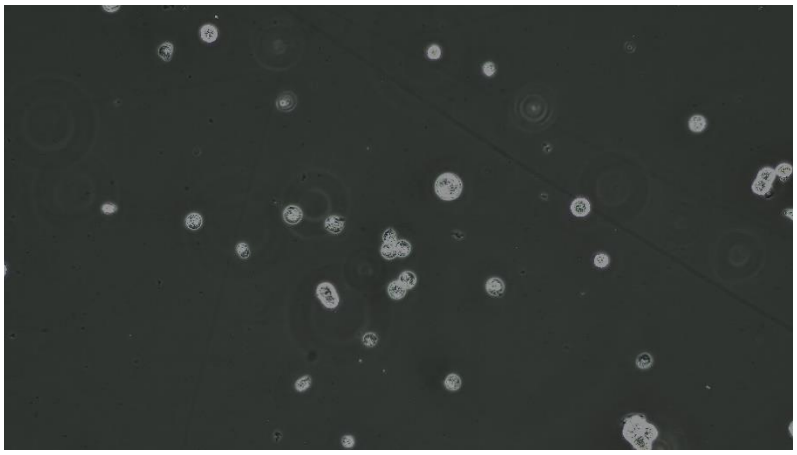


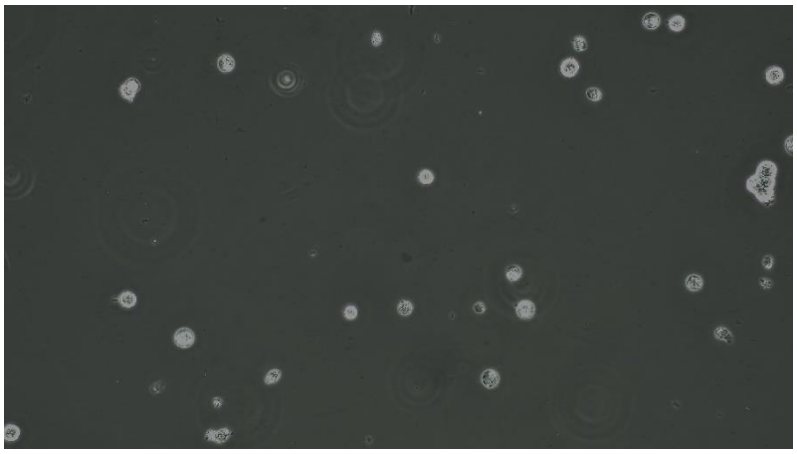
Figure 1C. *Cell Viability of Metformin or 5-FU Treatment in HT-29 and CAL-1 Cells after 72 h Incubation*



Control Group

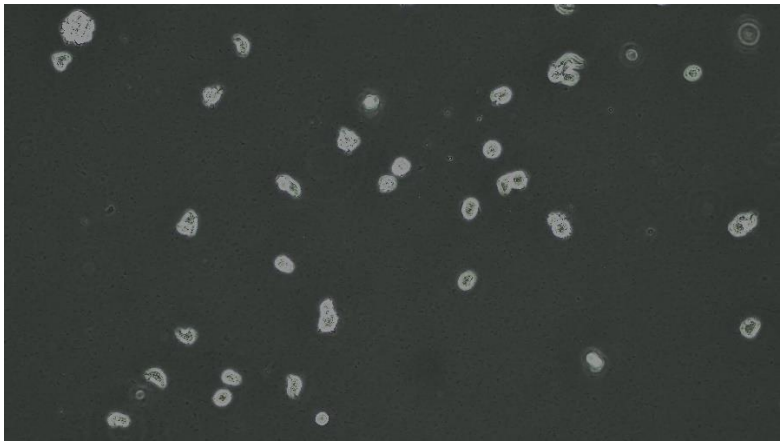


Metformin Group (10 mM)

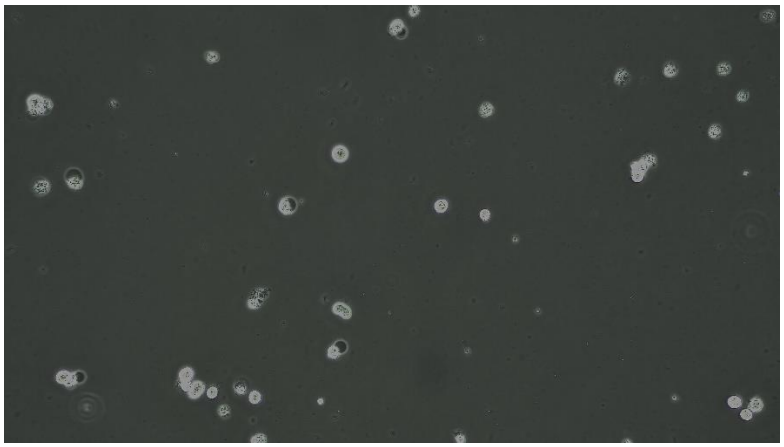


5-FU Group (50 µl)

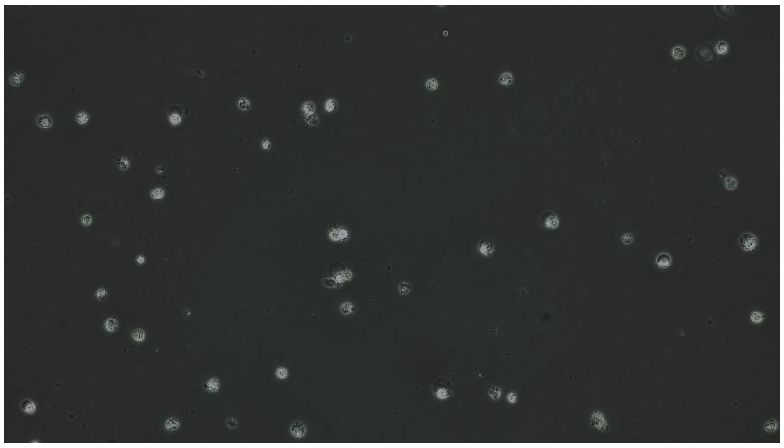
Figure 2A. *Cell Morphology of HT-29 cells of Control Group, Metformin Group (10 mM), and 5-FU Group (50 µl) (from up to down) after 48 h Incubation.*



Control Group

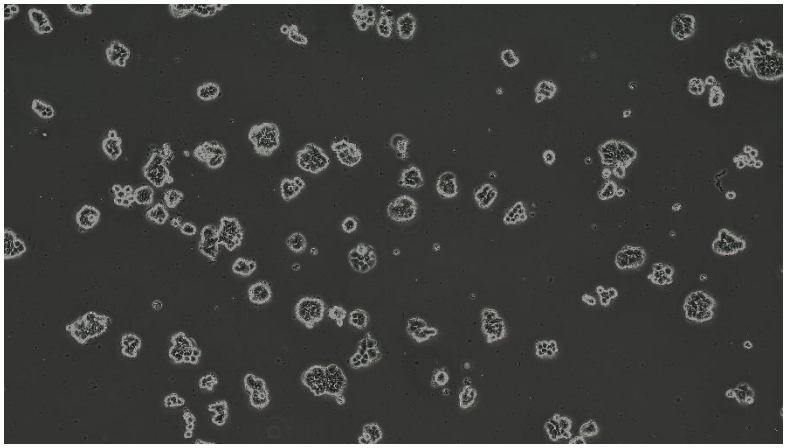


Metformin Group (10 mM)

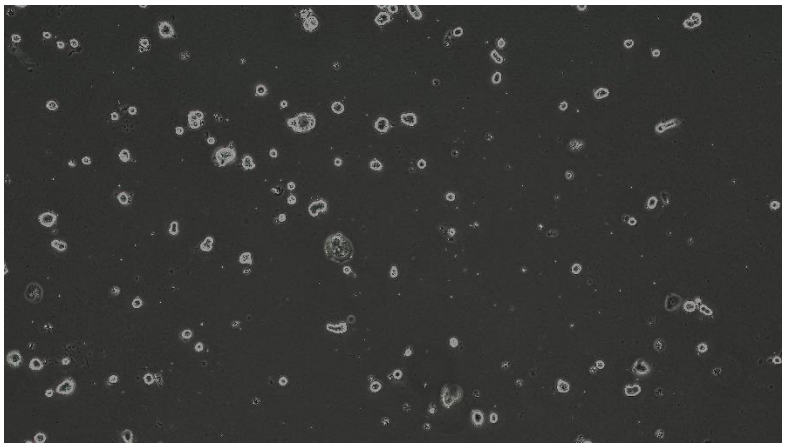


5-FU Group (50 µl)

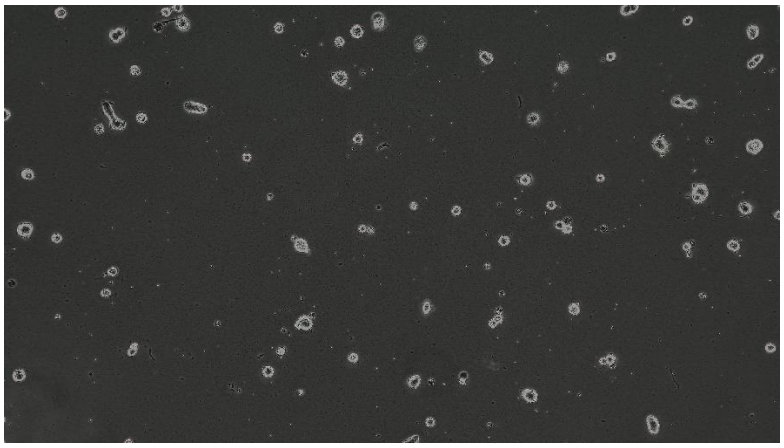
Figure 2B. *Cell Morphology of CAL-1 cells of Control Group, Metformin Group (10 mM), and 5-FU Group (50 µl) (from up to down) after 48 h Incubation.*



Control Group

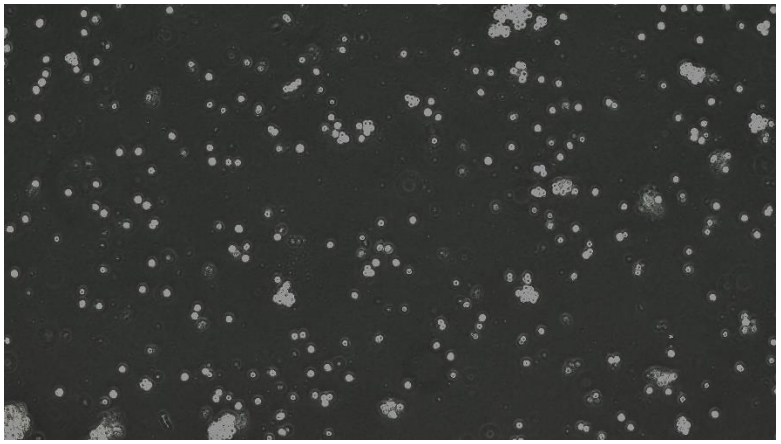


Metformin Group (10 mM)

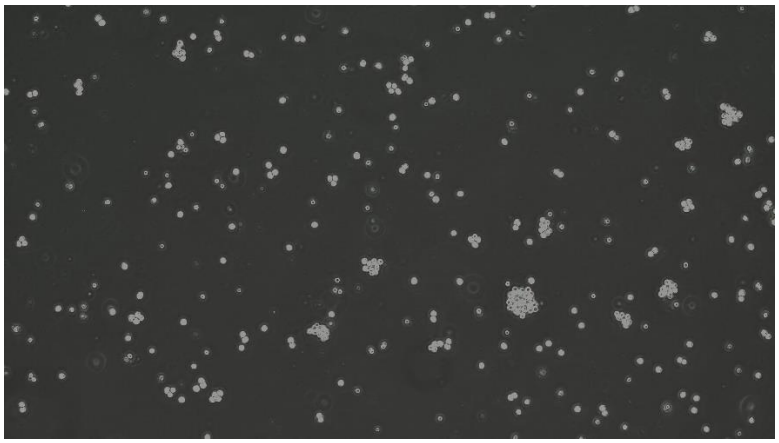


5-FU Group (50 µl)

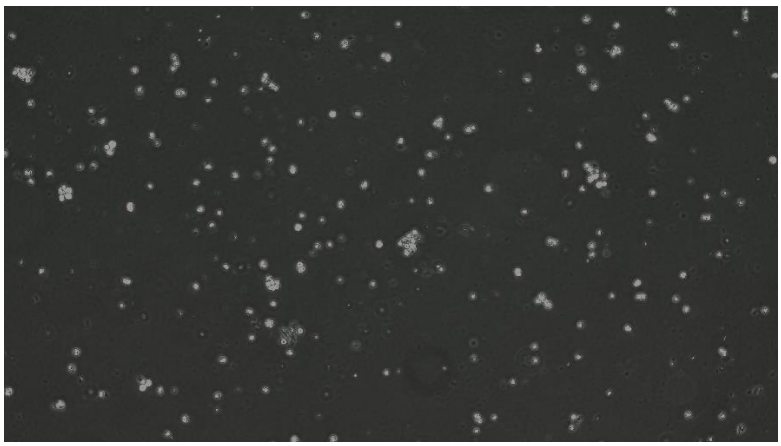
Figure 2C. *Cell Morphology of HT-29 cells of Control Group, Metformin Group (10 mM), and 5-FU Group (50 µl) (from up to down) after 72 h Incubation.*



Control Group

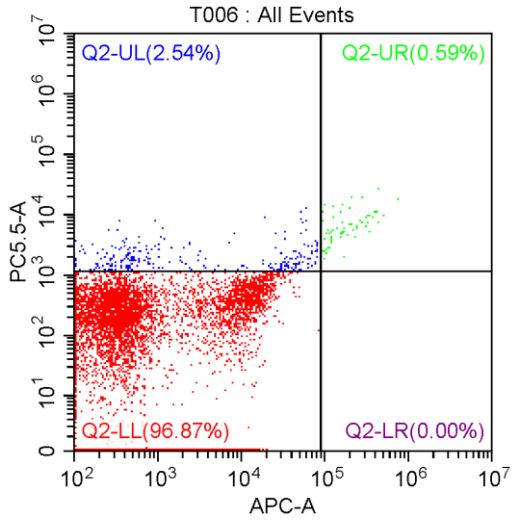


Metformin Group (10 mM)

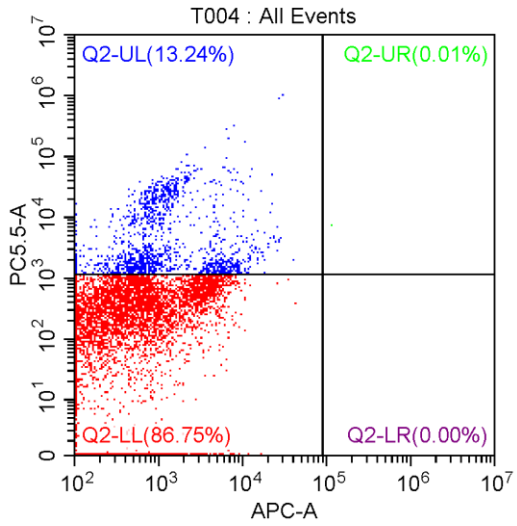


5-FU Group (50 µl)

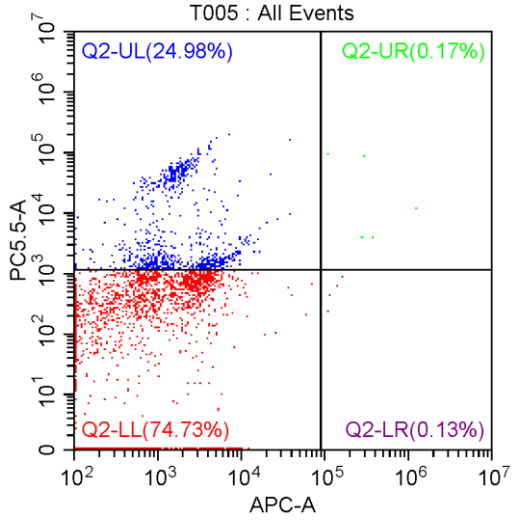
Figure 2D. *Cell Morphology of CAL-1 cells of Control Group, Metformin Group (10 mM), and 5-FU Group (50 µl) (from up to down) after 72 h Incubation.*



Control Group

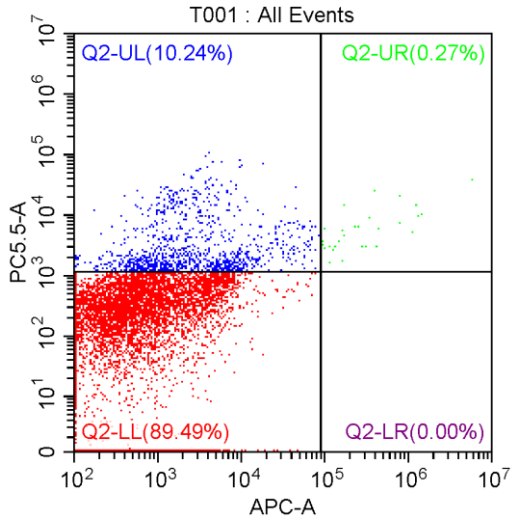


Metformin Group (10 mM)

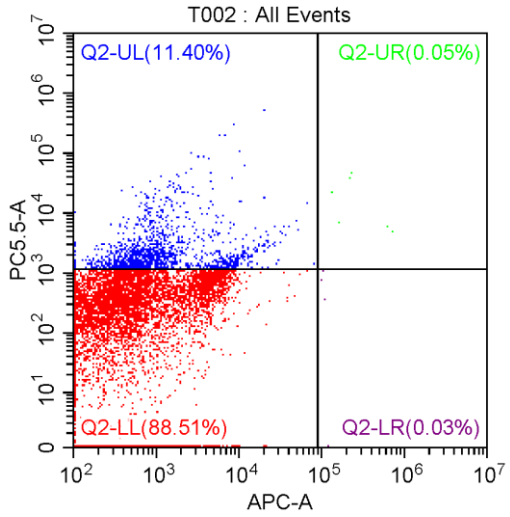


5-FU Group (50 µl)

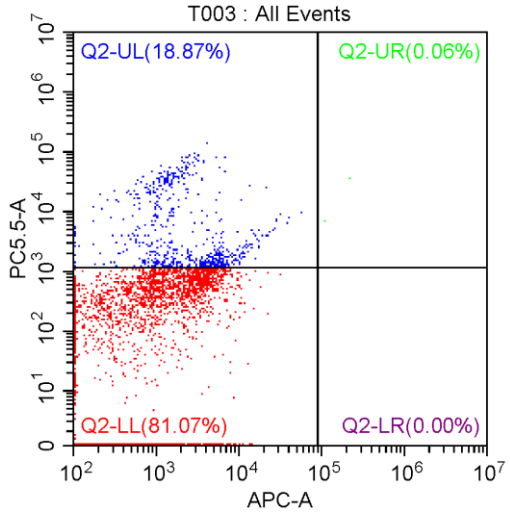
Figure 3A. *Cell Death of HT-29 Cell of Control Group, Metformin Group (10 mM), and 5-FU Group (50 µl) (from up to down) after 48 h Incubation.*



Control Group

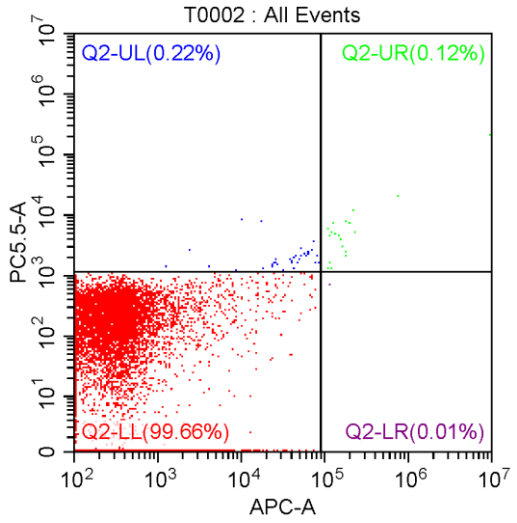


Metformin Group (10 mM)

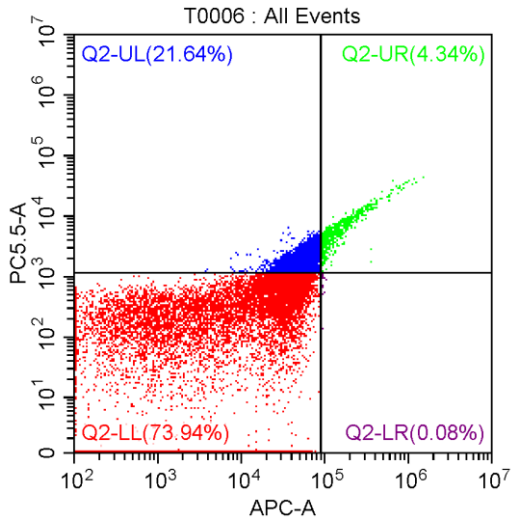


5-FU Group (50 µl)

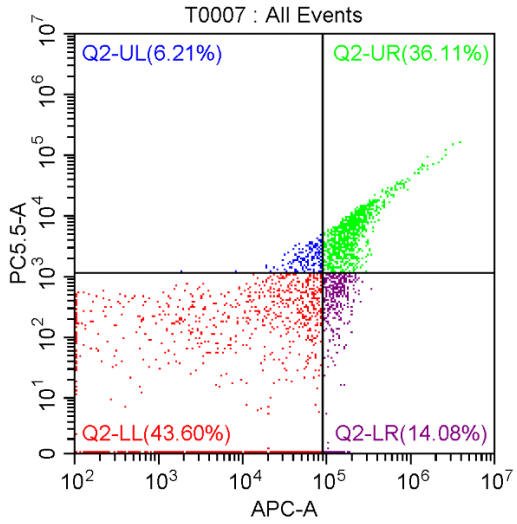
Figure 3B. *Cell Death of CAL-1 Cell of Control Group, Metformin Group (10 mM), and 5-FU Group (50 µl) (from up to down) after 48 h Incubation.*



Control Group

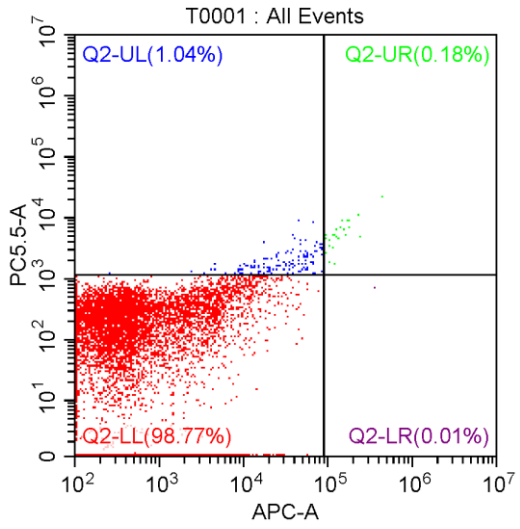


Metformin Group (10 mM)

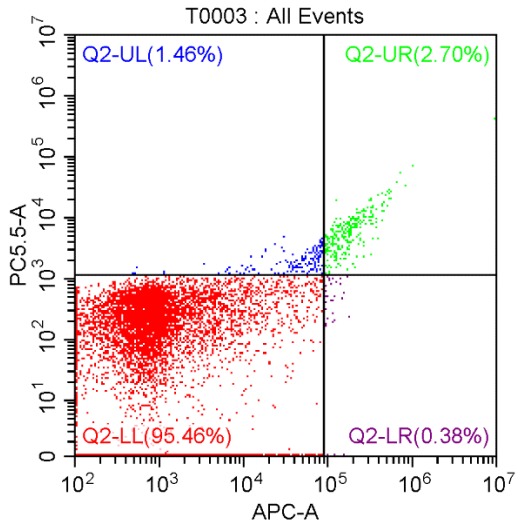


5-FU Group (50 µl)

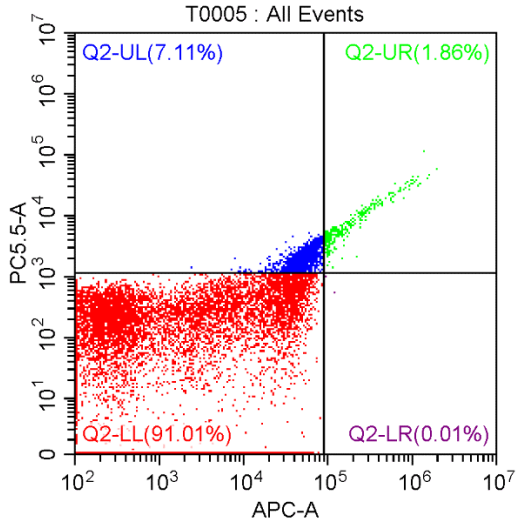
Figure 3C. *Cell Death of HT-29 Cell of Control Group, Metformin Group (10 mM), and 5-FU Group (50 µl) (from up to down) after 72 h Incubation.*



Control Group



Metformin Group (10 mM)



5-FU Group (50 µl)

Figure 3D. *Cell Death of CAL-1 Cell of Control Group, Metformin Group (10 mM), and 5-FU Group (50 µl) (from up to down) after 72 h Incubation.*

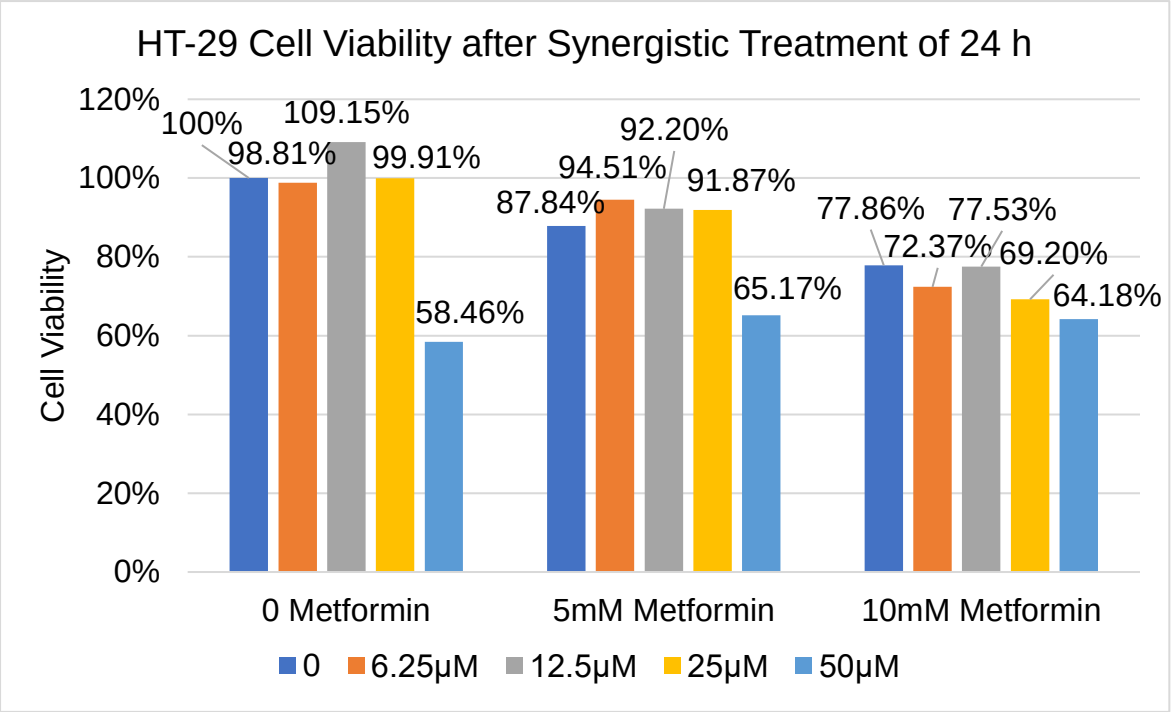


Figure 4A. *Cell Viability after Synergistic Treatment of 5 and 10 mM Metformin with various 5-FU concentrations on HT-29 Cells after 24 Incubation*

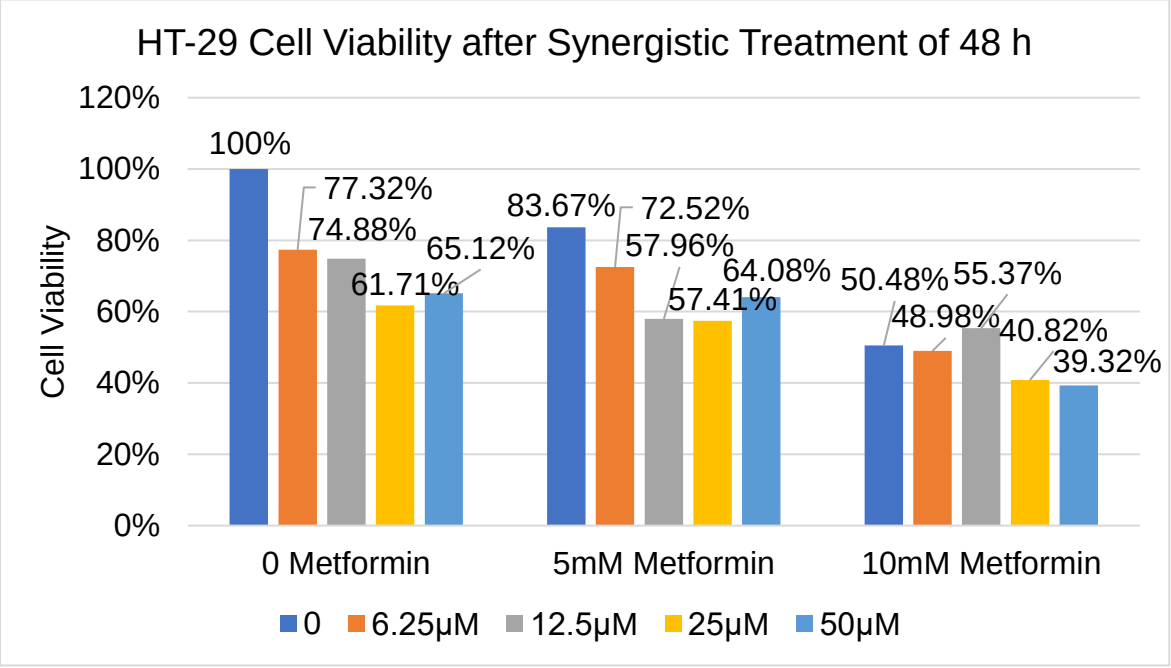


Figure 4A. *Cell Viability after Synergistic Treatment of 5 and 10 mM Metformin with various 5-FU concentrations on HT-29 Cells after 48 Incubation*

Supplementary Figures

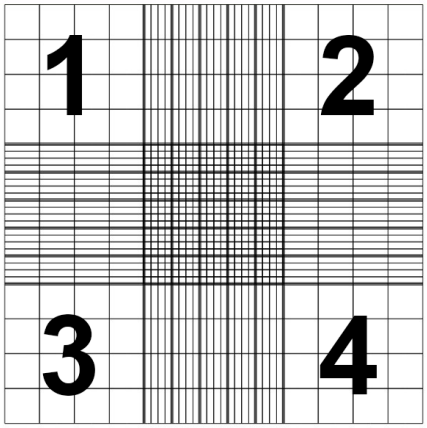


Figure S1. *Grids of Hematocytometer*

		5-FU					Metformin						Blank
24 h	C	50µM	25µM	12.5 µM	6.25 µM	5mM	50µM	25µM	12.5 µM	6.25 µM	5mM	10mM	0
	N	50µM	25µM	12.5 µM	6.25 µM	5mM	50µM	25µM	12.5 µM	6.25 µM	5mM	10mM	0
48 h	C	50µM	25µM	12.5 µM	6.25 µM		50µM	25µM	12.5 µM	6.25 µM	5mM	10mM	0
	N	50µM	25µM	12.5 µM	6.25 µM		50µM	25µM	12.5 µM	6.25 µM	5mM	10mM	0
72 h	C	50µM	25µM	12.5 µM	6.25 µM		50µM	25µM	12.5 µM	6.25 µM	5mM	10mM	0
	N	50µM	25µM	12.5 µM	6.25 µM		50µM	25µM	12.5 µM	6.25 µM	5mM	10mM	0

Figure S2. *Cell seeding and drug treatment on the 96-well plate for MTS assay for individual drugs (C = cancer cell line, HT-29; N = normal cell line, CAL-1)*

	Blank	Metformin	5-FU
HT-29 cells	0	10 mM	50 mM
CAL-1 cells	0	10 mM	50 mM

Figure S3. *Cell seeding and drug treatment on the 6-well plate for cell morphology exam and flow cytometry assay*

		5-FU									Blank
24 h	C	50μM	25μM	12.5μM	6.25μM	0	50μM	25μM	12.5μM	6.25μM	0	0
	N	50μM	25μM	12.5μM	6.25μM	0	50μM	25μM	12.5μM	6.25μM	0	0
48 h	C	50μM	25μM	12.5μM	6.25μM	0	50μM	25μM	12.5μM	6.25μM	0	0
	N	50μM	25μM	12.5μM	6.25μM	0	50μM	25μM	12.5μM	6.25μM	0	0
		Metformin (10 mM)					Metformin (5 mM)					0

Figure S4. *Cell seeding and drug treatment on the 96-well plate for MTS assay for synergistic effects of drugs (C = cancer cell line, HT-29; N = normal cell line, CAL-1)*

BACKGROUND AND SIGNIFICANCE

Background of Metformin

Metformin is an oral-administrative biguanide drug used to treat type 2 diabetes mellitus (T2DM). It is the most widely used antidiabetic drug all over the world and is

known as the first-line treatment of diabetes for its characteristics of safety, efficacy, and tolerability (Rizzo & Stoian, 2020).

Metformin has presented pleiotropic effects on various organs, including the liver, intestine, and muscles (Di Magno et al., 2022). The primary mechanism of metformin's action is the alternation of the energy metabolism in cells (Pernicova & Korbonits, 2014). According to Di Magno et al. (2022), metformin mainly activates the AMPK pathway to limit energy consumption in the liver, which results in the inhibition of high-energy-consuming processes, such as gluconeogenesis. Also, research suggests that metformin impairs the glucose genesis processes, especially gluconeogenesis, by limiting the mitochondrial redox state and activating the cytosolic redox state (Madiraju et al., 2014). Extending from these mechanisms, some researchers suggest that metformin may have several direct anti-cancer effects by targeting mitochondria and mediating AMPK to impair the growth and proliferation of cancer cells (Aljofan & Riethmacher, 2019). Moreover, based on the properties in treating hyperinsulinemic patients, metformin also has an indirect anti-cancer effect, for it has been proven that hyperinsulinemic patients have a higher risk of cancer (Di Magno et al., 2022). Therefore, the anti-cancer potential of metformin has drawn more and more attention nowadays.

Previous Studies of Metformin in Cancer

Several previous researches have revealed the anti-cancer potential of metformin. For example, Evan et al. (2005) suggested that metformin may have an effect on reducing the risk of cancer in diabetic patients. Also, research done by Zordoky et al. (2014) demonstrated that metformin has an anti-proliferative effect on triple-negative MDA-MB-231 breast cancer cells when the blood glucose level is at a normal state. Additionally, a study finished by meta-analysis found that metformin is associated with survival benefits in patients concurrent with pancreatic cancer and diabetes (Shi et al., 2020).

Apart from the anti-cancer potential coherent with diabetes, several studies reported encouraging anti-cancer results in non-diabetic conditions. Research done with a mouse model showed that metformin can reduce the acinar-to-ductal metaplasia and suppress the intraepithelial neoplasia in the pancreatic (Chen et al., 2017). Also, in lung cancer, the leading cause of cancer death worldwide, metformin presented a significant association with its risk decrease and survival increase (Xiao et al., 2020). Besides, targeting renal cell carcinoma, metformin also presented an excellent anti-cancer effect by inducing apoptosis (Jang et al., 2018).

Background of Colorectal Cancer

Colorectal cancer (CRC) is a highly prevalent cancer that develops from the colon or rectum region. It is known as the second leading cause of cancer death all over the world and has long been a significant public health burden (Siegel et al., 2023). Common treatments for CRC include surgery, chemotherapy, and radiotherapy, while chemotherapy is considered one of the established first-line treatment options (Bhattacharya et al., 2022). Many chemotherapeutic agents have been placed into clinical usage, including 5-fluorouracil (5-FU), Irinotecan, and Oxaliplatin. While these drugs have made significant progress in controlling tumor cell growth and improving patient outcomes, they are often associated with side effects, limitations, and cost burden (Bhattacharya et al., 2022). For example, 5-FU, one of the most effective and commonly used agents in CRC, may still present several severe toxicity effects, including nausea, diarrhea, and stomatitis (Vodenkova et al., 2020). Also, according to Gmeiner & Okechukwu (2023), chemoresistance emerges in 5-FU-based therapies due to multiple mechanisms. Therefore, the quest for a new, more effective, less toxic, and more cost-efficient chemotherapeutic agent for CRC patients remains an ongoing challenge.

Significance of the Study

The interaction of metformin, a well-established antidiabetic drug with anti-cancer potential, and CRC, a highly prevalent cancer, holds significant implications for both fields of cancer research and patient care.

For cancer research, metformin provides a new paradigm of using existing approved non-anticancer drugs in cancer treatment, which greatly saves the time and cost of clinical trials in new drug development. Also, metformin represents one of the metabolism-based therapies in CRC. Studying how metformin impacts energy metabolism and induces cancer cell death can help develop more targeted therapies and more accurate medication. Additionally, chemoresistance is a persistent challenge in CRC drug development. Investigating the anti-cancer potential of metformin assesses the drug resistance of CRC cells, implicating the direct or complementary treatment of metformin on CRC.

For patient care, if metformin is proven as an effective anti-cancer drug, it will broaden the treatment options of CRC patients with less drug toxicity and more cost-efficiency. Metformin possesses less drug toxicity compared to the existing chemotherapeutic drugs, like 5-FU. Applying or combining metformin to CRC patients will significantly

reduce the toxicity effects of chemotherapeutic drugs, providing a more safe and tolerable treatment process to CRC patients. In addition, cancer treatment can be a financial burden to some patients. The high cost-efficiency and accessibility of metformin will greatly relieve the financial burden of CRC treatment. Moreover, as mentioned before, it has been proven that metformin has an indirect anti-cancer effect on hyperinsulinemic patients. Hence, it can also be a tailored anti-cancer treatment approach for targeted high-risk populations, like diabetes patients.

Therefore, our research is beyond the field of cancer research. Due to the mature utilization of metformin in the market, not only will the study provide a significant implication in cancer research, but it will also imply strong practicability and immediate applicability in the field of patient care, if its anti-cancer properties are confirmed.

OBJECTIVES

The primary objective of this project is to investigate the apoptotic effects of metformin so as to assess its anti-cancer potential, especially for a specific focus on CRC. This study aims to assess the impact of metformin on human colorectal adenocarcinoma cells, HT-29, exploring its effect on cell survival and death over different time periods

and drug concentrations. Normal cell line CAL-1 is utilized to compare with the HT-29 cell line, while 5-FU is applied to compare with the effect of metformin.

Several aspects are aimed to be investigated during the research. Firstly, cell viabilities are measured in-depth by MTS assay to explore the anti-cancer effects of metformin by different dosages and times. In this way, the effective incubation times and drug concentrations on cells can be determined. After that, the morphological alterations of HT-29 cells are closely examined with or without exposure to metformin, which analyzes alterations in cell shape, size, and signs of death. In addition, an apoptosis assay is performed to specifically evaluate the metformin-induced cell death, assessing the predominant of apoptosis or necrosis. Last but not least, the potential synergistic effects of combining metformin and 5-FU are also examined.

By addressing these objectives, this project seeks to contribute valuable insights into the anti-cancer potential of metformin in the context of CRC. With significant implications for both cancer research and patient care, this project pursues the development of more effective and less toxic anti-cancer drugs.

METHODS AND MATERIALS

Cell Culture and reagents

HT-29 human colorectal adenocarcinoma cells and CAL-1 human plasmacytoid dendritic cells were procured from the American Type Culture Collection (ATCC). Roswell Park Memorial Institute (RPMI) 1640 Medium, Fetal Bovine Serum (FBS), Penicillin-Streptomycin Solution, HEPES, and 2-Mercaptoethnol were purchased from Thermo Fisher Scientific Inc. to prepare cell culture medium. For HT-29 cell culture medium, the medium was prepared by adding 10% FBS and 1% Penicillin-Streptomycin Solution into RPMI 1640 Medium. For CAL-1 cell culture medium, 25 ml FBS, 12.5 ml HEPES, and 0.5 ml 2-Mercaptoethnol were added into 500 ml RPMI 1640 Medium to prepare. All aseptic techniques were followed during all experiments. The cells were cultured in sterile culture flasks with a proper amount of culture medium (20 ml/flask) in an incubator at 37˚C with humidified 5% CO_2. Cell subculture was conducted regularly by removing the old culture medium, detaching (Trypsin-EDTA solution) and washing cells, and dividing and transferring to new flasks to maintain the healthy growth of cells. Metformin tablets (500 mg/tablet) are procured from the local hospital, and 5-FU (≥99% HPLC, powder) is purchased from Sigma-Aldrich. DMSO and PBS are procured from Thermo Fisher Scientific Inc., which are used for drug dissolution and cell washing, respectively.

Trypan Blue Exclusion Test

The trypan blue exclusion test was utilized to estimate the cell viability and determine the cell density. Following the cell harvest and cell suspension preparation, a few cell suspension (20 µl) was withdrawn and mixed with the same volume of Trypan Blue solution (0.4% w/v) (1:1 dilution, if the cell density is too high, the dilution factor can increase). The stained cell suspension was expelled into a hemocytometer. The number of unstained (viable) cells was observed and evaluated. Viable cells in the 16 squares of 4 corner grids were counted (labeled as 1, 2, 3, 4 in Figure S1), and the total viable cell number in the cell suspension was calculated using the formula: Number of viable cells per ml = average number of viable cells in grids × 10^4 × dilution factor.

Cell Viability Assay

MTS Assay Kit (Cell Proliferation) (Colorimetric) was procured from the supplier, Abcam. HT-29 and CAL-1 cells were seeded in a 96-well plate at a density of 1 × 10^4 cells/well. Due to the variances of effective concentrations of metformin and 5-FU (Khodaei et al., 2021; Tawfik et al., 2017), both drugs were tested at low concentrations (6.25 µM, 12.5 µM, 25 µM, and 50 µM) and high concentrations (5 mM,

10 mM). Different concentrations of metformin and 5-FU with negative control (culture medium) were applied to cells, respectively, for three different incubation time periods (24, 48, and 72 h) (Figure S2). As for the synergistic groups, two drugs with certain concentrations (Metformin: 5 mM and 10 mM; 5-FU: 6.25 μM, 12.5 μM, 25 μM, and 50 μM) were applied together in the same well of cells for 24 h and 48 h incubations (Figure S4). After the incubation, 10 μl of MTS solution was added to each well for a 4 h incubation. After finishing the incubation and briefly shaking the plate, the absorbance was measured at 492 nm with a reference wavelength of 620 nm using a microplate reader. The cell viability rate = (mean of sample absorbance / mean of control absorbance) × 100%.

Cell Morphology

Both HT-29 and CAL-1 cells were first seeded in 6-well plates at a density of 1×10^5 cells/well, and then they were treated either with the presence or absence of metformin (10 mM) and 5-FU (50 μM) (Figure S3). After a certain incubation period (48h or 72h), morphological changes were visualized by an inverted microscope with 200× magnification. Alterations, including cell density, cell size, and floating cells, were observed and compared. Digital images of cells were captured by the linked system.

Flow Cytometry Assay

Annexin V-FITC Apoptosis Staining / Detection Kit (ab14085) was procured from the supplier, Abcam. HT-29 and CAL-1 cells were seeded in a 6-well plate at a density of 1×10^5 cells/well. Metformin with a concentration of 10 mM, 5-FU with a concentration of 50 µl, and the same amount of negative control (culture medium) were applied to the cells with two different incubation periods (48 h and 72 h) (Figure S3). After the incubation, cells were harvested from the 6-well plate. 510 µl staining solution (500 µl binding buffer + 5 µl Annexin V-FITC + 5 µl PI) was added to resuspend the cells. After incubation in the dark, the signal of Annexin V-FITC and PI were measured by a flow cytometer with 10^4 cell counts in each sample. Both Annexin V-FITC and PI were excited by a 488 nm argon laser. The emitted fluorescence was monitored at 500-560 nm for Annexin V-FITC and at >670 nm for PI.

Statistical Analysis

All data from different experiments were well collected and analyzed. Cell densities from the trypan blue exclusion test and cell viability rates from the MTS assay were calculated according to the formulas. Histograms were generated by Excel. The proportion of different cells from flow cytometry was also compared and analyzed.

RESULTS

Dose-Dependent Impact of Metformin on HT-29 Cell Viability

The dose-dependent impact of metformin on HT-29 cells is first investigated to determine the most effective drug concentrations and incubation times for metformin treatment, as well as in comparison with 5-FU.

For the 24 h incubation, metformin presents a subtle trend in limiting HT-29 cell viability with different concentrations, where viability ranges from 71.63% to 80.33% (Figure 1A). Apart from that, metformin shows a slightly cytotoxic effect on CAL-1 cells, especially at a high concentration of 10 mM, where cell viability is around 73.39% (Figure 1A). On the contrary, 5-FU does not exhibit a significant anti-cancer effect at low concentrations after 24 h incubation. However, at high concentrations, 5-FU demonstrated an intense anti-cancer effect in limiting the development of HT-29 cells, where the viability rate is only 18.57% at 5 mM (Figure 1A). Unfortunately, it is also accompanied by a strong cytotoxic effect on CAL-1 cells, with only a 44.27% viability rate (Figure 1A). So, high concentrations of 5-FU are excluded in the following different incubation periods.

When extending the incubation period to 48 h, metformin's anti-cancer effects become more evident at high concentrations. The HT-29 cell viability drops to 41.71% at a high concentration of 10 mM (Figure 1B). However, it still remains at a high level with low concentrations, which are all more than 90%, and even some of them are over 100% (Figure 1B). Interestingly, metformin's cytotoxic effect on CAL-1 cells remains nearly the same as in 24 h incubation, where a sightly cytotoxic effect is presented (Figure 1B). On the other hand, 5-FU started to present a notable anti-cancer effect after 48 h incubation. At low concentrations, the HT-29 cell viability rate ranges from 61.71% to 77.32%, which is a potent anti-cancer effect (Figure 1B). Better yet, the viability rates of CAL-1 cells still remain at a high level, and some even over 100% (Figure 1B). This data indicates the high selectivity of metformin and 5-FU on HT-29 cells, where metformin is more effective at high concentrations, while 5-FU shows a better result at low concentrations.

In the 72 h incubation group, the anti-cancer trend of both metformin and 5-FU becomes more pronounced. As Figure 1C shows, metformin surprisingly presents an anti-cancer effect at a low concentration that HT-29 cell viability is 60.8% at 50 μM. Also, the viability of CAL-1 cells is even better than 24 h and 48 h incubation, with 79.49% at 10 mM and over 90% for all other concentrations (Figure 1C). As for the 5-

FU treatment, the result is even more significant when compared to 24 and 48 h incubation. All HT-29 cells treated with 5-FU significantly reduced their viability, with the lowest viability rate of 27.46% at 50 μM (Figure 1C). However, the viability rate of CAL-1 cells after 5-FU treatment is not as favorable as metformin treatment, as it registered a viability rate of 63.94% at 50 μM (Figure 1C).

Hence, the dose-dependent impact of metformin reveals that metformin will develop an inhibition effect on HT-29 cells over time at high concentrations, and it may also present effects on HT-29 cells at low concentrations (50 μM) as long as the time is long enough (>72 h). Also, compared to 5-FU, metformin appears to have lower cytotoxic effects on CAL-1 cells. By comparing all cell viability rates from different groups, 48 h and 72 h incubations demonstrate better anti-cancer results. Metformin at 10 mM and 5-FU at 50 μM present the best selectivity and suppression of cancer cells. Thus, 10 mM metformin and 50 μM 5-FU are utilized in the following metformin-induced morphological alteration and cell death studies, with incubation times of 48 and 72 h.

Metformin-Induced Morphological Alterations in HT-29 Cells

During cell morphology studies, our observation reveals distinct morphological alterations of HT-29 cells following exposure to metformin and 5-FU. The effect of these treatments is obviously observed after 48 and 72 h incubation with the comparison of CAL-1 cells.

After 48 h incubation, the cell density and size in the HT-29 cell culture treated with metformin and 5-FU have an observable decrease compared to the control group (Figure 2A). Also, floating cells are observed in the metformin and 5-FU treatments, indicating possible cell detachment and death. In contrast, under the same conditions, CAL-1 cells display subtle morphological changes. With the 5-FU treatment, CAL-1 cells also present a decrease in cell density and shrinkage of cell size to some extent (Figure 2B). However, compared to HT-29 cells, these changes are not pronounced. This suggests that metformin and 5-FU have a substantial effect on the cancer cell line, HT-29, compared to the normal cell line, CAL-1.

When extending the incubation period to 72 h, the morphological alterations in HT-29 cells become more significant. In the control group, HT-29 cells present vigorous growth, where cells display a high cell density and a tendency to grow in clumps (Figure 2C). On the contrary, metformin treatment results in a reduction in cell density

and a more dispersed cell morphology, while floating cells can also be observed (Figure 2C). More pronounced effects are presented in the 5-FU treatment. Significantly reduced cell density, notable cell shrinkage, and increasing floating cell count can be clearly observed (Figure 2C). Conversely, the morphological alterations of CAL-1 cells are not as conspicuous as HT-29 cells. The cell density of CAL-1 cells during 72 h incubation is increased compared to 48 incubation (Figure 2B & 2D). Although it presents that 5-FU still affects normal cell morphologies to some extent, the effects can be considered insignificant when compared to the effects on HT-29 cells (Figure 2D).

Therefore, these observations indicate that metformin and 5-FU significantly act on HT-29 cell morphologies over time, compared to the normal cell line, CAL-1. The alterations are pronouncedly presented as the decrease in cell density, shrinkage of cell size, and appearance of floating cells. These results imply that although not as strong as 5-FU, metformin still possesses a substantial impact on HT-29 cell morphology, which indicates its anti-cancer potential.

Metformin-Induced Cell Death in HT-29 Cells

In the investigation of metformin-induced cell death in HT-29 cells, our results demonstrate significant death-inducing effects of metformin after 48 h and 72 h incubations by drawing comparisons with 5-FU treatment and effects of CAL-1 cells.

After 48 h incubation, in the control group of HT-29 cells, we observed a high percentage of viable cells, where 96.87% of cells are alive (Figure 3A). However, in the metformin treatment group, the cell viability is reduced to 86.75%, with an increase of necrotic cells to 13.24% (Figure 3A). The 5-FU treatment group exhibits an even greater change that the percentage of viable cells is decreased to 74.73%, and 24.98% of cells are presented as necrotic (Figure 3A). These findings suggest that both metformin and 5-FU have the potential to induce HT-29 cell death, with a more pronounced effect shown by 5-FU. As for CAL-1 cells, the control group displayed 89.49% viable cells with a slightly higher necrotic cell count of 10.24% (Figure 3B). This discrepancy might be due to some degree of contamination. However, when compared to the metformin treatment group (88.51% viable cells and 11.40% necrotic cells) and the 5-FU treatment group (81.07% viable cells and 18.87% necrotic cells), the death-inducing effect of metformin and 5-FU is insignificant on CAL-1 cells (Figure 3B). Also, the percentage of necrotic CAL-1 cells is slightly higher in the 5-FU treatment group, suggesting its higher cytotoxicity.

The metformin-induced cell death on HT-29 cells is more significant when the incubation period extends to 72 h. In the control group of HT-29 cells, the cell viability is as high as 99.66%. On the contrary, with the metformin treatment, the percentage of viable cells decreases to 73.94% (Figure 3C). Interestingly, a significant portion of cells enter the necrotic stage (21.64%) or late apoptotic stage (4.34%) (Figure 3C). 5-FU has an even more significant death-inducing effect on HT-29 cells. Only 43.60% of cells remain alive, while 14.08% of cells enter the early apoptosis stage, and 42.32% of cells undergo late apoptosis and necrosis. These results illustrate a significant death-inducing effect of metformin and 5-FU, where metformin is more likely to induce necrosis, and 5-FU is more likely to induce apoptosis. Regarding CAL-1 cells, the cell mortality rate after drug treatment is insignificant compared to HT-29 cells. The cell viability remains at 98.77% in the control group, with a slight decrease in the metformin treatment group (95.46%) and the 5-FU treatment group (91.01%) (Figure 3D). Thus, compared with HT-29 cells, metformin does not induce significant cell death in CAL-1, which highlights the death-inducing effect of metformin on the cancer cell line HT-29.

Therefore, both metformin and 5-FU are effective in inducing cell death. However, compared to 5-FU, metformin is more likely to induce necrosis rather than apoptosis

of cell death. Nevertheless, from the aspect of cytotoxicity, metformin presents a less cytotoxicity effect on the normal cell line, CAL-1. In balance, although no strong apoptosis effect is presented, metformin still possesses an excellent anti-cancer potential, especially for its characteristic of low cytotoxicity on normal cells.

Synergistic Anti-Cancer Effects of Metformin and 5-FU in HT-29 Cells

In the investigation of the potential synergistic anti-cancer effects of combining metformin and 5-FU, the results of the cell viability assay illustrate a conspicuous synergistic effect that metformin can be a potent adjuvant for 5-FU treatment.

After 24 h incubation, the results show an apparent synergistic anti-cancer effect, especially at a low concentration of 5-FU (6.25 μM, 12.5 μM, and 25 μM). In these cases, with the addition of 10 mM metformin, the HT-29 cell viability rates are reduced to around 70%, compared to over 98.81% without metformin addition (Figure 4A). Besides, when the adjuvant metformin concentration reduces to 5 mM, the cell viability rates still present a slight decrease, ranging from 87.84% to 94.51% (Figure 4A).

The synergetic effect of metformin and 5-FU also shows in the 48 h incubation group. The HT-29 cell viability rates are reduced to approximately 39.32% to 55.37% with the

addition of 10 mM metformin, compared to around 61.71% to 77.32% observed in groups without metformin (Figure 4B). Also, the addition of 5 mM metformin shows a slight synergistic result, which is consistent with 24 h incubation results.

These findings in the synergistic effects of 5-FU and metformin manifest an excellent potential for metformin to be involved in anti-cancer drug development. In combination with a certain concentration (10 mM), metformin has the chance to accelerate and amplify the anti-cancer effect of 5-FU at low concentrations.

DISCUSSION

The primary objective of this project is to investigate the apoptotic effects of metformin on HT-29 cells so as to assess its potential for anti-cancer drug development. By integrating all the results, this discussion seeks to interpret and analyze results from different aspects, explore the possible underlying mechanisms, and illustrate its significance in CRC drug development.

To begin with, we can learn from MTS assay results that compared to 24 h incubation, metformin has a stronger anti-cancer after 48 and 72 h incubation. Also, at higher concentrations, such as 5 mM and 10 mM, metformin possesses a more substantial

effect in limiting HT-29 cell viability. These suggest that the anti-cancer effect of metformin is dose-dependent and time-dependent, where the effective dosage is at high concentrations (5 mM and 10 mM), and the effective time seems to be larger than 48 h. In addition, the positive control, 5-FU, possesses a more robust anti-cancer effect on HT-29 cells than metformin if the incubation time is over 48 h. However, metformin demonstrates a less cytotoxic effect on the normal cell line, CAL-1, compared to 5-FU. This suggests metformin's promise as a less toxic alternative to conventional chemotherapy.

On top of that, when delving into the impacts of metformin on HT-29 cells, it is found that metformin can induce morphological alterations and cell death in HT-29 cells. By comparing images of HT-29 cells, we can notice the viability and structure changes, including reduction of cell density, shrinkage of cell size, and presence of floating cells, after 48 and 72 h metformin treatment. However, these morphological variations are less pronounced in CAL-1 cells, indicating metformin's specificity to HT-29 cells. Although 5-FU presents stronger morphological changes on HT-29 cells, it also shows a relatively strong cytotoxic effect on CAL-1 cells. This also suggests metformin's anti-cancer role as a less cytotoxicity alternative. As for the cell death induction, after 48 h and 72 h incubations, metformin induces a significant amount of cell death. However,

the majority of death cells are classified as necrotic cells rather than apoptotic cells. This indicates that metformin has a poor potential to induce apoptosis, while its anti-cancer potential may mainly focus on necrosis or other mechanisms. Compared to metformin, 5-FU shows a strong apoptosis-inducing effect on HT-29 cells, especially after 72 h incubation, but for CAL-1 cells, more death cells are also presented. This suggests that 5-FU appears to be a more mature chemotherapy drug inducing cancer cell apoptosis, but it is also more cytotoxic to normal cells. The possible mechanism underlying these results needs further investigation, but the implications are clear: metformin may induce cell death by necrosis, while 5-FU appears to through apoptosis.

Moreover, the anti-cancer effect of both metformin and 5-FU seems hysteretic, for no significant effects appear after 24 h incubation. However, the combination of metformin and 5-FU shows a surprising synergetic anti-cancer effect. The HT-29 cell viability rate has a significant decrease after the combination treatment of metformin and 5-FU in both 24 and 48 h incubation times. This synergy suggests that metformin may potentiate the effect of traditional chemotherapy agents, but the possible molecular mechanism needs further exploration.

To interpret these findings, we need to consider the possible underlying molecular mechanism. As mentioned in the background, metformin may act on cancer cells through a direct, insulin-independent pathway or an indirect, insulin-dependent pathway (Di Magno et al., 2022). As for the insulin-dependent pathway, according to Mallik & Chowdhury (2018), the dysregulation of the AMPK pathway may have an essential role in cancer pathogenesis; thus, by activating the AMPK pathway, metformin appears to have multiple metabolic effects in suppressing cancer cells, including inhibition of cellular proliferation by reducing the expression of cyclin D1 (Mogavero et al., 2017). However, there is no evidence showing metformin will induce apoptosis in HT-29 cells, as shown in the research done by Mogavero et al. (2017) using Annexin V assay and Caspase-3 assay. These researches are consistent with the findings of this project that metformin can limit HT-29 cell viability rate and induce morphological alterations of HT-29 cells, especially the cell density, but cannot result in significant apoptosis.

Additionally, several researches reveal that there are two pathways that mediate the anti-cancer effects of metformin: one is AMPK activation, and the other is the increase of reactive oxygen species (ROS) that suppress the mTOR pathway (Mallik & Chowdhury, 2018; Mogavero et al., 2017; Nguyen et al., 2019). According to

Mogavero et al. (2017), metformin treatment on CRC cells leads to a significant increase in ROS, which causes oxidative stress and mitochondrial depolarization. Also, research done by Khodaei et al. (2021) reveals that metformin induces cell death through a mitochondrial energy stress mechanism, where it upregulates enzyme Sirt3 in HT-29 cells, and an increasing number of necrotic cells are observed. This energy suppression mechanism of metformin might be a reason for the observed metformin-induced HT-29 cell necrosis in this project.

Besides, the reason why the anti-cancer effect of metformin is dose-dependent (more effective at higher concentrations, 5 mM and 10 mM) and time-dependent (more effective at longer incubation times, 48 h and 72 h) may be due to the low intracellular accumulation level of metformin. As Dowling et al. (2017) suggest, the intracellular metformin level is as low as 10 – 15% of that in the medium, and the metformin level should be at least 1 mM in the medium for it to activate the AMPK pathway in cells. The dose- and time-dependent manner of metformin on HT-29 cells is also revealed by research done by Sena et al. (2018), which illustrates that metformin suppresses cell proliferation and alters NRF-2 and NF-κB expression in HT-29 cells.

Furthermore, the synergetic effect of the combination of metformin and 5-FU has also been illustrated by several studies. As Yip et al. (2022) suggest, metformin significantly enhances the therapeutic effects, where cancer cell growth is greatly inhibited, and cancer cell apoptosis is observed. Also, according to a study done by Harada et al. (2016), the combination of metformin and 5-FU suppresses tumor growth by inhibiting the Warburg effect, the effect that cancer cells tend to use glycolysis in cytosol rather than aerobic processes in mitochondria. These studies are in line with the findings in this project that metformin may be an excellent adjuvant agent during 5-FU therapy, where the advantages of both can be combined.

Limitations

While providing valuable insights, limitations still exist during the research. Although this research shows some attractive discoveries, it may not fully accord with the complex in vivo environment. Also, this project mainly focuses on HT-29 and CAL-1 cells; although representative to some extent, further investigation on other CRC cell lines and normal cell lines is still required. As for the synergetic of metformin and 5-FU, only cell viability rates by MTS assay are tested. Further apoptosis assay in synergetic groups is necessary. In addition, this research does not experiment with

the molecular mechanisms behind it, which are only speculated from previous studies. Further research on the underlying cell death mechanism is needed.

Apart from that, several technical issues were encountered during this research. For example, as mentioned earlier, during 48 h incubation, the mortality rate of CAL-1 cells of all three groups appeared slightly higher, which was probably caused by a slight contamination in the 6-well plate after drug treatment. Although it did not affect the cell culture process, the awareness of aseptic techniques needs to be implemented throughout the whole research. Also, from this research, I noticed that good time management and experiment design are crucial for the research to run smoothly. During the research, I encountered overtime usage of the flow cytometer, insufficient reagent preparation, and substandard waste management. These problems must raise alarms in future laboratory practices so that to maintain the standard laboratory operation and the integrity of results.

CONCLUSION

In conclusion, although metformin does not significantly induce the apoptosis of HT-29 cells, it still possesses excellent anti-cancer effects in limiting cancer cell viability, causing cancer cell morphological changes, and inducing cancer cell death. Also, by

combining with the existing chemotherapy drug, 5-FU, it demonstrates a strong synergy in enhancing anti-cancer effects. In addition, thanks to its less cytotoxic features, metformin has excellent potential as a more patient-friendly treatment option for CRC. These findings not only broaden the field of CRC research but also hold a promise in patient care. By comprehending underlying molecular mechanisms, further investigations can try to delve into the complexity of metformin mechanisms, its practicability as an adjuvant drug, and its clinical trials in vivo, which ultimately drive the development of anti-cancer drugs.

REFERENCES

Aljofan, M., & Riethmacher, D. (2019). Anticancer activity of metformin: a systematic review of the literature. *Future Science OA, 5*(8), FSO410–FSO410. https://doi.org/10.2144/fsoa-2019-0053

Bhattacharya, S., Page, A., & Maru, S. (Eds.). (2022). *Colorectal cancer diagnosis and therapeutic updates.* Bentham Science Publishers.

Chen, K., Qian, W., Jiang, Z., Cheng, L., Li, J., Sun, L., … Ma, Q. (2017). Metformin suppresses cancer initiation and progression in genetic mouse models of

pancreatic cancer. *Molecular Cancer, 16*(1), 131–131. https://doi.org/10.1186/s12943-017-0701-0

Di Magno, L., Di Pastena, F., Bordone, R., Coni, S., & Canettieri, G. (2022). The Mechanism of Action of Biguanides: New Answers to a Complex Question. *Cancers, 14*(13), 3220. https://doi.org/10.3390/cancers14133220

Dowling, R. J. O., Lam, S., Bassi, C., Mouaaz, S., Aman, A., Kiyota, T., Al-awar, R., Goodwin, P. J., & Stambolic, V. (2016). Metformin Pharmacokinetics in Mouse Tumors: Implications for Human Therapy. *Cell Metabolism, 23*(4), 567–568. https://doi.org/10.1016/j.cmet.2016.03.006

Evans, J. M., Donnelly, L. A., Emslie-Smith, A. M., Alessi, D. R., & Morris, A. D. (2005). Metformin and reduced risk of cancer in diabetic patients. *BMJ (Clinical research ed.), 330*(7503), 1304–1305. https://doi.org/10.1136/bmj.38415.708634.F7

Gmeiner, W. H., & Okechukwu, C. C. (2023). Review of 5-FU resistance mechanisms in colorectal cancer: clinical significance of attenuated on-target effects. *Cancer Drug Resistance, 6*(2), 257–272. https://doi.org/10.20517/cdr.2022.136

HARADA, K., FERDOUS, T., HARADA, T., & UEYAMA, Y. (2016). Metformin in combination with 5-fluorouracil suppresses tumor growth by inhibiting the

Warburg effect in human oral squamous cell carcinoma. *International Journal of Oncology, 49*(1), 276–284. https://doi.org/10.3892/ijo.2016.3523

Jang, J.-H., Song, I.-H., Sung, E.-G., Lee, T.-J., & Kim, J.-Y. (2018). Metformin-induced apoptosis facilitates degradation of the cellular caspase 8 (FLICE)-like inhibitory protein through a caspase-dependent pathway in human renal cell carcinoma A498 cells. *Oncology Letters, 16*(2), 2030–2038. https://doi.org/10.3892/ol.2018.8832

Khodaei, F., Hosseini, S. M., Omidi, M., Hosseini, S. F., & Rezaei, M. (2021). Cytotoxicity of metformin against HT29 colon cancer cells contributes to mitochondrial Sirt3 upregulation. *Journal of biochemical and molecular toxicology, 35*(3), e22662. https://doi.org/10.1002/jbt.22662

Madiraju, A. K., Erion, D. M., Rahimi, Y., Zhang, X. M., Braddock, D. T., Albright, R. A., Prigaro, B. J., Wood, J. L., Bhanot, S., MacDonald, M. J., Jurczak, M. J., Camporez, J. P., Lee, H. Y., Cline, G. W., Samuel, V. T., Kibbey, R. G., & Shulman, G. I. (2014). Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature, 510*(7506), 542–546. https://doi.org/10.1038/nature13270

Mallik, R., & Chowdhury, T. A. (2018). Metformin in cancer. *Diabetes Research and Clinical Practice, 143*, 409–419. https://doi.org/10.1016/j.diabres.2018.05.023

Mogavero, A., Maiorana, M. V., Zanutto, S., Varinelli, L., Bozzi, F., Belfiore, A., Volpi, C. C., Gloghini, A., Pierotti, M. A., & Gariboldi, M. (2017). Metformin transiently inhibits colorectal cancer cell proliferation as a result of either AMPK activation or increased ROS production. *Scientific Reports, 7*(1), 15992–12. https://doi.org/10.1038/s41598-017-16149-z

Nguyen, T. T., Ung, T. T., Li, S., Lian, S., Xia, Y., Park, S. Y., & Do Jung, Y. (2019). Metformin inhibits lithocholic acid-induced interleukin 8 upregulation in colorectal cancer cells by suppressing ROS production and NF-kB activity. *Scientific Reports, 9*(1), 2003–2003. https://doi.org/10.1038/s41598-019-38778-2

Pernicova, I., & Korbonits, M. (2014). Metformin--mode of action and clinical implications for diabetes and cancer. *Nature reviews. Endocrinology, 10*(3), 143–156. https://doi.org/10.1038/nrendo.2013.256

Rizzo, M., & Stoian, A. P. (Eds.). (2020). *Metformin.* IntechOpen.

Sena, P., Mancini, S., Benincasa, M., Mariani, F., Palumbo, C., & Roncucci, L. (2018). Metformin Induces Apoptosis and Alters Cellular Responses to Oxidative Stress

in Ht29 Colon Cancer Cells: Preliminary Findings. *International journal of molecular sciences, 19*(5), 1478. https://doi.org/10.3390/ijms19051478

Shi, Y. Q., Zhou, X. C., Du, P., Yin, M. Y., Xu, L., Chen, W. J., & Xu, C. F. (2020). Relationships are between metformin use and survival in pancreatic cancer patients concurrent with diabetes: A systematic review and meta-analysis. *Medicine, 99*(37), e21687. https://doi.org/10.1097/MD.0000000000021687

Siegel, R. L., Miller, K. D., Wagle, N. S., & Jemal, A. (2023). Cancer statistics, 2023. *CA: a cancer journal for clinicians, 73*(1), 17–48. https://doi.org/10.3322/caac.21763

Tawfik, E., Ahamed, M., Almalik, A., Alfaqeeh, M., & Alshamsan, A. (2017). Prolonged exposure of colon cancer cells to 5-fluorouracil nanoparticles improves its anticancer activity. *Saudi Pharmaceutical Journal, 25*(2), 206–213. https://doi.org/10.1016/j.jsps.2016.05.010

Vodenkova, S., Buchler, T., Cervena, K., Veskrnova, V., Vodicka, P., & Vymetalkova, V. (2020). 5-fluorouracil and other fluoropyrimidines in colorectal cancer: Past, present and future. *Pharmacology & Therapeutics (Oxford), 206*, 107447–. https://doi.org/10.1016/j.pharmthera.2019.107447

Xiao, K., Liu, F., Liu, J., Xu, J., Wu, Q., & Li, X. (2020). The effect of metformin on lung cancer risk and survival in patients with type 2 diabetes mellitus: A meta‐analysis. *Journal of Clinical Pharmacy and Therapeutics, 45*(4), 783–792. https://doi.org/10.1111/jcpt.13167

Yip, K.-L., Tsai, T.-N., Yang, I.-P., Miao, Z.-F., Chen, Y.-C., Li, C.-C., Su, W.-C., Chang, T.-K., Huang, C.-W., Tsai, H.-L., Yeh, Y.-S., & Wang, J.-Y. (2022). Metformin Enhancement of Therapeutic Effects of 5-Fluorouracil and Oxaliplatin in Colon Cancer Cells and Nude Mice. *Biomedicines, 10*(5), 955–. https://doi.org/10.3390/biomedicines10050955

Zordoky, B. N. ., Bark, D., Soltys, C. L., Sung, M. M., & Dyck, J. R. . (2014). The anti-proliferative effect of metformin in triple-negative MDA-MB-231 breast cancer cells is highly dependent on glucose concentration: Implications for cancer therapy and prevention. *Biochimica et Biophysica Acta. General Subjects, 1840*(6), 1943–1957. https://doi.org/10.1016/j.bbagen.2014.01.023