Effect of glibenclamide on N-nitroso-N-methylurea induced mammary tumors in diabetic and non-diabetic rats.

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ABSTRACT

The objective of this study was to evaluate the antitumor effect of glibenclamide (Gli) alone or in combination with tamoxifen (Tam) on experimental mammary tumors induced by N-Nitroso-N-methylurea (NMU) in non-diabetic and diabetic rats. For experimental diabetes induction, Sprague-Dawley rats were injected with streptozotocin (STZ) on the second day of life. For experimental mammary tumor induction, non-diabetic and diabetic rats were intraperitoneally injected with NMU at 50, 80 and 110 days of life. Non-diabetic and diabetic rats bearing mammary tumors were treated with 0.06 mg/day of Gli orally, Tam 1 mg/kg/day sc, or with the combined treatment (Gli+Tam).

After 20 days of treatment, different responses were observed. In non-diabetic rats, 64% of tumors were responsive to Gli treatment: they regressed or remained stable, whilst 57% of tumors under treatment with Tam exhibited a response. Results of the combined Gli+Tam treatment indicated that all tumors were responsive: 58% regressed and 42% remained stable. Diabetic rats receiving Gli treatment did not show response to this treatment, whilst 65% of the tumors of Tam treated diabetic rats showed regression. Histopathologic observation indicated an important intratumor secretion in all tumors of Gli, Tam or Gli+Tam treated rats. No secondary toxic effect was observed after treatment at any assayed doses.

In conclusion, the present data demonstrate the *in vivo* antitumor action of Gli treatment on the experimental mammary tumors employed, indicating that Gli exerted a direct effect on tumor cells in non-diabetic rats. The combined Gli+Tam treatment potentiates the antitumor effect of each drug alone. Future research will examine the molecular aspects of these findings.

KEY WORDS: glibenclamide, mammary tumors, N-nitroso-N-methylurea, rats, streptozotocin, diabetes.

ABREVIATIONS: Gli, glibenclamide; GTT, glucose tolerance test; I, insulin; IGFs, insulin-like growth factors; IGFBPs, insulin-like growth factor binding proteins; IGF-IR, insulin-like growth factor-I receptor; IGF-IIR, insulin-like growth factor-II receptor; NMU, N-nitroso-N-methylurea; STZ, streptozotocin; TAM, tamoxifen.

INTRODUCTION

It is known that breast cancer cell growth is dependent on several hormones such as estrogens, prolactin, progesterone, glucocorticoids, insulin, and growth factors. In earlier publications, Heuson and Legros and others researchers reported the importance of insulin in DNA synthesis of the hormone-dependent mammary carcinomas induced by 7,12-dimethylbenz(a)antracene (DMBA) and classified mammary tumors as dependent or independent to insulin for their growth (1). These pioneering experiments attributed a crucial role to insulin in the development of mammary tumors. Currently, it is undoubted that insulin, insulin-like growth factors type I (IGF-I) and type II (IGF-II), their corresponding receptors (IGF-IR and IGF-IIR) and binding proteins (IGFBPs) participate in the development of normal and neoplasic mammary tissue (2,3). Furthermore, clinical observations indicate that breast cancer patients with non-insulin-dependent diabetes mellitus (NIDDM) showed a better evolution and a longer survival time when were treated with the combination of the antiestrogen tamoxifen (Tam) plus hypoglycemic drugs (4-6). The second generation of hypoglycemic sulfonylureas includes glibenclamide (Gli). These drugs cause hypoglycemia by stimulation of insulin release from beta pancreatic cells, interacting with surface receptors of those cells and regulating the levels of glucose in type II diabetes (NIDDM) (7). Many sulfonylureas showed experimental evidence of antitumor activity (8-10) and some of these compounds advanced to phase I and II clinical studies, sometimes showing adverse effects (8,9,11).

In our laboratory we characterized an experimental mammary tumor induced by three ip injections of the carcinogen N-nitroso-N-methylurea (NMU) at 50, 80 and 110 days of life in normal (12,13) and diabetic rats (14,15). The objective of the present study was to evaluate the *in vivo* antitumor action of glibenclamide alone or in combination with Tam on induced mammary tumors in normal and diabetic rats. Therefore, we determined: a) glucose tolerance curves; b) blood insulin levels; c) plasma IGF-I levels; d) pancreatic and tumor histopathology; and e) mammary tumor growth.

MATERIALS AND METHODS

Reagents

NMU, STZ and citrate buffer, pH 4.8, were purchased from Sigma Chemical Co., (St. Louis, Missouri, USA). A blood glucose micromethod (Glucometer Gx, Ames S.A., Argentina) was used to determine glucose levels. The reagents for IGF-I determination by radioimmunoassay (RIA) were purchased from Diagnostic Systems Laboratories Inc. (Texas, USA) and the kit for insulin RIA was from International CIS (France). Glibenclamide was a gift from Raffo Laboratories SA, (Buenos Aires, Argentina). Tamoxifen was a gift from Gador Laboratories SA (Buenos Aires, Argentina).

Animals. Female Sprague-Dawley rats were randomly separated into batches and housed in stainless steel cages, with water and food *ad libitum,* temperature of 22-23°C, humidity around 56% and a 12 h light-dark cycle. In all the cases animals were kept at conditions recommended by the Guide for the Care and Use of Laboratory Animals, National Research Council, USA, 1996.

Diabetes induction. On the second day of life, one-half of the rats were injected sc with 90 mg/kg of STZ in 0.09 M citrate buffer, pH 4.8, as previously described (14,15).

Tumor induction. Rats, both non-diabetic and diabetic, were injected with three doses of NMU at 50, 80 and 110 days of life as previously described (12). The developed tumors were measured with a caliper three days a week to control their size and growth.

Glucose Tolerance Test (GTT). At day 45, rats were subjected to a glucose tolerance test (GTT); after basal blood glucose determination, 2 mg/kg of glucose was injected ip and glucose circulating levels were determined at 30, 60 and 120 min. Animals showing abnormal GTT were selected for diabetic groups.

GTT in rats under Gli treatment. ***Determination of the effective Gli dose.*** Doses of Gli capable of normalizing the GTT in diabetic rats were evaluated as we described above. The Gli concentration range was extrapolated from doses used in human diabetic patients (16). Four groups of 130 day-old diabetic rats were randomly separated (n = 5). Three groups were treated with Gli (suspended in 50% glycerin) at 0.02, 0.04, or 0.06 mg/day (STZ+Gli 0.02, STZ+Gli 0.04, or STZ+Gli 0.06 mg/day groups, respectively) for twenty days; the fourth group (STZ) was treated with vehicle. Also, five non-diabetic rats without treatment (n = 5) were employed as a control group (Control).

Effects of Gli, Tam and Gli+Tam treatment on tumor growth. Studies of the effects of 0.06 mg/day of Gli on tumor growth in diabetic and non-diabetic rats bearing NMU-induced mammary tumors were performed. Results were compared to those obtained in rats receiving 1 mg/kg/day of treatment with Tam (13,17), and the effects of combined Gli+Tam treatment were analyzed; Table 1 summarizes the treatment schedule. The parameters recorded were: *Relative tumor size*, calculated as the relationship between tumor size at different times versus the beginning of treatments; *Percentage of tumor regression:* each tumor was classified as regressing, growing, or stable according to the size in relation to the pre-treatment value. Tumors were considered regressing when their diameters were 80% lower than the diameter at the beginning of the treatment. Tumor size was determined by measurement of two

perpendicular diameters three days a week (12). *Tumor histopathology* was determined according to the classification of Russo et al. (18).

Insulin and IGF-I circulating levels. Blood samples from non-diabetic and diabetic rats, treated or non-treated with 0.06 mg/day of Gli or 1 mg/kg/day of Tam, were obtained from the tail of each rat pre- and post-treatment. Blood samples were centrifuged after collection and plasma aliquots were frozen at -20 ^{o}C until assayed. Insulin and total IGF-I, tIGF-I, (soluble IGF-I was separated from binding proteins using acid-ethanol extraction) were measured by RIA, performed in duplicate. Bound radioactivity was counted in a gamma scintillation spectrometer.

Histopathological studies. Histological observations of the pancreas and mammary tumors were performed. Specimens were fixed in 10% formaldehyde and embedded in paraffin. Slides were stained with haematoxylin-eosin (HE) for microscopic observation. Specimens of pancreas were also fixed in Bouin's solution and embedded in paraffin to evaluate the number and extension of Langerhans islets. Serial sections of the whole pancreas of each rat were examined**.** Gomori trichromic stained slides were microscopically examined to discriminate between alpha and beta pancreatic cells (19).

Apoptosis determination. Apoptotic cells in paraffin embedded tissues were detected in situ by peroxidase staining using the Apoptag®PLUS Peroxidase In Situ Detection Kit S701 (Chemicon International, CA, USA). The basis of the Apoptag® technique is to examine apoptosis via DNA fragmentation by the TUNEL assay.

Statistical analyses. In each figure and table the respective statistic test used is indicated (20).

RESULTS

Glucose Tolerance Test. Figure 1 describes the results obtained for rats lacking tumors. After glucose injection, only the STZ+Gli 0.06 mg/day treated rats had a recovery of glucose

levels to values similar of those of non-diabetic animals. The STZ+Gli 0.02 and STZ+Gli 0.04 groups did not change significantly their GTT versus diabetic rats (i.e., the STZ group) (Fig. 1a). Non-diabetic animals treated with 0.02, 0.04, or 0.06 mg/day of Gli did not change the GTT significantly versus the Control group (data not shown).

Non-diabetic and diabetic rats bearing mammary NMU induced tumors were treated with 0.06 mg/day Gli as described above. Results indicated that NMU injections did not affect GTT. Non-diabetic rats treated with 0.06 mg/day of Gli (NMU+Gli group) showed similar GTT to the NMU group. Also, diabetic rats bearing mammary tumors (STZ+NMU+Gli group) showed a similar GTT to non-diabetic ones (NMU) (Fig. 1b).

Insulin levels. To determine if the 0.06 mg/day level of Gli elevates the circulating levels of insulin, fast insulin values were determined pre- and post-treatment in rats bearing mammary tumors (Table 2). No significant differences in insulin circulating levels between NMU+Gli vs NMU rats were seen. Diabetic rats (STZ+NMU group) showed a non-significant lower insulin value than the NMU treated rats. However, in diabetic rats receiving 0.06 mg/day of Gli (the STZ+NMU+Gli group), the insulin levels were significantly increased vs STZ+NMU values ($P<0.05$).

tIGF-I circulating levels. Table 2 summarizes results obtained. Tam-treatment decreased significantly the mean circulating values of tIGF-I in all the cases.

Histopathological studies

Pancreas. The pancreas from non-diabetic rats always showed a normal structure with a normal number and size of islets. Alfa cells are located in the periphery of the islets and beta cells are in the center of the islets (Fig. 2a), according to reported observations (21). In contrast, all of the diabetic rats showed a pancreas with a low number of islets that were smaller and with scarce beta cells when were compared with normal ones (Fig. 2b). Pancreases from non-diabetic and Gli treated rats were of normal size with hypersecreting

alpha cells (Fig. 2c). Pancreases from diabetic rats treated with 0.06 mg/day of Gli, showed smaller and lower numbers of islets than normal pancreases and with scarce beta cells, similarly to that described for diabetic rats. Also, appreciable cytoplasmic depletion in beta cells was observed (Fig. 2d).

Tumors. All tumors from NMU treated rats were malignant adenocarcinomas with a cribriform, comedo or papillary pattern. Tumors of diabetic rats (STZ+NMU) showed predominantly a benign pattern, as we have previously described (15). Figure 3 illustrates the hypersecretion observed in the lumen of the mammary tumors of Gli treated rats (Fig. 3a, 3b and 3c). The appearance of these tumors was similar to that from Tam treated rats (Fig. 3d). Tumors Gli+Tam treated exhibited similar characteristics (data not shown).

Effects of treatments on tumors of non-diabetic rats.

Tumor growth. Table 3 summarizes the evolution of tumor growth. None of the tumors from the NMU group spontaneously regressed. Under Gli treatment (NMU+Gli group), only 36% of the tumors continued growing, whilst under Tam treatment, 43% of the tumors continued growing. The results indicate a very significant response of tumors to both drugs. The percentage of responsive tumors (regressed+stable) with Gli or Tam treatment was similar (64% vs 57%, *P*: NS).

Relative tumor size. After 20 days of Gli treatment (Fig. 4a), tumors that regressed (NMU+Gli(r)) showed only 20% of the mean size at the beginning of the experiment ($P<0.001$). Tumors considered stable (NMU+Gli(s)) were significantly smaller ($P<0.01$) than those that continued growing (NMU+Gli(g)). Tam treated tumors (Fig. 4b) showed clearly two types of response: 24/44 of tumors regressed to 20-50% of the size at the beginning of the experiments (NMU+Tam(r)), whereas the other tumors (19/44) continued growing (NMU+Tam(g)).

No significant differences between final mean sizes of tumors regressing with Tam treatment (NMU+Tam(r)) versus those regressing with Gli treatment (NMU+Gli(r)) were found (Fig. 4b and Fig. 4a).

Effects of treatments on tumors of diabetic rats.

Tumor growth. In diabetic rats no evidence of spontaneous tumor regression was seen (STZ+NMU group, Fig. 4c). When diabetic rats were treated with 0.06 mg/day of Gli (STZ+NMU+Gli), no response was observed (Table 4), although the final mean tumor size was smaller than the tumors without Gli treatment. With Tam treatment 65% of the tumors showed regression.

Relative tumor size. Figure 4c illustrates the mean size of Gli-treated tumors. The tumors from diabetic rats that regressed with Tam treatment (i.e., the STZ+NMU+Tam group) reached 10 to 55% of the size at the beginning of the treatment (Fig 4d).

Effect of combined Gli+Tam treatments in non-diabetic and diabetic rats.

Tumor growth and relative tumor size. When the combination treatments were administered, 100% of the tumors of non-diabetic rats were responsive (Table 3), showing that Gli potentiate the effect of Tam. In the case of diabetic rats (Table 4), the combined treatment (89% of regression) did not show significant differences with Tam alone treatment.

Apoptosis.

The effecs of Gli, Tam and Gli+Tam treatment on apoptosis, mitosis and necrosis in mammary tumors in non-diabetic rats were also studied. Table 5 summarizes the results obtained. Immunohistochemical observations indicate that control tumors did not show apoptotic cells. In contrast, tumors of Gli treated animals showed areas with a large number of apoptotic cells per field and a low number of mitotic epithelial cells. Tam treatments also induced an increase of the number of apoptotic cells in comparison to Control tumors; however, this effect was less important than the observed with the Gli treatment. In addition,

Tam treatments produced a large proportion of necrotic areas in regressed tumors. A very significant increase in the number of apoptotic cells was observed and a lower number of mitosis per field were detected with the combined Gli+Tam treatment (Table 5 and Fig. 5).

Finally, no evidence of toxic side effects were observed during the observation period. Also, studies in progress in our laboratory show that Gli treated rats have a longer survival time than non-treated ones.

DISCUSSION

It is known that the streptozotocin injection at the second day of life of rats provokes the destruction of pancreatic beta cells and the development of a diabetic syndrome (22). However, the survival time and the metabolic conditions of these animals are different to the experiments described in this paper. The first set of *in vivo* experiments showed that only 0.06 mg/day of glibenclamide restored GTT in diabetic rats and did not produce a significant increase in plasma insulin levels in non-diabetic rats. This finding may be explained by the existence of a reciprocal relationship between the rates of secretion of insulin and glucagon in normal pancreatic islets. Insulin and glucose levels may act on alpha cells regulating glucagon release, which in turn, may stimulate somatostatin liberation that decreases the secretion of insulin.

The dose of 0.06 mg/day of glibenclamide was selected to carry out the experiments of Gli action on tumor growth. Results of these experiments indicate that Gli induced the inhibition of growth of mammary tumors in non-diabetic rats without changes in the insulin or IGF-I plasmatic levels.

It is known that the effects of glibenclamide are initiated by its binding to surface receptors, producing a decrease in the conductance of ATP sensitive K^+ channels (K_{ATP}). This effect leads to an alteration in the membrane potential that may block Ca^{+2} influx (7,23). These

potassium channels are a large group of structurally and functionally diverse proteins that have been involved in the proliferation of many types of cells, including both normal and tumor cells (24-27).

K_{ATP} blockers like dequalinium, amiodarone and glibenclamide have been shown dose-dependent growth-suppressive effects on colon cancer cell lines (28). Also, Abdul et al.(29) examined the effects of K+ channel-blockers on cellular proliferation and reported that amiodarone and dequalinium potentiated the growth inhibitory effects of tamoxifen on human breast (MCF-7, MDA-MB-231), colon (Colo320DM, SW1116), and prostate (PC3, MDA-MC-2B) cancer cell lines. Our *in vivo* results are in agreement with these observations yet treatment with the combination of Gli+Tam markedly increased the antiproliferative action of tamoxifen alone. In addition, Zhou et al. (30) documented that glibenclamide could inhibit cell adhesion of hepatocarcinoma cells at high concentrations. Although the antitumor effects of sulfonylureas have been reported, the mechanism of their action is not fully understood. *In vivo* and *in vitro* studies are in progress in our laboratory to investigate the mechanism of action of glibenclamide. We hypothesize that Gli may exert a direct action on the malignant cells of ip experimental NMU mammary tumors, blocking K_{ATP} as described for the proliferation of other cancer cells (31,32). In the presence of Gli, tumor growth may be inhibited and cells arrested in the G_1/G_0 phase of the cell cycle; potassium dependent changes in membrane potential might be a possible cause of this arrest (29,30,33,34). In the experiments described herein, the proportion of tumors that regressed during Gli treatment (36%) was lower than those regressing from treatment with Tam. It is known that Tam decreases circulating levels of IGF-I (38), and that this mechanism contributes to the antitumor action of Tam (36,36). In our experiments, we demonstrate that all of the groups of rats receiving Tam treatment undergo a decrease in IGF-I levels. In contrast, Gli treatment did not cause a decrease in IGF-I circulating levels, indicating that the mechanism of action of Gli

is IGF-I- independent. When Gli+Tam were combined the inhibition of tumor growth may be due to arrest of cells produced by Gli and by the multiple ways of Tam action.

We also analyzed the number of mitotic cells, the number of apoptotic cells per field and the necrotic areas in tumors of non-diabetic rats. We observed that tumors of Gli treated animals showed a higher number of apoptotic cells per field than those treated with Tam. These effects were more pronounced in tumors from animals receiving combined treatment of Gli+Tam, where an increased number of apoptotic cells and a very low mitotic index were observed compared to Gli or Tam treatment alone. These results allow as to infer that glibenclamide potentate the anti-proliferative action of Tam leading to a better antitumor response than that induced by each drug separately. These observations also indicate that growth inhibitory responses to Gli treatment could be mediated by other mechanisms in addition to cell cycle arrest.

In diabetic rats, developed tumors showed a benign pattern; the contribution of insulin in the maintenance of mammary tumors induced in rats has been established earlier by Henson and Legros (1). In early experiments, Berger et al. (37) showed that 7,-12-dimethylbenzanthracene-induced mammary tumors in Sprague-Dawley rats decreased the mean tumor volume when rats were treated with diazoxide. In this experimental model, a significant decrease in serum insulin levels was observed (37). Under our experimental conditions, no significant changes in insulin levels were found using Gli, Tam, or the combined treatment. In diabetic animals, no response to Gli treatment was detected. The different histological patterns observed in these tumors, in concordance with our previous reports (15), may be the reason for the absence of response to the treatments. These tumors show a preponderant proliferation of stromal cells. One possible hypothesis may be that in these tumors a different K^+ channel may be expressed, or a different mechanism of action of glibenclamide is operative, like that Gli is not capable to excert its anti-proliferative action.

More studies will be necessary to clarify the mechanism of action of sulfonylureas on tumor cell growth in view of the possible employment of these drugs as antitumor agents alone or in combination with tamoxifen.

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LEGENDS

Figure 1: Glucose Tolerance Test. 1a: Blood glucose levels (mg/dl) in rats vs Time (min). Results are expressed as means ± SD (n = 5 each group). Error bars shows 95% confidence intervals. Two way ANOVA and Bonferroni post-test. (●) Control; (□) STZ; (▲) STZ+Gli 0.02 mg/day; (▼) STZ+Gli 0.04 mg/day; (◇) STZ+Gli 0.06 mg/day. *P*<0.001, STZ vs Control; *P*:NS, STZ vs STZ+Gli 0.02 and vs STZ+Gli 0.04; *P*<0.0001, STZ vs STZ+Gli 0.06; *P*:NS, STZ+Gli 0.06 vs Control. **1b:** Blood glucose levels in non-diabetic and diabetic rats bearing mammary NMU-induced tumours (mg/dL). (♦) STZ+NMU; (◇) STZ+NMU+Gli; (■) NMU; (□) NMU+Gli. *P*:NS, NMU+Gli vs NMU; *P*<0.001, STZ+NMU+Gli vs STZ+NMU; *P*:NS, STZ+NMU+Gli vs NMU.

Figure 2. Histopathology of pancreas. 2a: Normal alpha cells in the border of a pancreatic islet, as usually arranged in rats pancreatic islets (Gomory 400X). **2b:** Very reduced pancreatic islets with scarce beta cells and a group of alpha cells (Gomory 400X). **2c:** Histological appearance of a normal rat pancreas treated with Gli. Langerhans islets were normal in number and size; proliferation of alpha cells in the periphery of the islets was observed (upper, HE 100X ; down, hyperplasic alpha cells with scars cytoplasm, HE 400X). **2d:** Histological appearance of the pancreas from STZ diabetic rat treated with Gli. Scarce and small Langerhans islets were observed. Some of them were very reduced, showed loss of beta cells and persistence of scarce alpha cells located in the periphery of the islet, as usual in rats (Left, H.E. 100X; right H.E. 400X).

Figure 3. Histopathology of regressing mammary tumors. 3a: Regressing tumor following Gli treatment of non-diabetic rats, with intratumoral necrosis (H.E. 20X) and **3b** with hypersecretion (H.E. 100X). **3c:** Tumor regressing following Gli treatment of diabetic rats (H.E. 100X). **3d:** Tumor regressing following Tam treatment of a diabetic rat, with evidence of glandular hypersecretion (H.E. 100X).

Figure 4. Tumor growth rate in non-diabetic and diabetic rats under Gli or Tam treatment. Observation period: 20 days. **4a:** Results of Gli treatment in non-diabetic rats (NMU+Gli) group. (■) tumors of NMU non-treated rats; (□) Gli treated stable tumors; (Δ) Gli treated growing tumors; (▼) Gli treated regressing tumors. *P*<0.0001 (▼) vs (■). *P*<0.01 (□) vs (■). *P*:NS (Δ) vs (■). **4b:** Results of Tam treatment in non-diabetic rats (NMU+Tam) group. (■) tumors of NMU non-treated rats; (O) Tam treated growing tumors; (●) Tam treated regressing tumors. *P*<0.0001, (●) vs (■). *P*:NS, (○) vs (■). **4c:** Results of Gli treatment in diabetic rats (STZ+NMU+Gli group). (□) tumors of diabetic non-treated rats. (●) Gli treated stable tumors; (▲) Gli treated growing tumors. *P*<0.01, (▲) vs (□). *P*:NS, (●) vs (□). **4d:** Results of Tam treatment in diabetic rats (STZ+NMU+Tam group). (□) tumors of diabetic non-treated rats; (○) Tam treated growing tumors; (●) Tam treated regressing tumors. *P*< 0.0001, (●) vs (□). *P:*NS, (○) vs (□). **4e:** Results of Gli+Tam-treatment in non-diabetic rats (NMU+Gli+Tam group). (■) tumors of NMU non-treated rats; (◇) Gli+Tam treated stable tumors; (▽) Gli+Tam treated regressing tumors. *P*< 0.0001, (▽) vs (■). *P*< 0.0001, (▽) vs (◇). *P*< 0.0001, (◇) vs (■). **4f:** Results of Gli+Tam treatment in diabetic rats (STZ+NMU+Gli+Tam group). (□) tumors of diabetic non-treated rats; (▼) Gli+Tam treated regressing tumors; (●) Gli+Tam-treated stable tumors. *P*<0.0001, (▼) vs (□). *P:*NS, (●) vs (□).

In all the studies, two-way ANOVA and Tukey post-test

Figure 5. Apoptosis. 5a: Cribriform rat mammary adenocarcinoma totally negative staining for apoptotic nuclei in a control rat (Apoptag 100X). **5b:** Masive positive staining for apoptotic nuclei in a tumor treated with Gli (Apoptag 100X), **5c** and **5d** Larger magnification of figure 5b (Apoptag 400X). **5e:** Positive staining for apoptotic nuclei in local sector involving progressively necrotic area; tumor of rat treated with Tam (Apoptag 100X). **5f:**

Occasional staining for apoptotic nuclei. Tumor of rats treated with Gli plus Tam (Apoptag 400X).

Figure 1.

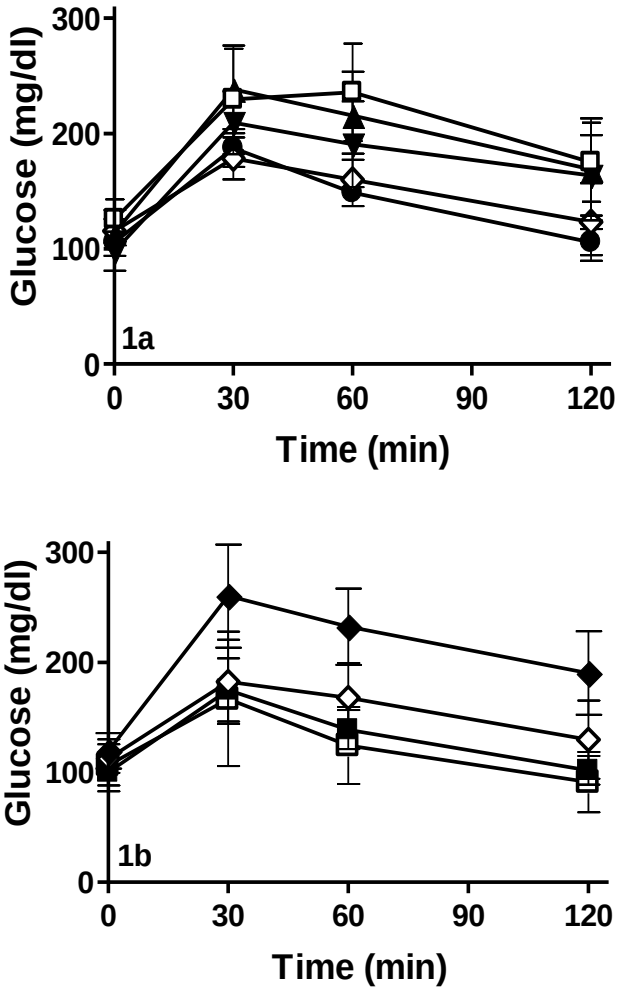
Glucose (mg/dl)
300
200
100
0
1a
0
30
60
90
120
Time (min)
Glucose (mg/dl)
300
200
100
0
1b
0
30
60
90
120
Time (min)

Figure 2.

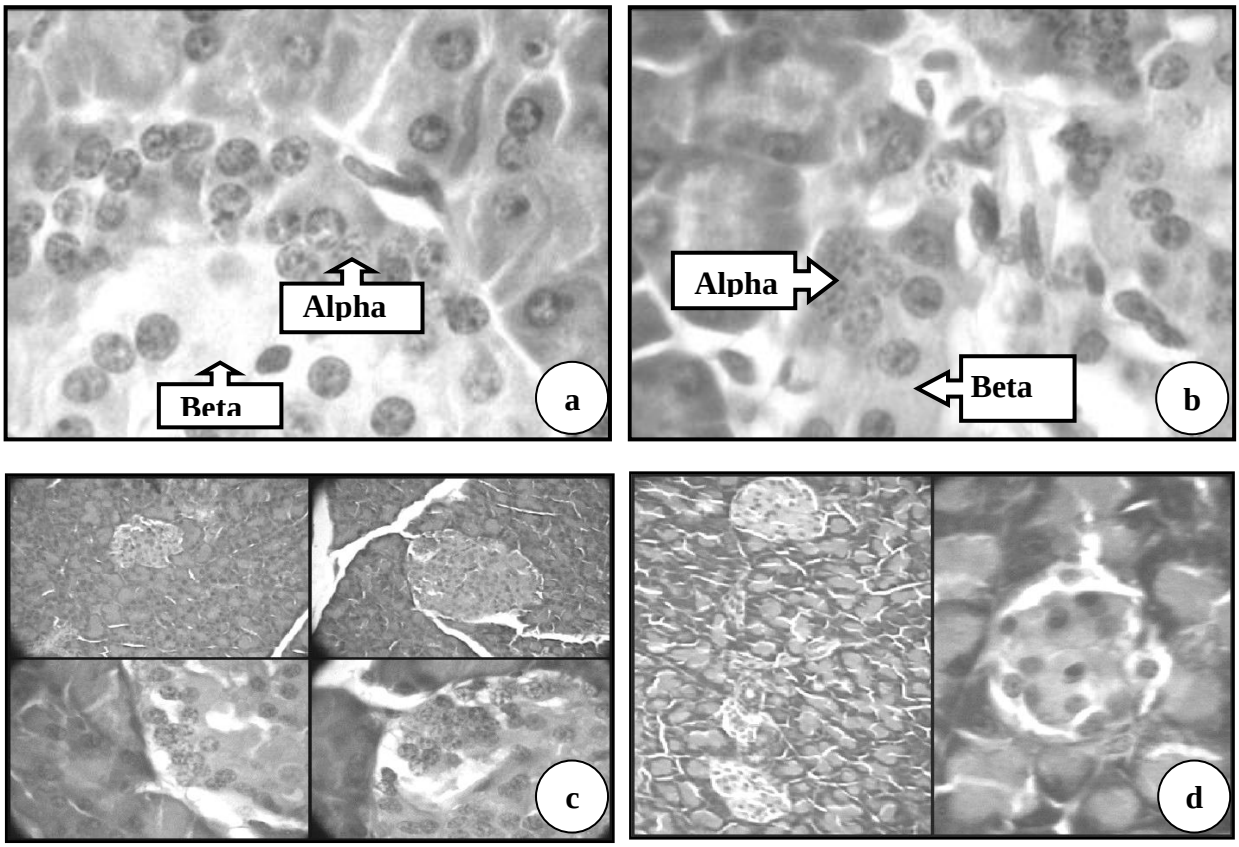

Figure 3

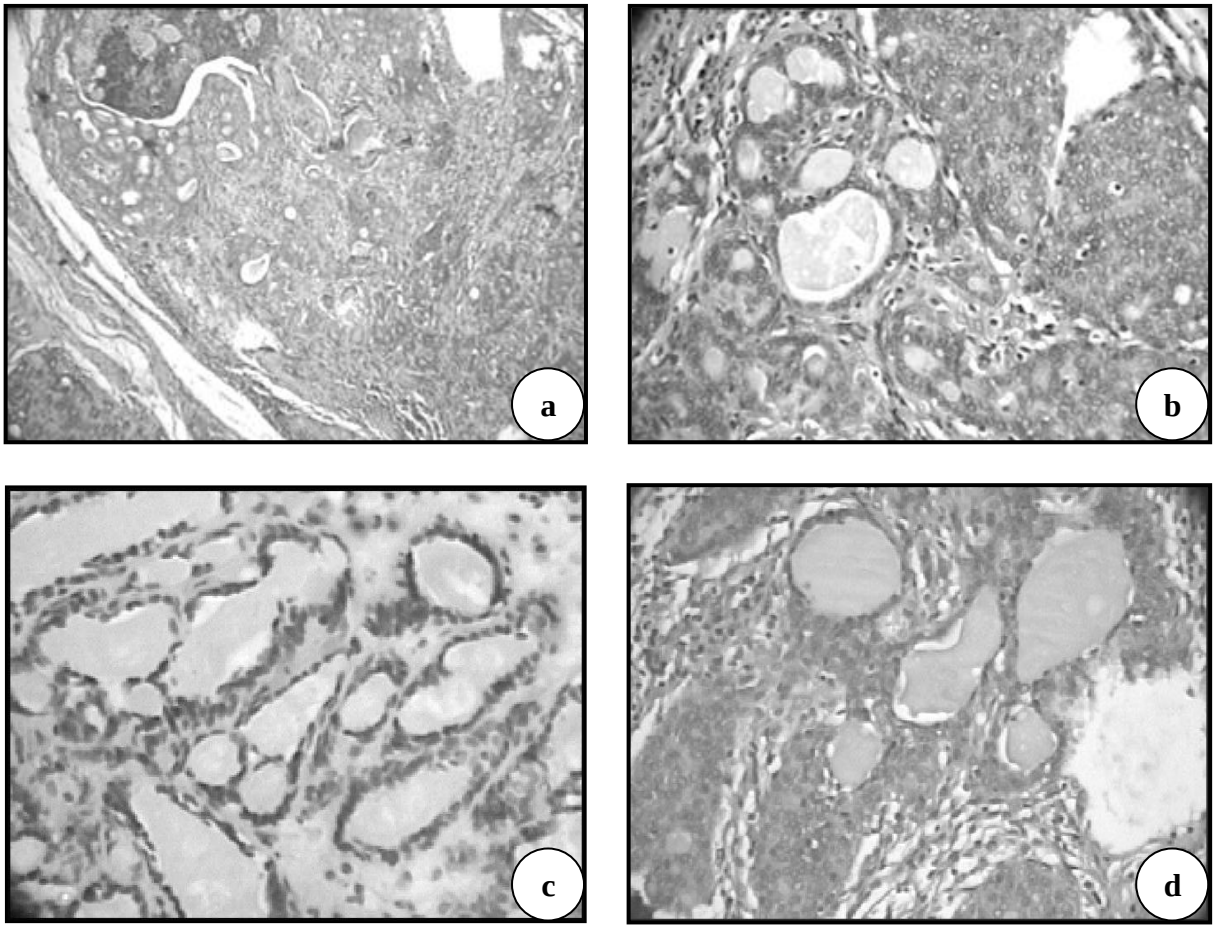

Figure 4.

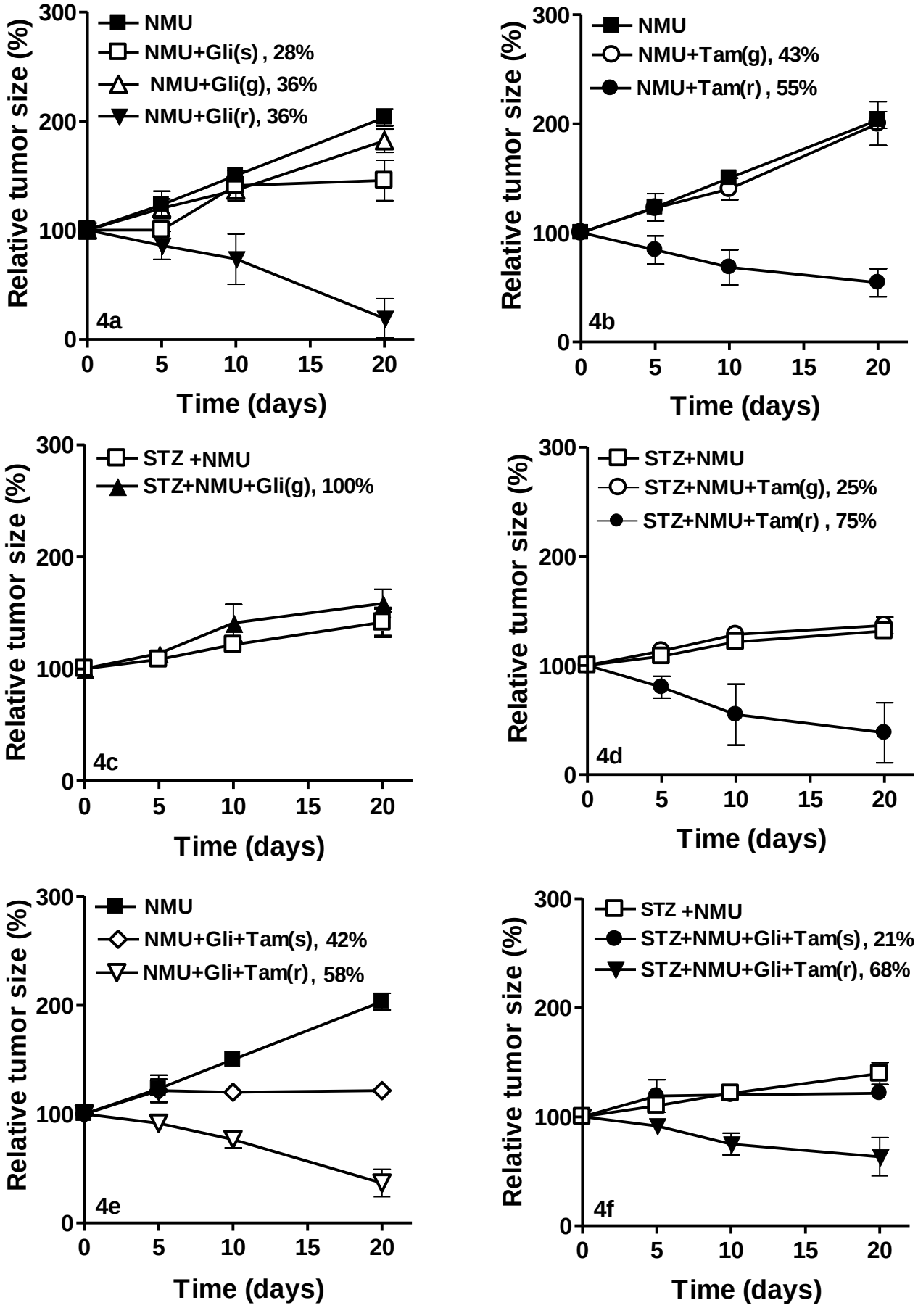
NMU
NMU+Gli(s) , 28%
NMU+Gli(g), 36%
NMU+Gli(r), 36%
Relative tumor size (%)
Time (days)
4a
NMU
NMU+Tam(g), 43%
NMU+Tam(r) , 55%
Relative tumor size (%)
Time (days)
4b
STZ +NMU
STZ+NMU+Gli(g), 100%
Relative tumor size (%)
Time (days)
4c
STZ+NMU
STZ+NMU+Tam(g), 25%
STZ+NMU+Tam(r) , 75%
Relative tumor size (%)
Time (days)
4d
NMU
NMU+Gli+Tam(s), 42%
NMU+Gli+Tam(r), 58%
Relative tumor size (%)
Time (days)
4e
STZ +NMU
STZ+NMU+Gli+Tam(s), 21%
STZ+NMU+Gli+Tam(r), 68%
Relative tumor size (%)
Time (days)
4f

Figure 5

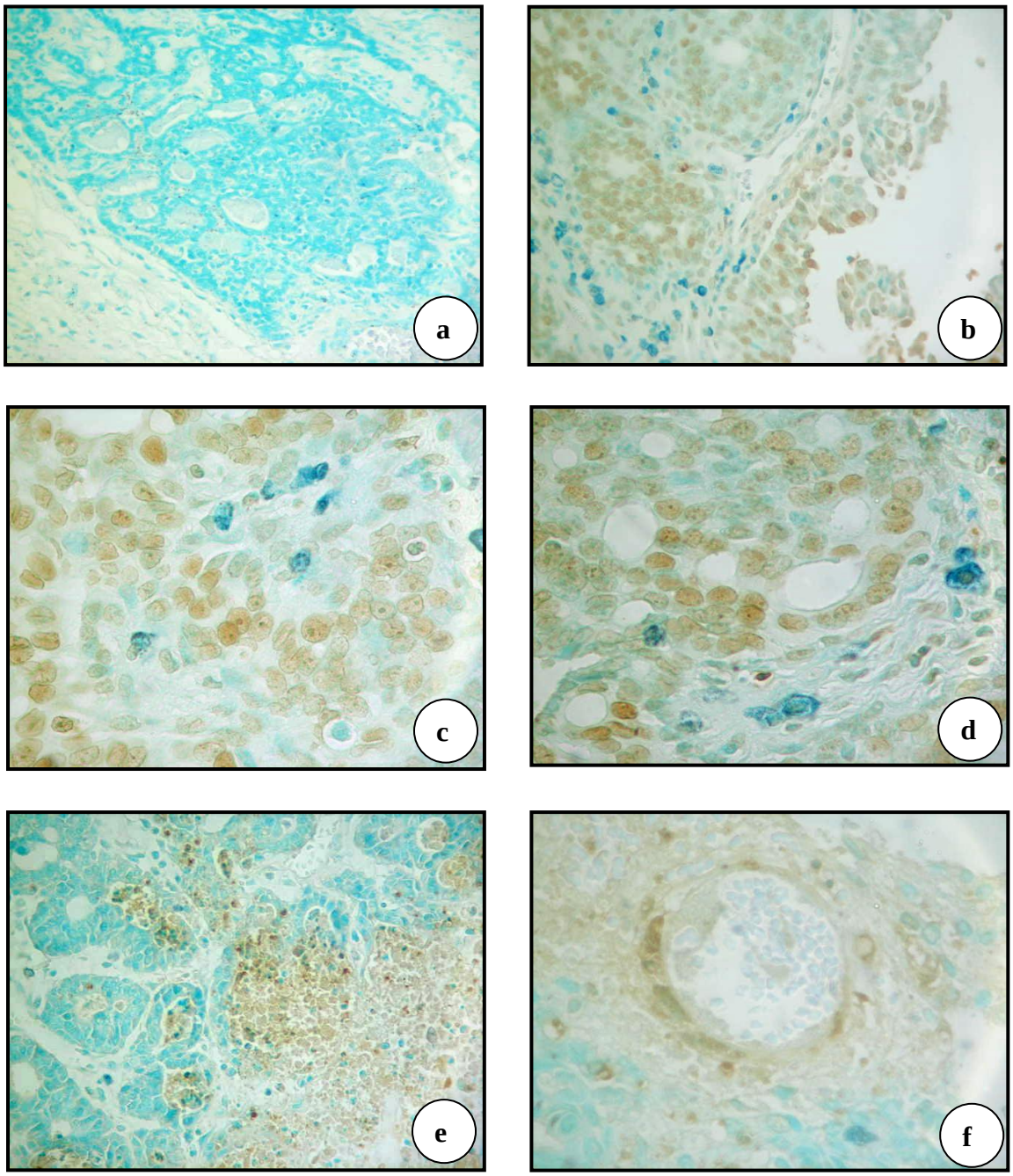

Table 1. Treatment schedule of non-diabetic (NMU) and diabetic (STZ+NMU) rats bearing NMU-ip mammary tumors.

Group	Drugs and route of administration	Doses (mg)
1. NMU	NMU ip	50/kg
2. NMU+Gli	NMU ip + Gli o	50/kg + 0.06/day
3. NMU+Tam	NMU ip + Tam sc	50/kg + 1/kg day
4. NMU+Gli+Tam	NMU ip + Gli o + Tam sc	50/kg + 0.06/day + 1/kg day
5. STZ+NMU	STZ sc + NMU ip	90/kg + 50/kg
6. STZ+NMU+Gli	STZ sc + NMU ip + Gli o	90/kg + 50/kg + 0.06/day
7. STZ+NMU+Tam	STZ sc + NMU ip + Tam sc	90/kg + 50/kg + 1/kg day
8. STZ+NMU+Gli+Tam	STZ sc + NMU ip + Gli o + Tam sc	90/kg + 50/kg + 0.06/day + 1/kg day

Rats in groups 1, 2, 3 and 4 are non-diabetic. Rats in groups 3, 4, 7 and 8 are diabetic. Groups 2, 4, 6 and 8 received glibenclamide. Groups 3, 4, 6 and 8 received tamoxifen. Gli and Tam treatments began when at least one tumor per rat reached a diameter of 1 cm and continued along 20 days. ip, intraperitoneal; sc, subcutaneous; o, oral; NMU, N-Nitroso-N-methylurea; STZ, streptozotocin; Gli, glibenclamide; Tam, tamoxifen.

Table 2. Plasma insulin levels and IGF-I circulating levels.

Group	Plasma Insulin levels (μUI/mL)	IGF-I levels (ng/mL)
1. NMU	17.6 ± 6.3	762 ± 53
2. NMU+Gli	20.8 ± 5.9	690 ± 53
3. NMU+Tam	19.3 ± 4.0	654 ± 44 [a]
4. NMU+Gli+Tam	19.0 + 5.1	647 + 59 [b]
5. STZ+NMU	14.4 ± 3.1	735 ± 50
6. STZ+NMU+Gli	21.8 ± 4.1*	740 ± 41
7. STZ+NMU+Tam	19.0 ± 5.2	659 ± 62 [c]
8. STZ+NMU+Gli+Tam	18.2 ± 4.9	639 ± 71 [d]

Rats in groups 1, 2, 3 and 4 are non-diabetic. Rats in groups 5, 6, 7 and 8 are diabetic. Groups 2, 4, 6 and 8 received glibenclamide. Groups 3, 4, 7 and 8 received tamoxifen. NMU, N-Nitroso-N-methylurea; STZ, streptozotocin; Gli, glibenclamide; Tam, tamoxifen.

Results are expressed as means ± SD. n=5 each group. *, $P<0.05$ STZ+NMU+Gli vs STZ+NMU. [a], $P<0.05$ NMU+Tam vs NMU; [b], $P<0.05$ NMU+Gli+Tam vs NMU; [c], $P<0.05$ STZ+NMU+Tam vs STZ+NMU; [d], $P<0.05$ STZ+NMU+Gli+Tam vs STZ+NMU.

One-way ANOVA and Tukey post-test.

Table 3. Tumor growth evolution of NMU-induced mammary tumors in normal rats under Gli or Tam treatment.

Group	Regressing N° (%)	Stable N° (%)	Growing N° (%)	Regres+Stable N° (%)
1. NMU (n = 49)	0 (0)	1 (2)	48 (98)	1 (2)
2. NMU+Gli (n = 42)	15 (36)	12 (28)	15 (36)	27 (64) [a]
3. NMU+ Tam (n = 44)	24 (55)	1 (2)	19 (43)	25 (57) [a, b]
4. NMU+Gli+Tam (n = 48)	28 (58)	20 (42)	0 (0)	48 (100) [a, c]

Groups 2 and 4 received glibenclamide (Gli). Groups 3 and 4 received tamoxifen (Tam). Observation period: 20 days; n, total number of tumors per group. NMU, N-Nitroso-N-methylurea; Tam, tamoxifen; Gli, glibenclamide. n, number of tumors. [a] , $P<0.0001$ vs NMU. [b] , *P*: NS vs NMU+Gli. [c] , $P<0.0001$ vs NMU+Tam. In all of the cases, Fisher test.

Table 4. Tumor growth evolution of NMU-induced mammary tumors in diabetic rats under Gli or Tam treatment.

Group	Regressing N° (%)	Stable N° (%)	Growing N° (%)	Regres+Stable N° (%)
5. STZ+NMU (n = 21)	0 (0)	0 (0)	21 (100)	0 (0)
6. STZ+NMU+Gli (n = 22)	0 (0)	0 (0)	22 100)	0 (0) [a]
7. STZ+NMU+Tam (n = 20)	18 (75)	0 (0)	5 (25)	15 (75) [b]
8. STZ+NMU+Gli+Tam (n = 19)	13 (68)	4 (21)	2 (10)	17 (89) [b, c]

Groups 6 and 8 received glibenclamide. Groups 7 and 8 received tamoxifen. Observation period: 20 days; n, number of total tumors per group. NMU, N-Nitroso-N-methylurea; STZ, streptozotocin; Tam, tamoxifen; Gli, glibenclamide. [a], *P:*NS vs STZ+NMU. [b], $P<0.0001$ vs STZ+NMU. [c], *P:*NS vs STZ+NMU+Tam. In all of the cases, Fisher test.

Table 5. Effects of Gli, Tam, or Gli+Tam treatments on apoptosis, mitosis and necrosis in NMU-induced mammary tumors in non- diabetic rats.

Group	Apoptosis [1]	Mitosis [2]	Necrosis [3]
1. NMU (n = 10)	-	+++	-
2. NMU+Gli (n = 8)	+++	+	-
3. NMU+ Tam (n = 7)	+	-	+++
4. NMU+Gli+Tam (n = 6)	++	-	+

[1]: -, < 2 apoptotic epithelial cells per field; ++, 60-100 apoptotic epithelial cells per field; **+++,** > 100 apoptotic epithelial cells per field (100X).

[2] : -, < 2 mitotic epithelial cells per field; +, 2-5 mitotic epithelial cells per field; +++, > 10 mitotic epithelial cells per field (100X).

3 : -, without areas of necrosis per field; +, 10% of necrotic areas per field; +++, 30-50% of necrotic areas per field (100X).

NMU, N-Nitroso-N-methylurea; Gli, glibenclamide; Tam, tamoxifen.