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Anticancer Effect of Metformin, an anti diabetic drug, on breast Cancer Cells

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ABSTRACT: Both diabetes and cancer are prevalent diseases whose incidence is increasing globally, in 2008 there were an estimated 12.4 million new cancer cases diagnosed. The most commonly diagnosed cancers are lung/bronchus, breast, and colorectal, whereas the most common causes of cancer deaths are breast, stomach, and liver cancer. Despite investigations into mechanisms linking type 2 diabetes and cancer, there is a gap in knowledge about pharmacotherapy for diabetes in cancer patients. Epidemiologic studies have shown that diabetic cancer patients on different antidiabetic treatments have different survival. The clinically relevant question is whether some antidiabetic pharmacotherapeutic agents promote cancer while others inhibit cancer progression. We investigated the hypothesis that Metformin, an antidiabetic drugs had differential direct impact on cancer cells using human cell line breast cancer (MCF7). Breast cancer is a type of cancer originating from breast tissue, that usually starts off in the inner lining of milk ducts or the lobules that supply them with milk. The present study shows insulin and glucose promoted cancer cell proliferation and contributed to chemoresistance. Metformin suppressed cancer cell growth and induced apoptosis. Metformin is found to affect signaling in the AKT/mTOR pathway; metformin activated AMPK. 500mg per day of metformin was sufficiently useful for inhibiting the breast cancer cells. In conclusion, Metformin, an antidiabetic pharmacotherapy drug had a direct impact on cancer cells. This study provides experimental evidence to support further investigation of metformin as first-line therapies for type 2 diabetes in cancer patients.

***Keywords*:** Metformin, Breast cancer, Type 2 diabetes, MCF7, Insulin, AMPK

INTRODUCTION

In Asia, the most commonly diagnosed cancers are prostate, lung/bronchus, and colon/rectum in men and breast, lung/bronchus, and colon/rectum in women. Of the world population between the ages of 20 and 79 years, an estimated 285 million people, or 6.6%, have diabetes in 2007, diabetes prevalence in the U.S. was 10.7% of persons aged 20 years and older (23.6 million individuals), with an estimated 1.6 million new cases per year. Type 2 diabetes is the most common form, accounting for ~95% of prevalent cases. In the relevant medical literature, type 2 diabetes has been linked to an increased risk of developing liver, pancreatic, colorectal, endometrial, kidney, urinary bladder and breast cancer as well as non-Hodgkin's lymphoma. However, men with diabetes mellitus have a slightly lower risk of developing prostate cancer than average. (Baur et al., 2010). Moreover, the impact of type 2 diabetes on the development of cancer in diabetic patients and the specific survival of those patients have most likely been underestimated because an estimated 3-5% of the adult population is thought to have undiagnosed type 2 diabetes. In newly diagnosed cancer patients, the prevalence of diabetes ranges from 8% to 18%, and diabetes is significantly associated with breast cancer in women, regardless of body mass. It should be acknowledged; however, that diabetes mellitus is not a homogeneous disease. Type 2 diabetes has metabolic and hormonal characteristics that differ from those in type 1 diabetes. Additionally, hyperglycemia and endogenous hyperinsulinemia can coexist for a long period, including during pre-diabetes. Importantly, strong evidence points

toward insulin resistance and associated mitogenic hyperinsulinemia as a direct pathway connecting diabetes, obesity and metabolic syndrome with cancer (Becker et al., 2009; Bianchi et al., 2007).

Breast cancer is a type of cancer originating from breast tissue that usually starts off in the inner lining of milk ducts or the lobules that supply them with milk. . Breast cancer occurs in humans and other mammals. While the overwhelming majority of human cases occur in women, male breast cancers can also occur.[1] Breast cancer can have a number of symptoms but usually shows as a lump or thickening in the breast tissue (although most breast lumps are not cancerous). If cancer is detected at an early stage, it can be treated before it spreads to nearby parts of the body (Dilman et al., 1982).

Metformin and pharmacogenomics

The anti proliferative and proapoptotic effect of Metformin in in vitro studies was observed in concentrations that are usually seen in diabetic patients treated with Metformin (approximately 1.5 grams per day). However, there is inter-individual variation in drug response and the role of drug-metabolizing enzymes and drug transporters are starting to form the focus of these research projects. Genetic polymorphisms have been identified in most transport proteins and many of them are now recognized as significant contributors to inter-individual variation in drug effects. Metformin uptake in the liver occurs mostly by OCTs. (organic cation transporters, transporters on sinusoidal membrane), while its efflux is facilitated by multidrug and toxin extrusion transporter 1 (MATE1). There were significantly higher glucose and insulin levels during OGTT in healthy volunteers with reduced-function OCT1 polymorphism compared to healthy volunteers with OCT1 reference alleles (after Metformin treatment). Moreover, there was significantly reduced hepatic accumulation and therapeutic response to Metformin in mice with OCT1 deletion. These findings suggest that OCT1 mediates the first step in the response pathway of Metformin and that genetic variation in OCT1 may modulate response to Metformin in humans. In addition, identification of OCT3, the novel transporter of Metformin, raises the possibility that genetic variation in OCT1 alone may not be sufficient to result in reduced response to Metformin in some patients. OCT3 may contribute to hepatic Metformin uptake when OCT1 is functionally impaired. Finally, the second highest OCT1 expression is in the kidneys, being five-fold higher than in the small intestine. As Metformin is a hydrophilic organic cation which is eliminated by the kidneys in more than 98% of the absorbed dose, the OCT1 polymorphism might play a significant clinical role in the therapeutic effect of Metformin analyzed genetic polymorphisms in OCT1, OCT2 and OCT3 and their effects on Metformin clearance. They found that the OCT1 polymorphism induces low or absent reabsorption of Metformin in distal tubules leading to increased Metformin excretion, this possibly accounting for a 10% variation of Metformin clearance (Wilcock and Bailey, 1994).

Effect of metformin on Breast cancer

Response rate to neo adjuvant chemotherapy (this means, chemotherapy given before surgery to shrink the size of the tumor): among a group of patients with type 2 diabetes, those taking metformin had a 24% chance of having a complete response (this means, no residual cancer found during surgery) versus only an 8% chance of having a complete response if not taking metformin (Evans et al., 2005). An important issue within the context of the effect of metformin on breast cancer is triple negative (TN) breast cancer (also called basal), consisting of cells with no expression of the steroid receptors [ER and progesterone (PR)] as well as of the tyrosine kinase receptor Her-2 (Va´ zquez-Martı´n et al., 2009; Liu et al., 2009), while overexpressing the EGFR. Epidemiological data have shown that TN cancers are more frequent in pre- and postmenopausal women with an elevated waist-hip ratio and body mass index, possibly signifying coexisting hyperinsulinemia and a positive therapeutic effect of metformin (Liu et al., 2009)).

MATERIALS AND METHODS

Cells and culture conditions

Breast cancer cells, which have already been characterized and their tumorigenicity has been previously tested was obtained from Cell repository, NCCS, Pune. They were used to imitate the *in vivo* conditions more closely than by using pure tumor cells. The cells were cultivated in EMEM medium (Himedia laboratories Pvt Ltd. Mumbai, INDIA) containing 10% fetal calf serum, antibiotics and phenol red for visualizing culture condition. The culture obtained had passage number as 54 and further it was trypsinized and passaged for 55^{th} time in animal cell culture lab, Triesta sciences. The cells were observed and counted by Heamocytometer after a period of 24 hr incubation.

Procurement and standardization of Metformin

Metformin was procured from (Prolab Marketing Pvt. Ltd, New Delhi, INDIA). It was obtained in the form of tablets and analyzed for its concentration per gram of the chemical obtained. The hydrochloride extract was used for Spectrophotometric analysis.

A simple and sensitive spectrophotometric method has been developed and validated for the estimation of metformin hydrochloride in bulk and in tablet formulation. The primary amino group of metformin hydrochloride reacts with ninhydrin in alkaline medium to form a violet color chromogen, which is determined spectrophotometrically at 570 nm. The proposed method was found to be accurate and precise for routine estimation of metformin hydrochloride in bulk and from tablet dosage forms.

Effect of Metformin on the breast cancer cell lines

Cells were homogenously suspended in the medium to determine viability through Heamocytometric Viability count using tryphan blue stain. The medium containing the suspended cells is then diluted to maintain final cell density to approximately 200 cells/100µl of the medium. The medium containing the cells was suspended in the MTT plate wells and incubated overnight for adherence of the cells to the surface of the wells as monolayers. After incubation, the cell layer was treated with different concentration of Metformin, and incubated over night and the media from the plate discarded and the monolayer treated with MTT dye and incubated for 3 hours. The MTT dye gets reduced by the viable cells to develop purple color crystals. The crystals were dissolved in cold isopropenol or DMSO and read at 260nm and 580nm. The readings were plotted on a graph and the IC50 value of the compound is assessed from the graph.

RESULTS AND DISCUSSION

Metformin is a biguanide, and a widely prescribed oral medication used as front-line therapy for type 2 diabetes. Population studies suggest that metformin decreases the incidence of cancer and cancer-related mortality in diabetic patients. At the level of cell signaling, several mechanisms of metformin action have been proposed; the most important one relates to the activation of AMPK. AMPK, the central cellular key energy sensor with a unique ability to directly sense cellular energy, places it in an ideal position to ensure that cell division, which is a highly energy-consuming process, only proceeds if cells have sufficient metabolic resources. Once activated, it leads to the suppression of many of the metabolic processes that depend highly on sufficient cellular ATP supply (gluconeogenesis, protein and fatty acid synthesis, cholesterol biosynthesis) and that promote catabolic processes (glycolysis, fatty acid beta oxidation)

1a. Viability test: Microscopic Method

Microscopic analysis using an inverted microscope, of the growing breast cancer cells which was transferred into fresh EMEM media from the master culture obtained from the repository is as shown in the Figureure 1. The growing culture was observed as densely growing cells which properly adhered to the base of the culture T flask. The cells were observed to be healthy and proliferating rapidly. The cells were seen as wedge shaped and densely populated and adhered to the material of the culturing container.

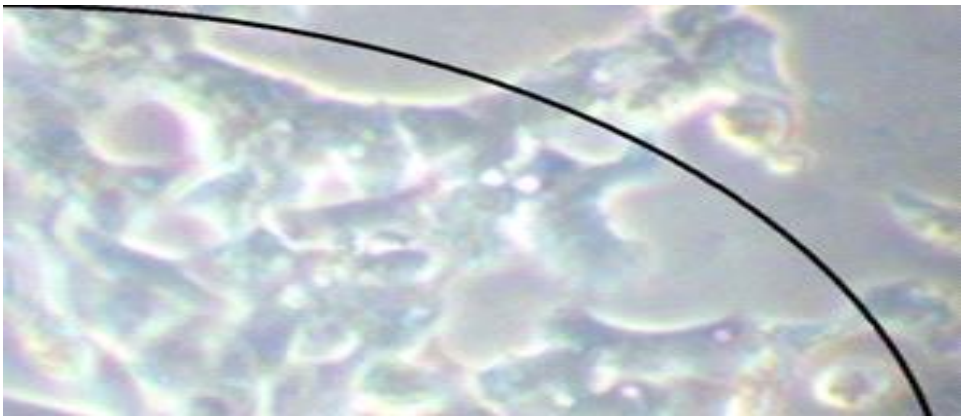

Figure 1. Microscopic analysis of the breast cancer cells under inverted microscope showing cells growing attached to the surface of the T-flask

1 b. Viability test: Trypan Blue Method

The densely growing cells were then trypsinized using 5% trypsin solution and properly separated by vigorous vortexing. The solution containing the cells was then stained with 0.5 % tryphan blue solution, which stains only the dead cells, where as the viable cells do not take up the stain. Microscopic counting of these cells was done using Heamocytometer, observation under compound microscope is as shown in the Figure 2, Heamocytometric counting was tabulated and the numbers of cells per µl were calculated.

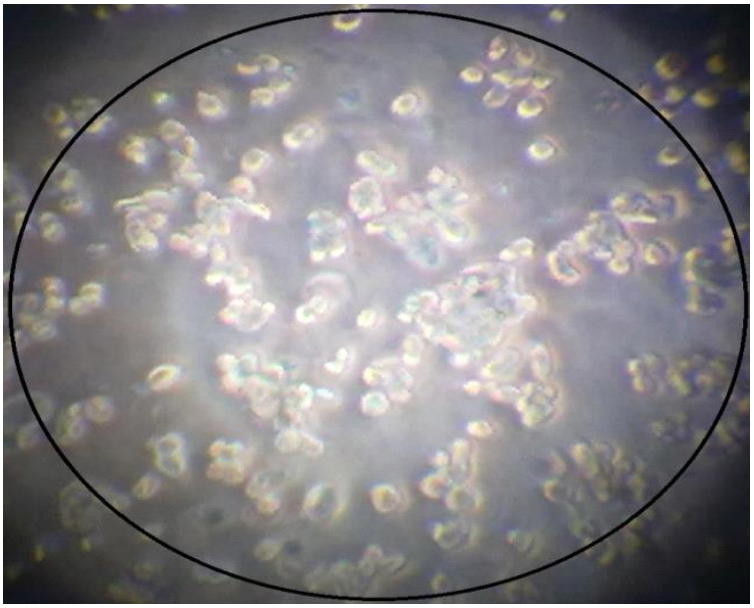

Figure 2. Showing trypsinized cells Stained with Tryphan Blue stain

The viability analysis of the proliferating cancerous cells after 54th passage was carried out using tryphan blue stain, which showed that the cell density in the solution was 95%. 95,000 cells / µl.
Upon calculation it was found out that the % of viability was 95.
The total cell count was 95,000 cells / µl.

This concentration of cells exceeded the required cell density for a single well (200 cells/100µl) for further MTT analysis. Thus the trypsinized culture was further diluted to obtain a density of 200 cells / µl using buffer as shown in the Figureure 3.

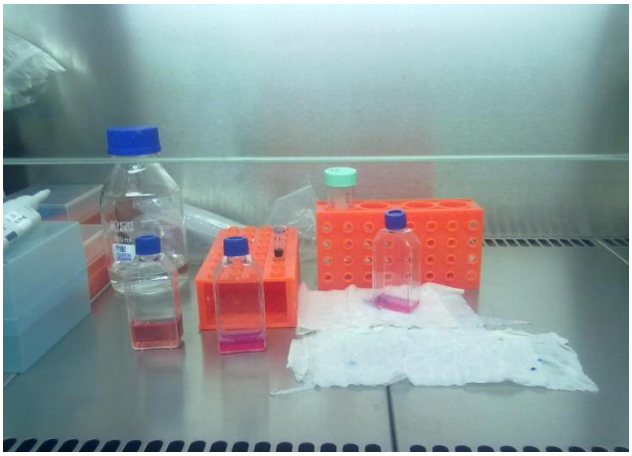

Figure 3. Methodology of trypsinization and dilution in Aseptic conditions

2. Anticancer Effect of Metformin on Proliferating Breast cancer cells

The cells obtained after trypsinization and proper dilution with EMEM buffer were then properly mixed and 100µl of the solution containing the cells were aseptically inoculated in 90 well MTT plate and were incubated overnight. After incubation, the cultures was observed for viability of the cells and the media was separated without disturbing the cells and was discarded, the adhered cells were washed slightly with buffer to remove the remnants of the media which would hinder the effect of the compound extracts when added. The cells were then treated with different aliquots of the metformin, as shown in the table 1. Metformin was dissolved in dilute hydrochloride which was found to be non toxic for normal human cells. And this concentration was also assessed in the experiment for analyzing the effect of the vehicle in which the compound is dissolved. After addition of the compound, the cells and the compound were incubated together for a period of 24 hours. This incubation period allow the effect of these compounds to occur on the cells, this incubation period is sufficient to assess the anticancer property of the compound. After this incubation period, the solution was discarded and the incubated cells along with the compound were treated with MTT dye prepared in methanol. This mixture was allowed to incubate at room temperature for a period of 2 hours and was allowed to react. The reaction leads to the development of purple color which indicated the viability of the cells and indicated the concentration of the cells in the wells. During the incubation period, the viable cells containing the mitochondrial cytochrome oxidized the dye and changed it into a purple colored formazan which can be read at 492 or 620nm.

After the reaction, it was observed that the dye reduction due to mitochondrial enzymes of the viable cells led to the development of a purple color as shown in the Figureure 4, which was read in UV range of light and the values were recorded and further the results were analyzed for the effect.

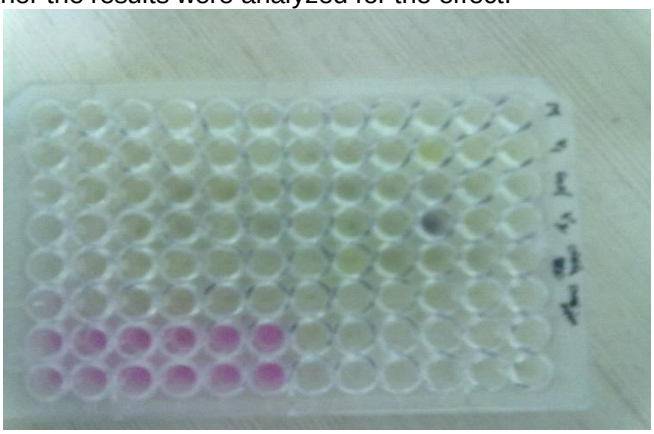

Figure 4. MTT Plate containing cells showing purple color development after incubation with the dye

The values obtained in the MTT assay experiment as recorded in the table 2 was studied and concluded that there was a maximum inhibitory effect of Metformin at 500mg in breast cancer cells. Effect of Metformin compound was analyzed from the graph and the IC50 value of 250mg was obtained. The Cytotoxic effect of the vehicle used for dissolving Metformin was also analyzed and the least toxic effect of the vehicle was found to be at a concentration of 0.5% that recorded in table 3.

Table 1. Values Recorded in ELISA reader after MTT Dye reduction reaction

Concentration of Vehicle in %	Metformin concentration in mg in Breast cancer cells	Well no
0.5	100	1
0.5	200	2
0.5	300	3
0.5	400	4
0.5	500	5
0.5	600	6
0.5	700	7
0.5	800	8
0.5	900	9
0.5	1000	10
0.5	1100	11

Table 2. Cytotoxicity assay of metformin in Breast cancer cells

0.088	0.083	0.088	0.084	0.087	0.088	0.085	0.087	0.085	0.088	0.087	0.088	Control
0.068	0.061	0.062	0.054	0.051	0.052	0.049	0.04	0.053	0.062	0.065	0.079	OD obtained after treating cells with metformin aliquotes

MTT assay of cell cytotoxicity of metformin on breast cancer cell lines

Table 3. Cytotoxicity assay of the vehicle (HCL) used to dissolve Metformin

1.2	1.1	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	Increasing percentage of the vehicle (HCL)
0.012	0.015	0.018	0.022	0.028	0.03	0.031	0.045	0.038	0.034	0.028	0.024	OD obtained after MTT treatment

MTT assay of cell cytotoxicity, of vehicle on breast cancer cell lines

Conclusion

Metformin is a widely prescribed oral medication used as front-line therapy for type 2 diabetes. It has been shown to inhibit the growth of cancer cell lines, including breast cancer cells, *in vitro* and *in vivo* tumor models. Population and retrospective studies showed that metformin decreases the incidence of cancer and cancer-related mortality, and increases the response to neoadjuvant chemotherapy in diabetic patients. Metformin induces AMPK activation, which decreases insulin levels and leads to inhibition of protein synthesis pathways, decreasing cancer cell proliferation and growth. As a result, metformin is being investigated as a therapeutic agent in different clinical settings for all breast cancer and Pancreatic subtypes. In the present study Metformin is found to inhibit the most widely occurring cancer cells in humans like the breast cancer cells and pancreatic cancer cells. It was found to inhibit Breast cancer cells at a concentration of 500mg dosage per day of Metformin, and 400mg of Metformin was known to inhibit pancreatic cell lines, suggesting that Metformin could be used as a potent inhibitor of cancer in patients suffering from Diabetes type II.

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