ORIGINAL ARTICLE

Metformin HCl Oral Preparation Exhibits Anticancer Activity In-vitro in a Human Non-small Cell Lung Tumour Cell Line

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ABSTRACT

Introduction: The most common variety of lung cancer is non–small cell lung cancer (NSCLC) accounting for 84% of new cases. Surgery, chemotherapy and radiation are the primary treatment option. Metformin has recently been demonstrated to have an anti-tumour impact on various cancer cells. The goal of this investigation was to determine the growth inhibitory, antiproliferative, cytotoxic, apoptotic and cell cycle arrest properties of metformin HCl oral tablets on the A549 lung carcinoma cell line. **Methods:** The cells were treated with different dosages of an oral preparation of metformin, with untreated cells used as a control. The Trypan Blue Exclusion Assay was used to determine metformin's inhibitory and cytotoxic effects. Flow cytometry was used to evaluate apoptosis and cell cycle arrest. **Results:** In a dose-dependent manner, metformin HCl was able to reduce the viability of treated cells compared to the untreated control. Cell proliferation was considerably inhibited in the treated group with the IC_{50} dose than in the untreated control group and the IC_{50} dose showed no cytotoxic effect on L929 cells. Induction of apoptosis and cell cycle arrest was observed in the IC_{50} dose-treated group by Flow cytometry analysis and data showed metformin oral drug causes early apoptosis and a considerable cell increase in the S phase of the cell cycle. **Conclusion:** Metformin inhibits cell growth and induces apoptosis and cell cycle arrest in the cell line. A comprehensive proteome examination is required to understand more about the mechanism of action of the oral metformin HCl on cancer cells.
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Keywords: Metformin HCl, Oral drug, Non-Small cell lung cancer, A549, Anticancer

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INTRODUCTION

According to GLOBOCAN 2020, around 10.0 million cancer deaths were recorded globally, with an estimated 19.3 million new cancer cases (18.1 million excluding nonmelanoma skin cancer) & (9.9 million excluding nonmelanoma skin cancer) (1). Lung carcinoma remained the leading cause of mortality, accounting for 1.8 million fatalities (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and mammary (6.9%) cancers (1). Lung cancer incidence and mortality are strongly connected to cigarette smoking routines. As smoking rates increase, the incidence and death of respiratory cancer rise - first in males, then in women. In comparison to developing countries, there are no disparities in cancer-related deaths among males, but there is an increased rate of lung cancer-related deaths among females in industrialized countries. Pulmonary cancer fatalities in women in developing nations trail those caused by breast cancer (2). NSCLC (non-small cell lung cancer) is the most frequent variety of lung cancer, accounting for 84% of new cases. Despite advancements in cross-sectional radiology and the introduction of screening programs, the majority of patients are diagnosed with stage IV cancer (3). In early-stage (I–IIIA) NSCLC, the majority of treatments include surgical excision with the goal of curing cancer, with final chemoradiation designated for individuals who are considered unresectable due to tumour features or surgical fitness (4). The majority of patients (30%-60%) develop metastases after treatment, with a typical 5-year life expectancy of 60% for stage IIA patients and 36% for stage IIIA patient populations (5).

Metformin, a major biguanide antihyperglycemic medicine that is commonly used to control type 2 hyperglycemia, has been shown to reduce the risk of disease progression. According to the recommendations of the major diabetic organizations, this is the first-

choice drug for usage as monotherapy in individuals with newly diagnosed type 2 diabetes. The active element of this medicine, galegine, was discovered in aqueous solutions of the herb Galega officinalis and since the late 1990s, metformin has been the preferred choice in the treatment of type 2 diabetes (6). Metformin is frequently used as part of a combination treatment when monotherapy is no longer beneficial (7). In addition according to an epidemiological study, people who use metformin had decreased cancer incidence and mortality (8). Several studies showed that metformin suppressed cell proliferation, activated apoptosis, caused cellular cycle disruption in vitro, and reduced the formation and progression of xenograft tumours in vivo (9). It has been widely researched in cell lines, indicating a range of biochemical processes that it affects directly or indirectly via other downstream effectors, limiting tumour cell proliferation (10). Metformin has anticancer signalling events such as mTOR inhibitory activity, antitumor activity, and immunomodulation, as per the studies (11). Metformin reduced IGF-1 (insulin-like growth factor-1) and mTOR in pancreatic cancer animal models while elevating phosphorylated AMPK as well as the tuberous sclerosis complex (TSC1, TSC2) (12). The drug also has been demonstrated to reduce the development of cervical cancer (13), breast cancer (14), oesophagal cancer (15), colon cancer (16), and hepatocellular carcinoma (17). Several clinical trials are now underway to assess metformin as a monotherapy or in combination with cytotoxic chemotherapy and/or radiation for the treatment of cancer at various stages (18-20). These previous studies have provided a foundation for additional analysis (21).

In our research, we hypothesize that metformin HCl oral preparation has the potential as anti-tumour, anti-proliferative and cell cycle arrest properties on cancer progression in vitro. Most of the research employed raw metformin used in the pharmaceutical industry however, we evaluated its tumour-limiting ability on A549 cells using a metformin HCl oral tablet that is easily available in pharmacies. Therefore, this research investigated the IC_{50} of the drug and whether this drug can produce antiproliferative activity with no cytotoxic effects on other normal cells and is responsible for cell cycle arrest and promote apoptosis in vitro, which would have a new choice of therapy for patients with NSCLC..

MATERIALS AND METHODS

Chemicals & Reagents

Metformin HCl 500 mg oral tablet was purchased from a regular pharmacy in Bartam, Penang, Malaysia, crumpled and ground into a fine powder, then mixed with 50ml (DD) double distilled water, filtered through a laboratory grade filter paper, and sterilized with a 0.22 mm microfilter at a concentration of 10mg/ml, and stored at -20°C. The drugs were diluted in a complete medium for each assay. Culture media was purchased from ATCC, USA and other chemical-related to culture were obtained from Invitrogen Inc. USA. Apoptosis and cell cycle analysis reagent kits were purchased from BD Pharmigen, USA.

Cell lines & culture condition

The human non-small cell lung cancer cell line A549 (ATCC Catalog No.CCL-185) and the mouse skin fibroblast L929 cells (ATCC Catalog No.CCL-1TM) were purchased from the ATCC in Manassas, Virginia, USA. At 37°C, the A549 cells were cultured in RPMI-1640 media with 10% Fetal bovine serum and 1% penicillin-streptomycin in a humidified environment with 5% CO2. In addition, the L929 cells were cultured in the same medium and under the same conditions. When the cells attained 80% confluency, they were passaged using 0.25% Trypsin-EDTA.

Determination of IC_{50} concentration of Metformin HCl

The A549 cells were plated at a density of $6.5x10^4$ cells on a six-well plate. After a 24-hour growth period, different doses of metformin (0.1, 0.3, 0.5, 0.7 and 0.9 mg/ml) were given in triplicate to the culture wells and incubated in a 37°C humidified environment with 5% CO2 levels. As a control group, untreated cells were given growth media but no metformin HCl solution. After 72 hours of incubation, assessed the viability of A549 was following treatment with various dosages of metformin. The cells were then trypsinized and collected, and the Trypan Blue Exclusion Assay (TBEA) was performed according to the manufacturer's procedures with slight adjustments to the quantity and dilution factor. The ratios of cell suppression were compared to the untreated control cells group. In contrast to untreated control cells, the IC_{50} was measured, and relative cell viability (%) was reported.

Antiproliferation assay

In a 6-well plate, the A549 cells were plated at $5x10^3$ cells. After 24 hours, the IC_{50} concentration of metformin solution was added to the media and nurtured at 37°C in a humid environment with 5% CO2 in the atmosphere. In the control group, cells were cultured in media without an IC_{50} dose of metformin HCl. The culture media was changed every 3 days (day 3 and 6) and cell viability was measured with TBEA on days 1, 3, 6, and 8, in triplicate.

Morphological observations on A549 cells

In a 6-well plate, the A549 cells were plated at a density of $6.5x10^4$ cells. After a 24-hour growth period, the measured IC_{50} doses of metformin HCl were added to the culture wells and then incubated at 37° Celsius in a humidified environment with 5% CO2. Untreated cells were given growth media in the absence of metformin as a control group. The cells were viewed under an inverted microscope after 72 hours of incubation, and any changes in the morphology of the treated cells were compared to untreated control cells and recorded. No

observation was recorded prior 72-hour period.

Cytotoxicity assay on L929 cells

In a 6-well plate, the L929 cells were plated at a density of $1x10^4$ cells. Metformin HCl at IC_{50} was administered to the culture wells after 24 hours of development and then incubated at 37° Celsius in a humidified environment with 5% CO2. Untreated cells were given growth media without metformin as a control group. The cell viability of L929 was measured compared to untreated control using the TBEA after 72 hours (3 days) of incubation, and graphs were produced accordingly.

Flow cytometric analysis of apoptosis

In 6-well plates, A549 cells were seeded and subsequently treated with the IC_{50} dosage of metformin HCl, after 24 hours of incubation time. The cells were Trypsinized and collected after being washed twice in cold PBS after 72 hours. After centrifuging the cells for 5 minutes at 1200 rpm, the supernatant was collected. Following the manufacturer's instructions, the Annexin V-FITC Apoptosis Detection Kit II was employed. Briefly, the collected cells were resuspended with 400 µl 1x Binding buffer and 100 µl of cells were relocated into 5 mL of flow cytometry tubes. Then 5 µl Annexin V-FITC and 5 µl Propidium iodide were added to the mix. For 15 minutes, the cells were gently vortexed and left in the dark (light-sensitive). The last step was to add 400 µl of 1x Binding buffer. Cells were analyzed within 1 hour using the Becton and Dickinson "FACanto II" flow cytometer coupled with CellQuest software.

Flow cytometric examination of the cell cycle

In 6-well plates, A549 cells were cultured and treated with metformin HCl at its IC_{50} dose after 24 hours of incubation. After incubation for 72 hours, the cell was trypsinized, harvested, and centrifuged for 5 minutes at 400 x g in a centrifuge tube. The supernatant was discarded, and about 1×10^5 cells/ml were resuspended in 250 µl Solution A and thoroughly mixed before incubating for 10 minutes at room temperature. Then, 200 µl of Solution B was added, mixed thoroughly, and incubated for 10 minutes at room temperature. Finally, 200 µl of Solution C (light-sensitive) was added and thoroughly mixed before being incubated for 10 minutes in the dark on ice. All the solutions were used according to the manufacturer from the CycleTest Plus DNA Reagent Kit 40T/Kit. The cells were analyzed within 3 hours for cell cycle analysis using the Becton and Dickinson "FACanto II" flow cytometer coupled with CellQuest software. Graphs were aligned using the ModFit software package.

Statistical analysis

SPSS 20 and Microsoft Excel 2019 were used to investigate statistical comparisons between groups and show the results as mean ±SD. To evaluate and identify any significant differences between groups, a one-way ANOVA and a post-hoc Tukey test were performed. Statistical significance was defined as P-values less than 0.05.

RESULTS

Determination of Half maximal inhibitory concentration (IC_{50}) of Metformin

The TBEA was used to assess the vitality of A549 cells that had been given varying doses of metformin. For subsequent tests, the IC_{50} of metformin at 72 hours was calculated. The metformin HCl stock solution (10 mg/ml) was diluted in complete RPMI-1640 media and concentrations of 0.00 mg per ml (untreated), 0.1 mg per ml, 0.3 mg per ml, 0.5 mg per ml, 0.7 mg per ml, and 0.9 mg per ml were used, respectively. The IC_{50} doses of 0.35 mg/ml were calculated, and metformin HCl solution was discovered to have a dose-dependent effect on treated A549 cells, with IC_{50} doses reducing 50% cell viability compared to the untreated control group. Data were summarized and the graph was plotted (Figure 1). The cells in the untreated control group were given growth media (RPMI-1640) in the absence of metformin HCl. Metformin HCl aqueous solution was found to have an effective inhibitory impact and potency on A549 cells. The experiment was carried out in three independent experiments with the value shown as a mean ± standard deviation.

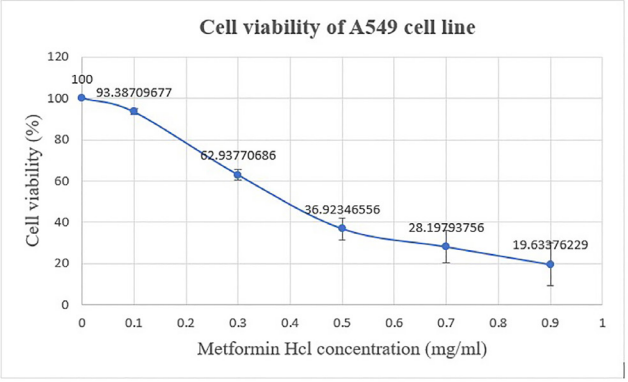

Figure 1: Effect of metformin HCl on cellular growth of A549 cells at different concentrations. Cells were treated with various concentrations of metformin for 72 hours which has reduced the cell growth as compared to untreated cells in a dose-dependent manner. IC_{50} at 0.35 ± 0.02 mg/ml. Data shown are mean ± standard deviation (SD) of triplicate experiments.

Observation of A549 cell morphological alterations

Figure 2(a) depicts untreated control cells, while Figure 2(b) depicts the morphological alterations in A549 cells after treatment with metformin HCl as viewed using phase-contrast inverted microscopy. Almost all of the treated cells underwent morphological alterations that suggested cellular apoptosis. The picture of untreated A549 cells in Figure 2(a) revealed well-differentiation, a typical nucleus-cytoplasm ratio, and a regular membrane shape. Following an IC_{50} dose of metformin HCl for 72 hours, inverted microscopy analysis revealed that some

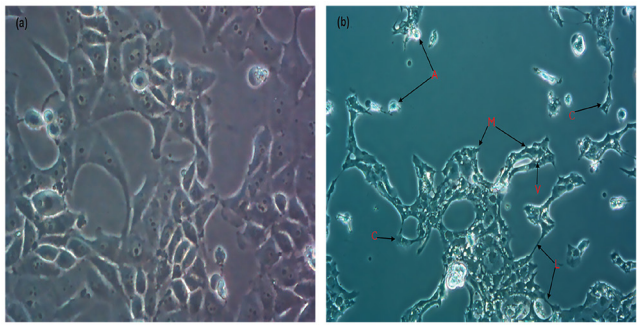

Figure 2: (a) Cell morphology of A549 cell line before treatment in 200x magnification. The untreated cells showed an 80% confluence in monolayer appearance. (b) Effect of metformin HCl on A549 cells indicating apoptotic-related features such as apoptotic bodies (A), cell shrinkage (C), membrane blebbing (M), vacuolization(V), cell elongation (L). Cells were treated with IC_{50} of metformin for 72 hours and observed under an inverted phase-contrast microscope at 200x magnification.

apoptotic features, such as apoptotic bodies (A), cell elongation (L), cellular shrinkage (C), vacuolization (V), and membrane blebbing (M), were observed in Figure 2(b). Furthermore, observations revealed that the metformin HCl-treated cells were reduced in number with identifiable morphological changes that distinguished them from the untreated control group of cells.

Antiproliferation analysis of Metformin on A549 cells

In order to determine whether metformin HCl could affect tumour cell proliferation, the antiproliferative effect of metformin was studied over 8 days. A549 cells were plated and subsequently treated with a determined IC_{50} dose of metformin HCl after 24 hrs of growth. The control cells were incubated with media without metformin HCl. The TBEA determines the number of viable cells and showed that IC_{50} doses of metformin HCl (0.35mg/ml) considerably suppressed the proliferation of A549 cells after 8 days of treatment compared to untreated cells. Figure 3 shows a positive antiproliferative effect after the number of cells harvested and calculated on 1, 3, 6, and 8 days of incubation. The study also demonstrates prolonged exposure to the metformin HCl at the IC_{50} doses did not cause total inhibition of the cells, but it suppressed cell growth consistently from day one and results indicated that it had suppressed cell growth considerably in treated cells compared to untreated cells after 8 days of incubation.

Cytotoxicity test of Metformin HCl on mouse skin fibroblast cell line (L929)

The cytotoxicity of metformin HCl on L929 cells was assessed by 72 hrs of incubation after treatment. The number of cells was counted, and the viability data were shown in Figure 4. After counting the cells, we calculated that 90.8% of the cells were viable after treatment. Microscopic observation shows that there was no significant variation in cell morphology when treated

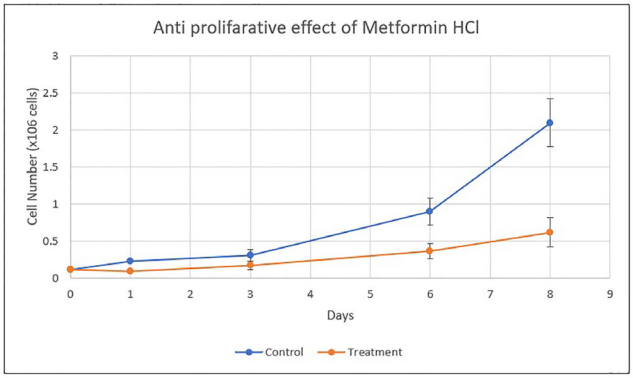

Figure 3: The Trypan Blue Exclusion Assay (TBEA) was used to measure cell proliferation, which demonstrated that proliferation of A549 cells was considerably suppressed in the treatment group by an IC50 dose of metformin HCl after 8 days of treatment. Values are presented in the mean ± SD manner of the triplicate experiment.

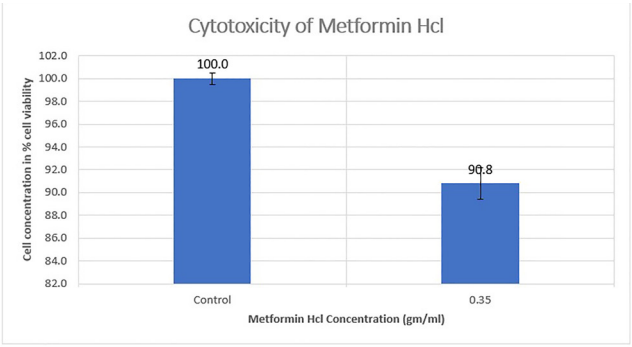

Figure 4: The cytotoxic effect of metformin HCl on the L929 cell showing percentages of cell viability upon treatment. Data shown are mean ± standard deviation (SD) of triplicate experiments.

with the IC_{50} dosages of metformin HCl compared to untreated cells, indicating that metformin HCl was not toxic to the cells.

Flow cytometric assessment of A549 cells for apoptosis

The inhibitory effect of metformin HCl was further confirmed with apoptosis analysis by flow cytometry after 72 hours of treatment. The annexin-V and propidium iodide (PI) assays were coupled to see if metformin HCl can cause apoptosis in A549 cells. Figure 5 showed the distribution of the apoptotic cells in the metformin HCl-treated and untreated A549 cells population obtained after data analysis from three independent experiments. Metformin HCl treated cells have reduced the percentage of live cells of A549 significantly ($p< 0.05$) from 86.33±1.00(untreated) to 59.00±1.00. This signifies that the treatment of metformin HCl has suppressed the proliferation of A549 cells and subsequently reduced the percentage of live cells. Metformin HCl also caused 24.00±1.00% ($p< 0.05$) of early apoptosis and produced 12.66±0.51% of late apoptosis.

Metformin HCl has an effect on A549 cell cycle analysis

We employed cell cycle analyses on the A549 cell

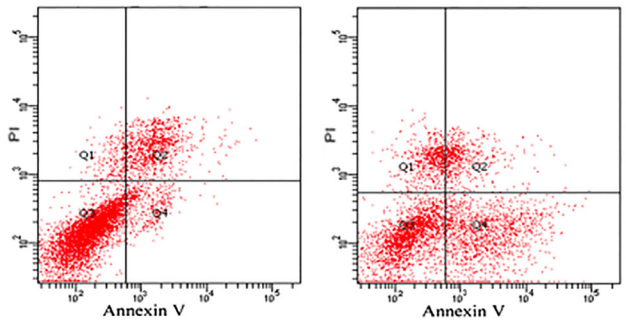

Figure 5: Metformin HCl showing apoptosis induction on A549 cell line after treatment with metformin HCl at IC50 dose for 72 hours. The upper left (Q1) debris cells, lower left (Q3) viable cells, lower right (Q4) and upper right (Q2) represent early and late apoptosis. After treatment cell number increased in the early apoptosis (Q4) quadrant compare to control.

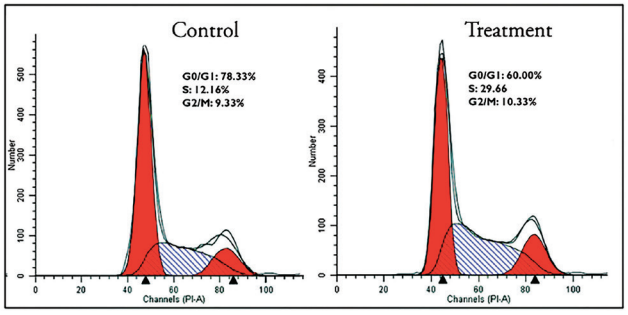

Figure 6: Effect of Metformin HCl on cell cycle of A549. The first peak represents G0/G1 phase, the second peak represents the G2/M phase, and the gap between the two peaks represents the S phase. Untreated cells mostly at G0/G1 phase. With IC50 dose of metformin HCl treatment for 72 hours, a significant transition block was observed in the S phase of the cycle.

line to find and observe which phase of the cell cycle was arrested by metformin HCl. After 24 hours of growth, the cells were treated with metformin HCl at the IC_{50} dose and incubated for 72 hours. Treated cells were then stained with Propidium Iodide (PI). PI is a fluorescent molecule that can cross the cell membrane and intercalate into cellular DNA, making it detectable and quantifiable using FACS (fluorescent-activated cell sorting) and Flow cytometry. The three phases in the cell cycle have been the G0/G1 phase, the S phase, and the G2/M phase. Figure 6 showed the histogram of cells in the cell cycle in metformin HCl-treated A549 cells. As in Figure 6, the untreated A549 cells were 78.33±1.0%, 12.16±0.06%, and 9.33±0.06% residing in the G0/G1, S and G2/M phase of the cell cycle respectively. Following exposure to IC_{50} of metformin HCl, the percentage had significantly ($p< 0.05$) increased in the S and G2/M phases which are 29.66±1.0% and 10.33±0.06 respectively, which indicate metformin HCl causes the S phage block of the A549 cell cycle.

DISCUSSION

Depending on the disease stage, resectability, and invasiveness, traditional lung cancer treatment options include surgery, chemotherapy, and radiation (22). Chemotherapy, either with or without radiation, is considered the treatment of choice for advanced lung cancer (23). Although therapies are available, they all have significant negative effects (24), furthermore, current therapy options are insufficient, necessitating the development of further effective therapeutic procedures (25). In our study, we used cell line A549 because adenocarcinoma is the most common type of NSCLC and accounts for approximately 40% of lung cancers (26) and this adenocarcinoma cell line A549 has been used for many in vitro and in vivo experiments for anti-cancer drug development and has characteristic features of Type II cells of the pulmonary epithelium, including lamellar bodies (27). Three of the following medications metformin, buformin and phenformin created for the management of hypoglycemia but buformin, and phenformin, were removed in the early 1970s due to cardiac mortality increased and the lactic acidosis risk (28). Metformin, on the other hand, has become the most often administered anti-diabetic medication in the world due to its lower risk and positive properties (29). It is a glucose-lowering medication with anti-cancer potential, and has been shown to reduce the occurrence and progression of a variety of human malignancies (30, 31) as well as increase cancer patients' overall survival rate (32).

In a previous study Larsson et al., (2006) proposed that synthetic drugs with IC_{50} values of less than 10μM be considered potential anticancer drugs in vitro (33). Ahinuma, H. et al., (2012) found that metformin has been explored in previous research in a variety of lung cancer cells, with results indicating that the IC_{50} of this drug ranges from 1-20mM (34). The current study found that metformin HCl aqueous solution significantly lowered the viability of the A549 cell line using the TBEA. Interestingly our study also found metformin HCl oral preparation lowers cell viability by 50% at 0.35 mg/ml, equivalent to 2.11mM, which is within the Ashinuma, H.; et al. described IC_{50} range. Recently Dong, J. et al., (2020) found metformin IC_{50} for 48 hours is 5mM by using the same cell line (35). Previously Wang, J. et al., (2015) and Zhou, X. et al., (2019) found that metformin reduces 50% of cell viability in 10mM of the drug by using a large cell lung cancer cell line (36, 37). In our investigation the difference in IC_{50} value found could be related to variations in the incubation period, media and testing procedure we used.

Metformin already has been tested as a single chemical in several lung tumour cells, and the results show that it has a broad range of anti-cancer characteristics. Metformin therapy reduced cell proliferation, activated apoptosis, and lowered colony formation in RERF-

LC-A1, IA-5, and WA-hT cells (38). In our study, we discovered that metformin HCl had an anti-proliferative impact on A549 cells. We discovered that the IC_{50} dose did not produce 100% inhibition of the A549 cells in an anti-proliferation assay over eight days, but it did considerably decrease cell growth from day one to day eight when compared to control cells that were solely exposed to a medium without treatment. It would be ideal to explore metformin's cytotoxic effect on normal lung cells but due to some limitations in material collection for our study, we had to use mouse skin fibroblast cell L929 for cytotoxic assay. Human or rodent cells are thought to be more useful, particularly if the study's goal is to test further in vivo or in vitro models (39). In a previous study, Kadoda et al., (2017) found metformin normally not toxic to normal cells but produces selective cytotoxicity on normal cells, especially in glucose-depleted conditions (40) but in our study, we used a glucose medium to provide a normal culture environment to the cells. Throughout the treatment period, cell viability was not appreciably affected at IC_{50} levels. This suggests that the IC50 dose of metformin HCl used in our investigation was harmless to L929 cells, implying that metformin aqueous solution could be a promising anti-cancer medication with low or no cytotoxicity in normal cells. In microscopic observation, however, the morphological study of the A549 cells revealed identifiable cell morphological alteration with noteworthy classical apoptotic signs (41). In the viewing field of the microscope illustrated in Figure 2(b), vacuolization, elongation, cellular shrinkage, apoptotic bodies, and membrane blebbing can all be seen.

On A549 cells, we used flow cytometry to identify whether the metformin aqueous solution could induce apoptosis and stop the cell cycle. However, some recent studies have found that metformin's ability to induce apoptosis in cancer cells varies (33, 42). In the present study, the treatment caused the ratio of apoptosis of A549 cells to increase by 13.67 % higher in the early apoptosis stage and 8.34 % higher in late apoptosis. Necrosis-induced cell death was ruled out because the therapy did not result in significant necrotic cells, as revealed in the results. Varied experimental circumstances and cancer cell line features are most likely metformin's various consequences in different cells to blame (42). In a recent study Xia et al., (43) found, that metformin stops the cell cycle in the G0/G1 phase but Yudhani et al., (44) found that metformin causes S and G2/M checkpoint arrest. The result of the apoptotic findings of our study leads us to do flow cytometry analysis for cell cycle arrest and we also found that upon treatment with metformin there is the number of cells within the S phases of the cell cycle has increased to 17.50%. Velma, V.et al., (2016) found that Cisplatin therapy delayed the cell cycle at the DNA synthesis phase (S phase), and as time passed, the cells gradually accumulated in the sub-G1 phase when HL-60 cells were employed (45). Our findings demonstrate that metformin HCl oral preparation has a similar effect on the S phase in A549 cells, implying that metformin can be used to treat non-small cell lung cancer alongside or instead of commonly used platinum-based chemotherapeutic medications.

CONCLUSION

This study provides initial experimental evidence that the oral metformin HCl tablets have the potential to be used as an anti-cancer treatment for NSCLC. According to this study, an oral tablet of metformin exhibited a lower IC50 value and a considerable amount of cell suppression. However, metformin's antiproliferative actions did not suppress cell proliferation completely after 8 days of incubation, but morphological observations revealed that the aqueous solution triggered apoptotic cell death and flow cytometry examination for apoptosis confirmed that the cells had undergone apoptosis. Further analysis for the cell cycle arrest revealed an accumulation of cells in the S phase in metformin-treated cells. Interestingly, despite all of the aforementioned findings, metformin had no adverse effect on normal mouse fibroblast cells. These results support the further development of a pre-clinical experiment to evaluate specific protein expressions, explore the apoptotic pathway, and comprehend the cell cycle arrest mechanism by metformin oral preparation on NSCLC.

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