Glucose deprivation enhances the antiproliferative effects of oral hypoglycemic biguanides in different molecular subtypes of breast cancer: An *in vitro* study

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Extensive *in vitro* studies have been conducted to evaluate the anticancer activity of oral hypoglycemic agents. Many of these studies experienced detrimental limitations, since they were conducted on cancer cells commonly grown in culture media consisting of extremely high concentrations of growth factors and glucose. The present study was aimed at exploring the antiproliferative effects of the commonly studied metformin and the less frequently reported phenformin oral hypoglycemic agents on different molecular subtypes of breast cancer under rich glucose and glucose deprived conditions. Our results indicate that under glucose deprived conditions, which better reflect the factual glucose-starved solid tumors *in vivo*, biguanides exert more antiproliferative activities against the three molecular subtypes of breast cancer cell lines examined in this study. In addition, the observed antiproliferative activities of biguanides appear to be mediated by apoptosis induction in breast cancer cells. This induction is significantly augmented under glucose deprived conditions.

It has recently been reported that morbidity and mortality have arisen from breast cancer in diabetic patients in contrast to nondiabetic control patients, excluding all other diseases (1). Globally, cancer and diabetes are among the most prevailing chronic diseases (2), with a notable relation between the two diseases (1, 3). Obesity, which is frequently linked to diabetes, significantly elevates breast cancer risk (4). In addition, poor outcomes in diabetic women with breast cancer have been often encountered compared to their nondiabetic counterparts (5).

Interestingly, interruptions in metabolic pathways that relate diabetes and cancer have been lately observed. Fundamentally, cancer cells endure adjustment of their conventional metabolic functions to support aerobic glycolysis for energy requirements as an unorthodox pathway to mitochondrial oxidative phosphorylation (6). Even though aerobic glycosylation is unbeneficial in terms of energy production, rapid DNA replication and cancer cell division favor superfluous glucose for prompt bursts of energy (7). Consequently, tumor cells are substantially reliant on the increased rates of glucose absorption and catabolism for growth and viability. It has therefore been proposed that high glucose abundance in the bloodstream, as manifested in diabetic patients, may supply the nutritional requirements to sustain the hasty growth of cancer cells; hence the suggestion that glucose lowering treatments may be rewarding for cancer prevention and treatment (8).

Metformin, a biguanide oral hypoglycemic agent that is frequently used to treat type II diabetic patients, combats insulin resistance by reducing the available amount of serum glucose. On the other hand, phenformin, the first commercialized biguanide, was

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expeditiously withdrawn from the market due to a high risk of lactic acidosis. It was replaced by the safe metformin, which is associated with fewer than 1/10,000 cases of lactic acidosis, primarily in patients with impaired renal function (9).

Metformin has recently earned special interest as a potential anticancer therapeutic and chemotherapy adjuvant. Evidence from *in vitro* and epidemiological studies supports the beneficial role of metformin in improving the overall survival for cancer/diabetes comorbidity in patients (1, 3). Metformin's promising anti-cancer activity was supported by lower prevalence of cancer in type II diabetics in contrast to other glucose regulating drugs (10). It was initially suggested that the anti-cancer activity of metformin was mainly related to its systemic glucose and insulin regulating properties. However, several other mechanisms, at the cellular level, by which metformin exerts its anti-cancer activity, have recently been proposed and investigated (11, 12).

Interestingly, the anti-oncogenic properties of the anti-diabetic biguanide metformin of crucially inhibiting cell proliferation in cultured cancer cells *in vitro* can be observed only at its supra-pharmacological concentrations of the millimolar range (13). It is noteworthy that *in vitro* studies of metformin were carried out on tumor cells commonly grown in culture media consisting of extremely high concentrations of growth factors and glucose. While the normal range of fasting glucose level in healthy non-diabetic adults is maintained at about 5 mmol L^{-1}, and 7 mmol L^{-1} to 10 mmol L^{-1} in metabolic syndrome and diabetic patients, regular tissue culture media contain glucose concentrations between 10 and 25 mmol L^{-1} in addition to the insulin-rich fetal bovine serum (5–10 % *v/v*). Hence, the exceptionally high, non-physiological pre-clinical concentrations of metformin commonly claimed to exert the anti-cancer effect can reflect

the artefactual interference of faulty, glucose-rich cell culture experimental conditions deviating from the actual glucose-starved solid tumors *in vivo*.

In this report, we evaluated the anticancer effects of metformin and phenformin on the breast cancer cell lines of different molecular subtypes, including estrogen receptor [ER] positive, HER-2 negative MCF-7 cell line, ER positive, HER-2 positive T-47D cell line, and triple-negative TN; ER negative, progesterone receptor [PR] negative and HER2 negative MDA-MB-231 cell line, under physiological conditions reflecting the glucose-deprived microenvironment of cancer cells *in vivo*.

EXPERIMENTAL

Materials and cell lines

Low glucose Dulbecco's modified eagle medium (DMEM), high glucose Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin-EDTA solution (0.25 % trypsin, 1 mmol L^{-1} EDTA), amphotericin B and penicillin-streptomycin solutions were obtained from Biochrom AG (Germany). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) glycine, trypan blue, metformin, phenformin and Raloxifene were purchased from Sigma-Aldrich (USA). MCF-7, T-47D and MDA-MB-231 cell lines were obtained from the American Type Culture Collection (ATCC) (USA). Phospho-AMPKa (Thr 172, #2531) was purchased from Cell Signaling Technology, Danvers, MA, USA. Secondary anti-rabbit antibody was purchased from Sigma- Aldrich (USA).

Cell viability assay

Cells were cultured at a cell count of 3×10^4 cells in 24-well plates in 500 μL low glucose (5.5 mmol L^{-1}) DMEM medium or high glucose (25 mmol L^{-1}) DMEM medium containing 10 % FBS and 2 mmol L^{-1} L-glutamine in triplicate. The plates were maintained in humidified air containing 5 % CO_2 at 37 °C for a 24 h incubation period, after which 1 mmol L^{-1} concentration of either metformin or phenformin was added to the medium. During the treatment period, cells were stained with trypan blue, collected and counted at 24 and 48 h using a hemocytometer.

MTT-based cytotoxicity assay

MTT-based antiproliferative assay was performed as previously described (14). In short, the cell lines were transferred to 96-well plates, 8,000 cells were seeded per well in 200 μL of DMEM medium and allowed to adhere to wells for 24 h. Cells were incubated in humidified air containing 5 % CO_2 at 37 °C. After that, cells were treated with different concentrations of either metformin or phenformin. Both test and control samples were allowed 48 h incubation at 37 °C in a 5 % CO_2 incubator. At the end of the exposure period, the MTT assay was carried out and the viable cell count was determined using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The yellow tetrazolium dye [MTT, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] was reduced by metabolically active cells into an intracellular purple formazan product. The quantity of formazan product, as determined by the absorbance at 490 nm, is directly proportional to the number of living cells in the culture. Cell viability was calculated based on the

measured absorbance relative to the absorbance of the cells exposed to negative controls, which represented 100 % cell viability.

Western blotting

MDA-231 cell line was sub-cultured, split, counted, and plated at 1.5×10^6 cells/plate in suitable tissue culture plates and incubated for 24 h at 37 °C with 5 % CO_2 humidified air. Cells were then treated as duplicates with 2 mmol L^{-1} phenformin and incubated with the corresponding controls for 48 h at 37 °C with 5 % CO_2 humidified air. Treated and control cell lysates were then collected and subjected to the protein assay. Equal amounts of cell protein per well were separated using 10 % (*v/v*) SDS-PAGE in a maximum volume of 30 µL/well. Proteins were then separated by electrophoresis and transferred to a nitrocellulose membrane. Blocking of the membrane with 5 % BSA (Bovine Serum Albumin) for 1 h at room temperature was followed by incubation overnight at 4 °C with rabbit derived phospho-AMPKa (Thr 172). The membrane was washed with TBST three times, 5 minutes each, and then incubated for 1 h at room temperature with goat derived anti-rabbit IgG (whole molecule)-peroxidase conjugate secondary antibody. The membrane was then washed again with TBST three times, 5 minutes each, preceded by addition of a luminescence substrate. Detection of band density was then performed using a C-DiGit Blot Scanner 3600 (Li-Cor, Lincoln, NE, USA).

Flow cytometry analysis

To evaluate the variable effects of biguanide treatment on the induction of apoptosis in the examined cell line under different medium glucose levels, the Annexin V-FITC Apoptosis Detection Kit was used according to the manufacturer's protocols. MCF-7 cells were seeded into 6-well plates at 5×10^5 cells/dish and treated for 48 h with either 1 mmol L^{-1} metformin, 1 mmol L^{-1} phenformin, or the control drug doxorubicin. Cells were maintained at 37 °C in a 5 % CO2 incubator. For apoptosis analysis, the cells were treated with trypsin for 7 min, washed with cold PBS and then resuspended in 500 µL cold binding buffer. After that, cells were stained using the Annexin V-FITC reaction reagent (5 µL of Annexin V-FITC, 5 µL of propidium iodide) at 37 °C for 30 min protected from light. Stained cells were analyzed using flow cytometry analysis.

RESULTS AND DISCUSSION

The effect of biguanides on cell viability

As shown in Table 1, metformin does not affect cell viability at any exposure time and in any of the examined cell lines under hyperglycemic conditions. On the other hand, phenformin appears to be more potent in reducing cell viability in the three examined cell lines and at both exposure times of 24 and 48 hours. Interestingly, when grown under normoglycemic conditions (5.5 mmol L^{-1}), the three cell lines used in this study appeared to become more responsive to biguanide treatment. For example, while maintaining the viable cell count above 80 % of the control when treated with 1 mmol L^{-1} metformin under hyperglycemic conditions, MCF-7 and T47D cell lines failed to sustain a viable cell count of more than 52 % of the control under normoglycemic and otherwise similar treatment conditions (Table 2). It is noteworthy that phenformin consistently exerts a

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more potent effect in reducing cell viability compared to the metformin effect regardless of the medium glucose concentration. Similarly, biguanide treatment demonstrated preferential cytotoxicity against MDA-MB-231 cells under glucose-deprived normoglycemic conditions (Table 2).

The antiproliferative effect of biguanides in different subtypes of breast cancer cell lines

Although the antiproliferative effects of metformin have been previously reported, in the present study, we have evaluated the consequences of treating diverse molecular subtypes of breast cancer under normoglycemic conditions with pharmacological doses of two biguanides, metformin and phenformin. According to the results (Table 3), IC_{50} values for both metformin and phenformin, defined as the concentration at which 50 % of cellular population is killed by drug treatment, were significantly enhanced in ER-positive cell lines when the treatment was carried out under glucose deprived conditions. For example, compared to the glucose rich media, MCF-7 cells demonstrated better sensitivity to metformin and the IC_{50} value dropped nearly 7 fold when grown in low glucose media. Interestingly, triple-negative cells MDA-MBA-231, a highly aggressive and invasive tumor very resistant to many anticancer treatments, appear to be less responsive to drug in both high and low glucose media. This may be explained by the deficiency of the tumor suppressor kinase LKB1 in these cells (11). Phosphorylation of LKB1 is required for activation of the AMPK pathway, which has been broadly accepted as the proposed mechanism for the observed biguanide anticancer activity (11).

The effect of glucose concentration on the potential antiproliferative activity of phenformin on different molecular subtypes of breast cancer

In accord with the literature, our results indicate that glucose level regulates the susceptibility of different tumor cell lines to the cytotoxic effects of metformin. To the best of our knowledge, this is the first report that describes the effect of glucose concentration on the potential anticancer activity of phenformin on three molecular subtypes of breast cancer. While physiological variations of glucose level are restricted under normal conditions, provocation of glucose dispossession in breast cancer cells could potentially sensitize cancer cells to biguanide treatment and thus introduce an effective strategy to improve treatment outcomes.

The effect of phenformin on the AMPK activity under low glucose conditions

Evidence supporting the involvement of AMP-activated protein kinase (AMPK), a cellular energy sensor and a key controller of energy metabolism, in anticancer as well as antidiabetic activities of metformin has begun to accumulate (11). Indeed, it has been reported that metformin stimulates AMPK indirectly by inhibiting complex I of the mitochondrial respiratory chain (15). Inhibition of complex I provokes energy exhaustion, increases AMP concentrations, and results in AMP binding to the nucleotide-sensing AMPK γ subunit which will trigger AMPK activity (15). In an attempt to assess different levels of AMPK activation, in response to biguanide treatment, western blot analysis was performed to detect phosphorylation of AMPK (Thr172). Interestingly, in the medium containing 5.5 mmol L^{-1} glucose, considered a low glucose medium, phenformin resulted in a decrease in the level of phosphorylated AMPK compared to the

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control (Figure 1). This can be explained by the possible reduction of total AMPK in low glucose medium (not detected) and thus total phosphorylated AMPK was decreased with phenformin treatment compared to the control.

The effects of biguanide on cell apoptosis

Furthermore, to study biguanide-induced apoptosis effects, flow cytometric analysis was carried out. The number of apoptotic cells was examined after a 48-hour incubation period with either 1 mmol L^{-1} of metformin or phenformin under the different medium glucose conditions. Interestingly, a significant increase in the number of apoptotic cells after 48 h of drug treatment was observed (Figure 2). Statistical analysis shows that the percentages of early apoptotic and late apoptotic/necrotic cells were significantly increased over the control parameters in MCF-7 cells, suggesting that biguanide treatment promotes apoptosis preferably under low glucose levels (Figure 2).

CONCLUSIONS

Taken together, in this study we evaluated the effect of pharmacological concentrations of metformin and phenformin on the proliferation of the different molecular subtypes of breast cancer under glucose rich and glucose deprived conditions. Glucose deprived medium, which resembles better the *in vivo* compartment, was shown to sensitize the three examined molecular subtypes of breast cancer cell lines towards the metformin and phenformin antiproliferative activity through an induced apoptotic pathway. Our results highlight the significance of establishing a proper glucose level in clinical settings for the appraisal of biguanide anticancer effects. Further research is

required to elucidate the mechanism through which the antiproliferative effects of biguanides take place.

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Table I. Effect of metformin and phenformin on viability of breast cancer cell lines in hyperglycemic conditions

Different molecular subtypes of breast cancer cell lines including MCF-7, T47D, and MDA-MB-231 were cultured in a medium with 25 mmol L^{-1} glucose concentration and treated with pharmacological concentration of biguanides (1 mmol L^{-1}). Cell viability was assessed every 24 h using trypan blue. Data are represented as % mean ± SD.

Metformin	Phenformin

Cell Line	24 h	48 h	24 h	48 h
MCF-7	85 ± 2	81 ± 3	73 ± 1	69 ± 4
T47D	87 ± 3	82 ± 2	71 ± 2	68 ± 3
MDA-MB-231	94 ± 3	89 ± 4	87 ± 4	82 ± 3

Table II. Effect of metformin and phenformin on viability of breast cancer cell lines in normoglycemic conditions

Different molecular subtypes of breast cancer cell lines including MCF-7, T47D, and MDA-MB-231 were cultured in a medium with 5.5 mmol L^{-1} glucose concentration and treated with biguanides (1 mmol L^{-1}). Cell viability was assessed every 24 h using trypan blue. Data are represented as % mean ± SD.

	Metformin		Phenformin	
Cell Line	24 h	48 h	24 h	48 h
MCF-7	51 ± 2	49 ± 4	36 ± 3	31 ± 4
T47D	52 ± 3	47 ± 2	34 ± 5	30 ± 4
MDA-MB-231	78 ± 2	71 ± 3	65 ± 3	69 ± 3

Table III. Metformin and phenformin IC$_{50}$ values against breast cancer cell lines in hyperglycemic and normoglycemic conditions

Different molecular subtypes of breast cancer cell lines including MCF-7, T47D, and MDA-MB-231 were cultured in either medium with 5.5 or 25 mmol L^{-1} glucose concentration and treated with variable concentrations of biguanides. Cell proliferation was assessed after 48 h using the MTT assay. Data are represented as mean (mmol L^{-1}) ± SD.

Drug	Metformin IC$_{50}$ (mmol L^{-1})		Phenformin IC$_{50}$ (mmol L^{-1})	
Cell Line	25 mmol L^{-1} Glucose	5.5 mmol L^{-1} Glucose	25 mmol L^{-1} Glucose	5.5 mmol L^{-1} Glucose
MCF-7	4.9 (± 0.9)	0.75 (± 0.7)	2.1 (± 0.2)	0.58 (± 0.5)
T47D	5 (± 0.6)	0.90 (± 0.5)	2.2 (± 0.2)	0.43 (± 0.3)
MDA-MBA-231	7.88 (± 0.3)	6.4 (± 1)	3.5 (± 1.2)	3.1 (± 1.3)

Fig. 1. Phenformin inhibits phosphorylation of AMPK in glucose-deprived conditions. Extracts of MDAMB231 cells were harvested after 48 h treatment with phenformin in 5.5 mmol L^{-1} glucose medium and were used for western blotting for the detection of phosphorylated AMPK and B-Actin.
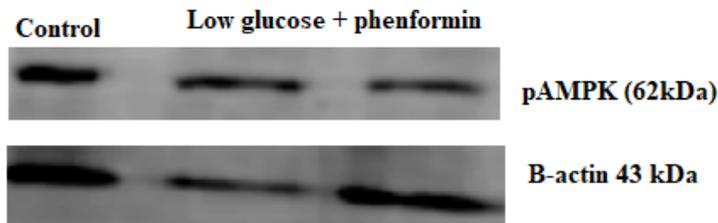
Fig. 2. Biguanides induce apoptosis in MCF-7 breast cancer cells. a) control cells, b) MCF-7 cells treated with 1 mmol L^{-1} of metformin for 48 h in high glucose medium, c) MCF-7 cells treated with 1 mmol L^{-1} of metformin for 48 h in low glucose medium, d) MCF-7 cells treated with 1 mmol L^{-1} of phenformin for 48 h in high glucose medium, e) MCF-7 cells treated with 1 mmol L^{-1} of phenformin for 48 h in low glucose medium.