Original Paper

Chronic Glibenclamide Treatment Attenuates Walker-256 Tumour Growth in Prediabetic Obese Rats

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Key Words

Diabetes • Glibenclamide • Walker-256 tumour • Prediabetic rats

Abstract

Background/Aims: The sulphonylurea glibenclamide (Gli) is widely used in the treatment of type 2 diabetes. In addition to its antidiabetic effects, low incidences of certain types of cancer have been observed in Gli-treated diabetic patients. However, the mechanisms underlying this observation remain unclear. The aim of the present work was to evaluate whether obese adult rats that were chronically treated with an antidiabetic drug, glibenclamide, exhibit resistance to rodent breast carcinoma growth. **Methods:** Neonatal rats were treated with monosodium

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L-glutamate (MSG) to induce prediabetes. Control and MSG groups were treated with Gli (2 mg/kg body weight/day) from weaning to 100 days old. After Gli treatment, the control and MSG rats were grafted with Walker-256 tumour cells. After 14 days, grafted rats were euthanized, and tumour weight as well as glucose homeostasis were evaluated. **Results:** Treatment with Gli normalized tissue insulin sensitivity and glucose tolerance, suppressed fasting hyperinsulinaemia, reduced fat tissue accretion in MSG rats, and attenuated tumour growth by 27% in control and MSG rats. **Conclusions:** Gli treatment also resulted in a large reduction in the number of PCNA-positive tumour cells. Although treatment did improve the metabolism of pre-diabetic MSG-rats, tumour growth inhibition may be a more direct effect of glibenclamide.

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Introduction

Obesity is a worldwide epidemic that is closely associated with non-communicable diseases (NCDs) and leads to a huge impact on health systems. Cardiovascular diseases (CVD), cancers, diabetes and respiratory diseases are the main chronic diseases linked to obesity, contributing to a great number of deaths [1, 2]. Among these diseases, type 2 diabetes is one of the most prevalent NCDs. Beyond its association with obesity, diabetes is also related to several types of cancer, including cancer of the kidney, bladder, pancreas, liver, breast, colon, endometrium and rectum [1, 3-5]. The association between type 2 diabetes and cancer is well known.

Diabetic patients have a high propensity to develop cancer due to metabolic alterations, such as hyperinsulinaemia and hyperglycaemia, or even drugs that affect the cell cycle, which may contribute to mechanisms promoting cancer initiation or progression [6, 7]. Nevertheless, the role of metabolic dysfunctions, such as hyperinsulinaemia, which might induce cell proliferation and the consequent increased risk for cancer development, remains incompletely understood [3, 6].

Gli is an oral antidiabetic drug in a class of medications known as sulphonylureas, which have been widely used for 6 decades in the treatment of type 2 diabetes mellitus. Gli is a secretagogue agent that blocks ATP-dependent potassium channels (K_{ATP}) after binding to a receptor located on the beta cell plasma membrane, allowing pancreatic beta cells to depolarize and leading to the opening of voltage-gated calcium (Ca^{+2}) channels, promoting an increase in the cytoplasmic Ca^{+2} concentration and subsequent stimulation of the exocytosis of insulin-containing granules. The increased insulin levels reduce hyperglycaemia in diabetic patients, improving their health condition [7, 8].

Epidemiological studies have demonstrated a low incidence of certain types of cancer in diabetic patients given antidiabetic therapies; however, the mechanisms underlying this association are still unclear. Gli may inhibit tumour growth directly or may act through its ameliorating effects on metabolism. [7, 9]. Furthermore, the lack of experimental studies showing the anticancer long-term effects of Gli makes it difficult to understand its effects.

The experimental model of obesity used in this work was generated by early postnatal administration of monosodium glutamate (MSG), which induces neonatal neuro-intoxication, causing hypothalamic lesions in the arcuate nucleus and neuroendocrine alterations such as hyperinsulinaemia and increased adiposity, among other effects. MSG treatment of animals has been reported to be a suitable model to study metabolic dysfunction [10-13].

The aim of the present work was to evaluate whether obese adult rats that were chronically treated with an antidiabetic drug, glibenclamide, exhibit resistance to rodent breast carcinoma (Walker-256 tumour) growth.

Material and Methods

Animals and experimental groups

To induce the obese and pre-diabetic rat model, during the first 5 days of life, neonatal male Wistar rats were subcutaneously injected with MSG (Sigma-Aldrich®, St. Louis, MO, USA) at a dose of 4 mg/kg body weight (bw). Control animals received injections of an equimolar saline solution [14, 15].

At 21 days old, the male Wistar rats were divided into four groups: control and MSG, both treated with or without Gli.

During all procedures, animals were kept under controlled temperature conditions ($23 \pm 2^\circ\text{C}$) and a light/dark cycle of 12 h with ad libitum access to water and a standard diet (Nuvital®, Curitiba, PR, Brazil). The Ethics Committee on Animal Use of the State University of Maringá approved the protocols.

Experimental groups and Gli treatment

To evaluate the potential of chronic Gli treatment as a preventive strategy against tumour growth, we administered the treatment by gavage once a day from weaning until 100 days of age. A batch of animals from both the control and MSG groups was treated with Gli (Glibenclamide, Medley, Brazil), which was dissolved in water, at a dose of 2 mg/kg of bw, and another batch of animals from the control and MSG groups received water. The drug dose used in the current protocol was previously tested in rats [16]. Twenty-four hours after the last glibenclamide gavage, animals from both groups, control and MSG, were used in the subsequent experimental procedure, in which a portion of the animals were inoculated with tumour cells. Figure 1 summarizes the experimental protocols.

Inoculation of Walker-256 cells

The Walker-256 tumour is a well-established experimental model that is often used to study tumour growth. This tumour was first described in 1928 by George Walker as a spontaneous carcinosarcoma from the mammary gland of rats [17]. Since then, the Walker-256 tumour has been perpetuated by various techniques.

In our laboratory, Walker-256 cells, kindly provided by the Laboratory of Cellular Metabolism Physiology (Federal University of Parana, Brazil), have been maintained in the intraperitoneal cavity of Wistar rats (ascitic form) [18] through weekly passages, every 7 days, by aseptic intraperitoneal injection of 1.0×10^6 cells per rat. After 7 days of ascitic growth, the peritoneal exudate is withdrawn and subjected

Fig. 1. Schematic representation of the experimental protocol.

to differential centrifugation at 4°C to obtain tumour cells. These cells are resuspended in phosphate-buffered saline (PBS; 16.5 mmol l⁻¹ phosphate, 137 mmol l⁻¹ NaCl and 2.7 mmol l⁻¹ KCl, pH 7.4). Cell viability is evaluated using the trypan blue exclusion method.

At the end of the treatment period, the 101-day-old animals from each experimental group were inoculated subcutaneously with 8.0×10^7 viable tumour cells/animal in the right rear flank [19, 20]. The inoculation of the Walker-256 tumour cells was performed 24 h after the end of treatment with Gli. After 14 days of tumour inoculation, animals were euthanized for blood and tissue collection. Figure 1 summarizes the experimental protocols.

Obesity assessment

Obesity was assessed based on bw and nasoanal length (NAL), which was used to calculate the rodent body mass index, or Lee index, using the following equation: Lee index = (body weight [g]^{1/3} / NAL [cm]) x 100 [21]. Retroperitoneal (RET), epididymal (EPI) and brown (BAT) adipose tissues were removed and weighed to estimate corporal adipose tissue.

Analysis of metabolic and biometric parameters

During the 14 days of tumour growth, body weight and food intake of all the groups were evaluated. After 14 days of tumour cell inoculation and 12 h of fasting, animals from all groups were weighed and subsequently euthanized by decapitation, and blood samples were collected and centrifuged to obtain the plasma for further analysis. Glucose concentration was determined by the glucose oxidase method using a commercial kit (Gold Analisa®, Belo Horizonte, MG, Brazil), and insulin levels were evaluated using a radioimmunoassay (RIA) with a gamma counter (2470 Wizard² Automatic Gamma Counter; PerkinElmer). The intra- and interassay coefficients of variation were 12.2% and 9.8%, respectively, for insulin. The detection limit for the insulin level was 0.006 ng/ml [22].

Evaluation of cachexia

The tumour mass was carefully dissected and weighed to calculate the percentage of body weight loss (BWL) using the following equation:

$$\text{BWL (\%)} = 100 \times (\text{bwi} - \text{bwf} + \text{tm} + \text{gbw}) / (\text{bwi} + \text{gbw}),$$

where bwi: initial body weight (g), bwf: final body weight (g) of rats with tumours; tm: tumour mass (g); and bwg: body weight gain (g) of rats without tumours during the 14 days of the experiment. The rats were considered cachectic when the BWL was higher than 10% [19].

Immunohistochemical assay

Tumour samples were fixed in 4% buffered paraformaldehyde, embedded in histological paraffin and sectioned (5 µm) in non-serial cuts. The tissue sections were deparaffinized, rehydrated, blocked against endogenous peroxidase with a 3% hydrogen peroxide solution ([v/v]:H₂O₂ in methanol; 3 ml/97 ml) for 15 min, washed in 0.01 mol l⁻¹ phosphate-buffered saline (PBS, pH 7.4) and incubated with 10% non-immune goat serum blocking solution for 10 min. The sections were then incubated with a monoclonal primary antibody against proliferative nuclear cell antigen (PCNA) at a dilution of 1:100 (Zymed®, San Francisco, CA, USA) for 60 min. After the sections were washed (0.01 mol l⁻¹ PBS), they were incubated with biotinylated secondary antibody for 10 min, washed, incubated with diaminobenzidine chromogenic solution (DAB) for 15 min, washed and counterstained with haematoxylin. Analyses of PCNA-positive cells (brown nuclei) were performed using 40 digital images (x400 magnification) from each animal (n=5 animals/group), for a total of 200 images per group. The quantitative analyses were performed by calculating the percentage of PCNA-positive cells over the total cell number for each digital image. Cell counts were performed manually using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Data are given as the mean ± SEM and were subjected to variance analysis (one-way ANOVA) followed by Bonferroni's test. A p value of less than 0.05 was considered statistically significant. Tests were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, CA, USA).

Results

Effects of MSG and Gli on biometric parameters

Table 1 shows that neonatal MSG treatment caused a 30.8% reduction in bw and a 15.8% reduction in body length compared to control rats ($p < 0.0001$). However, chronic Gli treatment was not able to change these parameters in either control or MSG animals. MSG treatment induced a 12% increase in the Lee index ($p < 0.0001$), while Gli treatment provoked a decrease of 7% in the MSG rats ($p < 0.0001$) without changes in control animals. Chronic Gli treatment and tumour grafts did not alter the final food intake between the groups. The RET, EPI and BAT weights were higher in the MSG group compared to the control group ($p < 0.0001$), whereas Gli treatment reduced the EPI and BAT fat pads ($p < 0.0001$).

Effects of MSG and Gli on progression of cachexia

The rate of cachexia was 19.5% and 13.6% in rats from the control and MSG groups, respectively. However, chronic Gli treatment decreased this parameter in the animals of groups C-Gli (16.0%) and MSG-Gli (12.1%), as shown in Figure 2 ($p < 0.0001$).

Effects of MSG and Gli on glucose homeostasis

Figure 3 shows the fasting glycaemia and insulinaemia for all experimental groups. There was no difference in glycaemia among the control groups that were treated or untreated with Gli and inoculated or not with the Walker-256 tumour cells (Fig. 3A). However, the tumour-bearing MSG rats showed a decrease of 15% in the plasma glucose concentration compared to the MSG rats that were not grafted with tumour cells, as shown in Figure 3B ($p < 0.0001$). The fasting glycaemia was reduced in animals of both tumour-grafted MSG groups, regardless of the Gli treatment.

Table 1. Biometric parameters of the Control and MSG animals treated with or without Gli and without tumour cells. The data represent the mean $\pm$ SEM obtained from 12 rats of each experimental group. Values within a row with unlike superscript letters were significantly different based on one-way ANOVA ($p < 0.0001$). ^a control; ^b control treated with Gli; ^c MSG; and ^d MSG treated with Gli. (Ret) retroperitoneal, (Epi) epididymal and (Bat) brown adipose tissues

Biometric Parameters	Control ^a	Control-Gli ^b	MSG ^c	MSG-Gli ^d
Final Body Weight (g)	353.2 $\pm$ 7.4 ^{cd}	342.4 $\pm$ 10.8 ^{cd}	233.7 $\pm$ 16.6 ^{ab}	270.7 $\pm$ 8.5 ^{ab}
Body Length (cm)	21.5 $\pm$ 0.3 ^{cd}	21.3 $\pm$ 0.4 ^{cd}	18.1 $\pm$ 0.5 ^{ab}	18.5 $\pm$ 0.2 ^{ab}
Lee Index	33.5 $\pm$ 0.5 ^c	33.3 $\pm$ 0.8 ^c	37.4 $\pm$ 1.0 ^{abd}	34.0 $\pm$ 0.4 ^c
AUC Food Intake (g/100 g)	82.0 $\pm$ 3.87	74.9 $\pm$ 8.30	81.9 $\pm$ 9.40	72.4 $\pm$ 3.06
Ret Fat Pad (g/100 g)	1.03 $\pm$ 0.09 ^{cd}	1.08 $\pm$ 0.09 ^{cd}	1.65 $\pm$ 0.13 ^{ab}	1.67 $\pm$ 0.08 ^{ab}
Epi Fat Pad (g/100 g)	0.92 $\pm$ 0.05 ^{cd}	1.07 $\pm$ 0.07 ^{cd}	2.13 $\pm$ 0.06 ^{abd}	1.66 $\pm$ 0.08 ^{abc}
Bat Fat Pad (g/100g)	0.02 $\pm$ 0.002 ^{cd}	0.03 $\pm$ 0.002 ^{cd}	0.10 $\pm$ 0.007 ^{abd}	0.06 $\pm$ 0.003 ^{abc}

Fig. 2. Effect of Gli treatment on the percentage of cachexia. The bars represent the mean $\pm$ SEM of the percentage of rats with a bw loss of more than 10%. The letters over the bars represent statistically significant differences using one-way ANOVA ($p < 0.0001$) among the groups. ^a control; ^b control treated with Gli; ^c MSG; and ^d MSG treated with Gli.

Fig. 3. Effect of chronic Gli treatment on the fasting glycaemia and insulinaemia of the animals. The bars represent the mean $\pm$ SEM of the fasting plasma glucose concentration in control rats (A) and MSG rats (B), healthy or with tumours, and fasting plasma insulin levels from control rats (C) and MSG rats (D), healthy or with tumours. The letters over the bars represent statistically significant differences using one-way ANOVA ($p < 0.0001$) among the groups. ^a control; ^b control treated with Gli; ^c MSG; and ^d MSG treated with Gli.

Fig. 4. Representative photomicrographs (A – F, $\times 400$ magnification) show Walker-256 tumour sections immunostained with an anti-PCNA antibody. (A) control group, (B) Gli-treated control group, (C) MSG group and (D) Gli-treated MSG group. Arrows in the images show nuclei immunolabelled for PCNA in the tumour (brown). (E) Tumour growth. (F) Quantitative analysis of the PCNA-positive cells. Data are presented as the mean $\pm$ SEM, $p < 0.05$. The letters over the bars represent statistically significant differences using one-way ANOVA ($p < 0.05$) among the groups.

Fasting insulinaemia was increased 3-fold in the MSG animals compared with the control animals (Fig. 3C and 3D; $p < 0.0001$). While chronic Gli treatment did not change fasting insulinaemia in the control animals, the treatment did cause a 60% decrease in the MSG rats ($p < 0.0001$). Tumour transplantation decreased the fasting plasma insulin levels in both the control ($p < 0.0001$, Fig. 3C) and MSG groups ($p < 0.0001$, Fig. 3D); however, there was a 4-fold reduction in the control rats and an 8-fold decrease in the MSG rats ($p < 0.0001$). Tumour-grafted control and MSG animals treated with Gli showed decreases of 8- and 10-fold in fasting insulinaemia, respectively, as also shown in Figure 3C and 3D ($p < 0.0001$).

Effects of MSG and Gli on growth and histological analysis of Walker-256 tumours

The tumour growth was attenuated by approximately 27% in Gli-treated animals from both groups, as shown in Figure 4E ($p < 0.0001$).

The histological examination of the tumour tissue revealed a large reduction in PCNA-positive cells in the Gli-treated control group, MSG group and Gli-treated MSG group compared to the control group (Fig. 4A - D). Furthermore, the quantitative morphometrical analysis showed a significant reduction in PCNA-positive cells (Fig. 4F) in MSG rats and in both groups treated with Gli. As seen in Figure 4F, the number of immunolabelled PCNA-positive cells was reduced by 65% in the control Gli-treated group and 44% in the MSG group compared to the control group ($p < 0.0001$ and $p < 0.001$, respectively). In addition, Gli promoted a 49% reduction of PCNA-positive cells in the Gli-treated MSG group in relation to the MSG group (Fig. 4E; $p < 0.05$).

Discussion

The prediabetic obese rats displayed moderate hyperglycaemia and severe hyperinsulinaemia, similar to previous studies [23, 24]. In addition, chronic Gli treatment improved the metabolism of prediabetic obese MSG-rats. For example, Gli treatment normalized the tissue insulin sensitivity and glucose tolerance, suppressed the fasting hyperinsulinaemia and reduced adipose tissue accretion, effects that were previously reported by other studies with antidiabetic drugs [25, 26]. Gli treatment did not change the biometric and metabolic parameters of control rats.

It has been shown that Gli was able to inhibit growth in human and rodents tumour cell culture [27]. In the current study, Gli previous treatment was also inhibited tumour growth in rats bearing Walker-256 cancer cells. The literature shows that Gli inhibits cell growth by inducing G0/G1 arrest in the human breast cancer, cell line mda-mb-231 [28-30]. Indeed, for the first time, our findings show that chronic treatment with Gli is capable of protecting both control and prediabetic obese MSG-rats against tumour growth, even when Gli treatment was stopped one day before tumour cell grafts. Our results suggest that Gli has an antiproliferative effect. Using another antidiabetic drug, metformin, our group recently demonstrated that Walker-256 tumour growth was inhibited [26]. In the present study, the rats did not receive Gli during tumour growth. Tumour cells were grafted 24 h after the last dose of Gli treatment, which implies that no traces of the antihyperglycaemic drug could be found in the blood stream of the rats. According to other studies, the Gli plasma elimination half-life, after oral administration of multiple dosages, is approximately 15 h [31], indicating that Gli could protect the organism against tumour growth.

Studies have shown that rats grafted with Walker-256 tumours display low levels of plasma insulin and decreased glucose-induced insulin secretion in pancreatic β cells [32, 33]. In the present work, Walker-256 tumour growth decreased insulin levels 4-fold in control rats and 8-fold in prediabetic obese rats, which is in agreement with previous reports [26, 32, 33]. Tumour requires lot of energy consuming very rapidly blood glucose. Like other cancer cells Walker-256 need anaerobic sources and it induces insulin resistance and at same time inhibits insulin secretion to guarantee that mostly of glucose available will be feeding tumour. Cachexia observed in rats bearing Walker-256 tumour is metabolic expression of low insulin blood levels [33]. As can be observed our results show that rats bearing tumour exhibit very low insulin blood concentration, even glibenclamide treated ones. It is surprising that glucose levels are unchanged in those conditions, it means peripheral tissues from rats grafted with Walker-256 tumour present reduction of glucose uptake regarding, ungrafted ones [26, 32, 33]. Regarding those observation, including insulin tissues resistance of tumour-bearing rats, it was expected hyperglycaemia; however, glucose levels are maintained similar to rat that were not transplanted with tumoral cells. It can be suggested that exceeding glucose is used by the tumour cells.

The magnitude of tumour growth was the same in both prediabetic obese MSG-rats and the control group, which suggests that the insulin decrease did not alter the tumour

aggressiveness. We also cannot attribute the anti-tumour effects of Gli to the decrease in insulin, considering that the magnitude of growth inhibition was the same in both groups.

Our results show that the population of PCNA-positive cells in the Walker-256 tumour cells was greatly reduced in both the control and MSG groups treated with Gli compared with the control group, suggesting that Gli exerts a direct inhibitory effect on tumour growth. One explanation for this direct effect is the blocking of K_{ATP} channels [7, 34]. However, the number of PCNA-positive cells was also markedly reduced in the tumours of prediabetic MSG-obese rats not treated with Gli.

It has been shown that administration of MSG to newborn rodents produces hypothalamic lesions in the arcuate nucleus causing obesity, hyperinsulinaemia, insulin resistance, low sympathetic activity, high parasympathetic tone, increased DNA synthesis in some organs and the stimulation of cell proliferation. [35, 36]. However, studies have reported that the turnover of some cells in MSG-treated rats was decelerated [35]. These findings allow us to suggest that the cell turnover of the Walker-256 tumour may have decreased in grafted rats with hypothalamic obesity.

Several studies have demonstrated the effects of Gli on tumour growth arrest in different cancer types [27, 28, 37]. It has been shown that different subtypes of potassium channels are involved in normal and malignant cell proliferation [38]. Moreover, previous studies showed that Gli has a wide range of anti-inflammatory effects [39, 40]. It has been shown that compounds with anti-inflammatory action also present anticancer effect [41].

Furthermore, the sulphonylurea receptor (SUR) is a regulatory subunit of ATP-sensitive potassium channels and belongs to the ATP-binding cassette (ABC) protein superfamily, a group of transmembrane proteins that utilize ATP to transport substrates, such as metabolic products, lipids, sterols, and drugs, across extra- and intracellular membranes. Thus, Gli can bind to SUR and block the activity of numerous ABC transporters, including the multidrug transporter involved in anticancer drug resistance, which may be a promising target for anticancer therapy [7, 28].

Gli is a low-cost drug widely used for the treatment of type 2 diabetes, and to the best of our knowledge, there are no published studies reporting the toxicity of Gli when it is used at therapeutic concentrations. Recently, our group published results that indicate no cytotoxicity and genotoxicity effect of metformin and glibenclamide in eukaryotic cells. These results, at least, suggest that these antidiabetic drugs can be used in chemotherapy to cancer tumours, without risk to aggravate the disease, such as secondary cancer [42, 43].

The results showed in the present study are consistent to conclude that previous treatment with Gli, prior to the tumour cell grafts and/or their growth, induced an imprinted effect that attenuates the cancer growth independent of the positive effects on metabolism, indicating a direct effect of Gli on the Walker-256 tumour growth. Independent of mechanisms of action, Gli might be faced as potential prophylactic anticancer therapy.

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Disclosure Statement

The authors declare no conflicts of interest associated with this manuscript.

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