Effect of Rosiglitazone on N-nitroso-N-methylurea-induced Mammary Tumors in Rat

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Abstract

The objective of this study was to evaluate the *in vivo* antitumor action of rosiglitazone (Rosi) alone or in combination with tamoxifen (Tam) on experimental mammary tumors induced by N-nitroso-N-methylurea (NMU) in Sprague-Dawley rats. Animals bearing mammary tumors were treated with 0.06 mg/kg/day or 0.12 mg/kg/day of Rosi orally, 1 mg/kg/day of Tam sc, or with the combined treatment (Rosi+Tam). After 25 days of treatments, the following responses were observed: 45% of tumors were responsive to 0.06 mg/kg/day of Rosi treatment, while 55% of tumors under Tam treatment responded. Results of the combined Rosi+Tam treatment indicate that 75% of tumors were responsive. Similar results were obtained with 0.12 mg/kg/day of Rosi. Apoptosis, necrosis and glandular hypersecretion were observed in Rosi-treated tumors. In all cases, the combined Rosi+Tam treatment potentiates the antitumor effect of Tam alone. No side-effect was observed after treatment at any assayed dose.

Key Words: Rosiglitazone, mammary tumors, N-nitroso-N-methylurea, tamoxifen.

Abreviations: GTT, glucose tolerance test; I, insulin; IGF-I, insulin-like growth factor-I; NMU, N-nitroso-N-methylurea; Rosi, rosiglitazone; STZ, streptozotocin; Tam, tamoxifen.

Introduction

Currently, it is believed that several hormones, such as estrogens, insulin and insulin-like growth factor systems (IGFs) participate in the development of normal and neoplastic mammary tissue (1-4). Thiozolidinediones (TZDs), such as rosiglitazone, are compounds that improve the insulin sensitivity in patients with non-insulin-dependent diabetes mellitus (NIDDM) as well as in rodent models of NIDDM and are extensively used for their antidiabetic properties in the treatment of the diabetes (5). It is known that these compounds act as high affinity ligands for a number of the nuclear hormone receptor super-family PPARs (peroxisome-proliferator activated receptors), which has been reported to play an important role in lipid and glucose metabolism as well as in adipocyte differentiation (6,7). The PPARs are a group of 3 nuclear receptor isoforms encoded by different genes (7). Among them, PPARγ is of particular interest because, in addition to diabetes (8), it has been implicated in several other pathological conditions including cancer (9). Much evidence associates the PPARγ ligands with the differentiation, and inhibition of tumor growth of established tumors, the chemopreventive effects in animal models and the inhibitory effect on cell growth in most cell types studied (10-14). Troglitazone, a class of TZDs, has also been reported as effective in the treatment of 7,12 dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats (15).

We previously characterized an experimental mammary tumor induced in rats by 3 *ip* injections of the carcinogen N-nitroso-N-methylurea (NMU) at 50, 80 and 110 days of life (16). The objective of the present study was to evaluate the *in vivo* antitumor action of rosiglitazone (Rosi) alone or in combination with tamoxifen (Tam) on these experimental mammary tumors. Therefore, the effect of the treatments was determined by: a) glucose tolerance; b) mammary tumor growth; c) histopathological characteristics of the tumors; d)

blood insulin and total IGF-I levels; e) other physiological parameters in rats during treatments.

Materials and Methods

Reagents. NMU was purchased from Sigma Chemical Co., (St. Louis, Missouri, USA). A blood glucose micromethod was used to determine glucose levels (Glucometer Gx, Ames S.A., Argentina). The kit for IGF-I determination by radioimmunoassay (RIA) was purchased from Diagnostic Systems Laboratories Inc. (Texas, USA) and the kit for insulin detection (RIA) was from International CIS (France). Rosi was a gift from GlaxoSmithKlein Laboratories SA, (Buenos Aires, Argentina). Tam was kindly provided by Gador Laboratories SA (Buenos Aires, Argentina). Anti PCNA (proliferating cell nuclear antigen), was a mouse monoclonal antibody from DakoCytomation (Clone PC10, Denmark). The second antibody was antimouse IgG peroxidase conjugate and for signal detection the 3,3'diaminobencidine tablets (DAB) were from Sigma Chemical Co. The synthetic balsam for microscopic observation was from Alwik (Poland). Other reagents were of analytical grade.

Animals. Female Sprague-Dawley rats (from National University of La Plata, Animal Production Division, Argentina), 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 cases the animals were kept at conditions recommended by the Guide for the Care and Use of Laboratory Animals, National Research Council, USA, 1996.

Experiment 1. Glucose tolerance test (GTT) in rats under treatment. The effect of 0.06 and 0.12 mg/kg/day of Rosi were analyzed. The employed doses were extrapoleted from those utilized in human patients for the treatment of diabetic desease (17). In order to examine if Rosi treatments affected the circulating levels of glucose, GTT was performed pre- and post-treatment in all treated rats. Six groups of 120-day-old animals were randomly separated (n=5

rats each) and treated as follows: i) rats received vehicle (control); ii) rats received 0.06 mg/kg/day of Rosi orally dissolved in 15% glycerin/water; iii) rats were treated with 1 mg/kg/day of Tam *sc* suspended in corn oil (Tam); iv) rats received combined 0.06 mg/kg/day of Rosi plus 1 mg/kg/day of Tam (Rosi 0.06+Tam); v) rats received 0.12 mg/kg/day of Rosi (Rosi 0.12); vi) rats received combined 0.12 mg/kg/day Rosi+1 mg/kg/day Tam (Rosi 0.06+Tam). On days 120 and 145 of life of the animals and after basal glucose determination, 2 g/kg of glucose was injected *ip* into rats and glucose circulating levels were determined at 30, 60 and 120 min post-glucose injection.

Experiment 2. Effects of Rosi, Tam, and Rosi+Tam treatments on tumor growth. For malignant mammary tumor induction, rats were injected with three *ip* doses of NMU at 50, 80 and 110 days of age as previously described (15). The developed tumors were measured with a caliper 3 days a week to control their size and growth. Studies of the effects on tumor growth of 0.06 mg/kg/day and 0.12 mg/kg/day of Rosi, alone or combined with Tam, were performed. Results were compared to those obtained in rats receiving Tam alone and to controls receiving placebo (vehicle). Treatments began when at least one tumor per rat had a diameter of 0.6 cm. The treatment schedule of this experiment is summarized in Table I. The parameters recorded were: i) *percentage of tumor responsive to treatments:* each tumor was classified as responsive or growing according to its size in relation to the pre-treatment value; tumors were considered responsive when their diameters were similar to the initial value or when were lower than its diameter at the beginning of the treatments (18,19); ii) *relative tumor size*, calculated as the relationship between tumor size at different times *versus* the one at the beginning of each treatment. Tumor size was determined as the mean of the measurement of 2 perpendicular diameters 3 days a week (18,19); iii) *histopathology:* the histopathological characteristics of all tumors were determined according to the classification of Russo *et al* (20). Histological observations of all mammary tumors were performed. The

specimens were fixed in 10% formaldehyde and embedded in paraffin. The slides were stained with hematoxylin-eosin (HE) for microscopic observation (Axiolab Karl Zeiss S.A., Zurich, Switzerland, microscope, carried out with Canon G5 digital camera and Nowell Canon remote capture 2.7, Image Browser 3.0, Photo Stich 3.1); iv) *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 employed technique is to examine apoptosis *via* DNA fragmentation by the TUNEL assay; v) *PCNA expression:* samples from all tumors were fixed for 24 h in formaldehyde 10% (PBS 10 mM pH 7.4) and embedded in paraffin. Serial sections (5 μm) were cut with a microtome and mounted on glass xylanized slides. The PCNA expression was determined with the anti PCNA antibody 1:50 overnight at 4^{0}C. The slides were then incubated with rabbit antimouse HRP conjugate antibody 1:100. The immune complex was visualized with the chromogenic substrate DAB. All experiments included positive and negative controls.

Experiment 3. Effects of Rosi, Tam, and Rosi+Tam treatments on insulin and total IGF-I circulating levels. In order to determine if the treatments produced changes in insulin or in total IGF-I circulating levels, both pre- and post-treatments value were determined in a new set of experiments. Blood samples (n=4 each group) were obtained from the tail of each rat pre- and post-treatment and centrifuged after collection; serum 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.

Experiment 4. Effect of treatments on body weight, water and food intake of rats. During the treatments, the evolution of body weight (BW) and water and food consumption of rats were monitored. Rats 120-days-old were randomly separated into 4 groups (n=5 rats each): i)

control, normal rats without any treatment; ii) rats received 0.06 mg/kg/day of Rosi; iii) rats received 1 mg/kg/day of Tam; and iv) rats that received 0.06 mg/kg/day of Rosi plus 1 mg/kg/day of Tam. The animals were individually housed in stainless steel cages equipped with an individual feeder and a water bottle; water and food were provided *ad libitum* and their intake was monitored at daily intervals through the experiment. The body weight was determined every fifth day using an adequate scale and expressed as g. Water and food consumption of each rat were expressed as ml/100 g BW and g/100 g BW, respectively.

Statistical analyses. In each figure and table, the respective statistical test used is indicated.

Results

Experiment 1. Glucose tolerance test. After glucose injection, rats treated with Rosi, Tam, or combined treatment showed a normal recovery of glucose levels (Table II). The results also indicate that NMU injections did not affect GTT (data not shown).

Experiment 2. Effects of treatments on tumor characteristics.

i) *Tumor growth.* The evolution of tumor growth after 25 days of treatment is summarized in Table III. None of the tumors from the NMU control group spontaneously regressed. In the NMU+Rosi 0.06 group, 45% of the tumors were responsive, while 55% continued growing. With the Tam treatment (NMU+Tam group), 55% of tumors showed regression and 45% continued growing, similar to our previous observations of the same experimental model (18,19). In the combined treatment (NMU+Rosi 0.06+Tam), a higher proportion of tumors showed response (75%). In the NMU+Rosi 0.12 group, 50% of the tumors were sensitive, while 50% continued growing. When 0.12 mg/kg/day Rosi was combined with 1 mg/kg/day Tam, a greater proportion of tumors showed response (80%).

ii*) Relative tumor size.* After 25 days of 0.06 mg/kg/day Rosi treatment (Figure 1a), tumors that were sensitive to Rosi (Rosi 0.06(r)) display a significant different evolution to those treated with placebo and their growth, either regressed or stabilized. When rats were treated

with 0.12 mg/kg/day of Rosi, the final mean tumor sizes observed in responsive tumors (Rosi 0.12(r)), were similar to those responsive at 0.06 mg/kg/day Rosi (Figure 1b). Tumors regressing under the Tam treatment, (Tam (r)), reached a mean size of 50% at the end of the experiments (Figure 1c).

When rats bearing tumors were treated with 0.06 or 0.12 mg/kg/day of Rosi plus 1 mg/kg/day of Tam, the responsive tumors had similar sizes to those responsive to Rosi alone (Figures 1d and e). In summary, the combined Rosi-plus-Tam treatment produced a significant increase in the number of responsive tumors (Table III).

iii) *Histological results.* In Figure 2 mammary tumor histology and morphology, apoptosis and PCNA expression are presented. Tumors from NMU rats were always malignant adenocarcinomas, with a cribriform, comedo or papillary pattern, as previously described (18). A typical malignant pattern of a cribriform tumor is illustrated in Figure 2a. Tumors of rats treated with Rosi showed also malignant pattern but with areas of glandular secretory differentiation, fibrous tissue reaction and inflammatory infiltrating cells (Figures 2b and c). Tumors from Tam-treated rats showed secretory gland differentiation (Figure 2d) and necrotic sectors (19), and the tumors from animals treated with Rosi+Tam also exhibited important intraglandular secretion, moderate fibrous, inflammatory reaction and extended necrotic areas (Figures 2e and f).

iv*) Apoptosis.* The effects of Rosi, Tam, and Rosi+Tam treatments on apoptosis, mitosis and necrosis in mammary tumors are summarized in Table IV. Immunohistochemical results indicate that the NMU-control tumors did not reveal apoptotic cells (Figure 2g). On the contrary, tumors from Rosi-treated animals showed areas with a large number of apoptotic cells per field and a low number of mitotic epithelial cells (Figures 2h and i). Tumors that regressed under Tam treatment showed extended apoptotic (Figure 2j) and necrotic areas, as previously reported (19). With the combined Rosi+Tam treatment, a significant increase in

the number of apoptotic cells *versus* controls (placebo treatment) was observed and a lower number of mitosis per field was detected (Figures 2j and k).

v) *PCNA expression*. An large number of cells in the NMU tumors (80-90%) were found stained (Figure 2l). In contrast, in the Rosi-treated tumors (Figure 2m), the proportion of PCNA-positive cells was significantly lower (15-25%). The low expression of the PCNA antigen was similar in the Tam or Rosi+Tam-treated tumors (Figure 2o and p), as shown in Figure q.

Experiment 3. Insulin and tIGF-I circulating levels. No significant differences, either in insulin or in tIGF-I circulating values were seen between the Rosi-treated and the control rats (Table V). Neither the Tam nor the combined treatments changed the baseline values.

Experiment 4. Body weight, water and food intake. At the baseline, the Control, Rosi, Tam and Rosi+Tam rats had similar body weights. At the end of the study, no significant differences were observed (Table VI). Also, no differences were observed either in water or in food consumption under the different treatments in comparison to the control rats. The data obtained with the doses of 0.12 mg/kg/day Rosi or Rosi 0.12+Tam did not differ significantly (data not shown).

Discussion

Breast cancer is highly prevalent in women all over the world (21). Most breast cancer is estrogen-dependent for its growth and consequently, endocrine ablation either by removal of the ovary or by the administration of anti-estrogenic drugs has been one of the major therapy options (22). An important group of drugs has a selective effect on hormone responsive tissue and the most successful of these is Tam, which acts like an estrogen antagonist in the mammary epithelium (23). Once bound to the estrogen receptor (ER), Tam blocks transcriptional and post-transcriptional events. However, an important proportion of breast

cancer is not responsive to Tam treatment, or develop resistance (23). Consequently, a focus of interest is on the development of new drugs for the management of this illness.

The thiazolidinediones, such as rosiglitazone are synthetic antihyperglycemic drugs that act as insulin sensitizers by binding to and activating a specific transcription factor in the cell nucleus (24); when activated, this factor binds to specific genes. Many of these genes regulate proteins involved in lipid metabolism, adipose tissue differentiation and intracellular insulin signaling cascade (24). As mentioned above, it is known that PPARγ ligands not only exert antidiabetic effect in type 2 diabetes but also induce cell growth arrest, apoptosis and differentiation in many cancers and cell types (10,12-14,25-27), suggesting that PPARγ may be a potential therapeutic target for treatment of certain human cancers.

Based on this background, the experiments presented in this paper conformed to the *in vivo* mammary tumor model induced in rats by NMU (16). To test the hypothesis that rosiglitazone has a direct action on tumor growth, we administered doses to rats extrapolated from those employed in human type 2 diabetic patients (17). In the first set of experiments, we determined that these treatments did not affect the glucose tolerance in rats (Table I). The same doses of Rosi were employed to assay the possible action of the drug on tumor growth. Twenty-five days of treatment produced interesting results on the treated rats: an important proportion of tumors was sensitive to Rosi treatment, and growth regression or stabilization was observed. These results correlate with those reported by other researches. Pighetti *et al* in their study of experimental mammary tumors induced by DMBA, showed that troglitazone induced the regression or stabilization of tumor growth (15). Ohta *et al* showed a very significant decrease in tumor growth of BHP18-21 thyroid carcinoma cells injected into *nude* mice when the animals were treated with the PPARγ ligand (12). Our results clearly indicate that Rosi treatment reduced the growth rate in 40-50% of the tumors.

When the number of apoptotic cells was analyzed, Rosi was found to provoke a higher number of apoptotic cells than the Tam or placebo treatments. The process of apoptosis is well recognized as playing an important role in the maintenance of normal tissue homeostasis and in tumorigenesis. It is known that tumor growth rate is a balance between both proliferative activity and cell death. Increasing evidence indicates that the activation of PPARγ inhibits tumor growth by the induction of apoptosis. Yang *et al* showed in renal cells that TZDs causes massive apoptosis with increasing *bax* and decreasing *bcl-2* levels (27). Similar results were obtained by Liu *et al* with K562 and HL-60 cells (28). In our experimental model, a significant proportion of cells underwent apoptosis (Table III). To investigate the molecular mechanism of this process, we assessed the expression of the apoptosis-regulatory genes *bcl-2* and *bax.* Preliminarly, *bax* was upregulated by Rosi treatment (data not shown), suggesting that the apoptosis course *via bax* up-regulation. PPARγ activation was also found to induce apoptosis in human and rat glioma with a transient up-regulation of *bax* and *bad* protein levels (29). Conversely, Boggazi *et al*, did not show that Rosi affected *bax* expression in GH3 cells (30). Chen *et al* reported that apoptosis induced by PPAR ligands was sequentially accompanied by reduced levels of *bcl-2* (31). On the other hand, Shiau *et al* reported that thiazolidinediones provoke apoptosis independently of PPARγ activation (32). Other investigations also support the existance of PPARγ-independent pathways to mediate the anticancer effects of TZDs (33,34).

In our experimental model, the Rosi treatment clearly diminished PCNA expression. The expression of PCNA, a nuclear protein related to the cell cycle and used as marker of cell proliferation (35), was found significantly diminished in tumors from Rosi-treated rats in comparison to controls. It is known that the *PCNA* gene executes cellular responses to stress-repair or apoptosis. Absence or low levels of functional PCNA may drive cells into apoptosis (36).

As mentioned above, many investigations indicated that PPARγ ligands promote cellular differentiation. Mueller *et al* showed that the activation of PPARγ causes extensive lipid accumulation and changes associated with a more-differentiated and less malignant state of the cells (37). Haydon *et al*, in their study on human osteosarcoma cell lines, showed that PPARγ agonists can induce apoptosis and differentiation in human osteosarcoma (38). In pancreatic carcinoma cell lines, TZDs cause ductal differentiation, but not apoptosis (39). Animal model studies demonstrated that rosiglitazone redifferentiates thyroid cancers (40). In our experimental model, a clearly more differentiated pattern was observed in tumors after Rosi treatment, with important glandular hypersecretion and apoptotic sectors. The results presented in this paper show a clear additive effect on tumor response, apoptosis and differentiation when the rats were treated with the combination Rosi plus Tam.

One possible hypothesis to explain the action of rosiglitazone on the growth of a proportion of treated tumors may be the different expressions of estrogen receptors (ER) found on them. In this experimental model, ER are expressed in a wide range of well-differentiated cribriform tumors, while no expression was found in poorly-differentiated tumors (41). Wang and Kilgore suggested that a signal cross talk exists bidirectionally between PPARγ and ER in breast cancer cells (42). Bonofiglio *et al* showed that the ERα and PPARγ pathways have an opposite effect on the regulation of the PI3K/AKT cascade (43). Recently, Papadaki *et al* analyzed 170 human breast cancer biopsies, 51% of them PPARγ-positive (44) and found that PPARγ correlated with ERβ expression, associated with the inhibition of proliferation and invasiveness of breast cancer cells (45). The greater response observed with the Rosi-plus-Tam treatment could be due to the Tam treatment *per se* and to the effect of PPAR ligand through ER. Currently, experiments are in progress in our laboratory to quantify PPARγ expression on Rosi-treated and non treated mammary NMU-induced tumors.

Rosiglitazone is also known to be orally effective in decreasing plasma glucose levels in non-insulin-dependent diabetic mellitus patients (24). Results of the recent investigation indicate that prolonged Rosi administration decreased insulin levels in human patients (46). In previous experiments, we demonstrated that the IGF-I plays an important role in the development of the mammary model used in these experiments (3). In the experiments presented here, we demonstrate that Rosi did not increase IGF-I circulating levels, indicating that under these conditions, the mechanism of action of Rosi is IGF-I-independent. When Rosi was combined with Tam, the inhibition of tumor growth may have been due to the Rosi *per se* action plus the multiple actions of Tam (22,23). Macroscopic and microscopic observations of animals and organs did not evidence any change. Rosiglitazone did not show cytotoxicity or hepatic injury, in concordance with Yamamoto *et al* (47). Stout *et al* (48), reported that Rosi was well tolerated in a clinical trial and that it has an improved safety profile in terms of liver toxicity. No effect on body weight, water or food intake in the rats under treatment was found in agreement with the parameters reported by the Charles River Laboratories for Sprague-Dawley rats (http://www.criver.com).

Even though recent clinical trials using a PPARγ ligand failed to show a clinical benefit in metastasic breast cancer (49,50), several studies reported the existence of links between certain metabolic disorders and cancers (51).

In conclusions, our experimental results signal the potential benefit of combined rosiglitazone plus tamoxifen treatment on breast cancer.

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References

1. Heuson JC and Legros N: Effect of insulin on DNA synthesis and DNA polymerase activity in organ culture of rat mammary carcinoma, and the influence of insulin pretreatment and of alloxan diabetes. Cancer Res *31*: 59-65, 1971.

2. Werner H and Le Roith D: New concepts in regulation and function of the insulin-growth factors: implications for understanding normal growth and neoplasia. Cell Mol Life Sci *57*: 932-934, 2000.

3. Cocca C, Gutierrez A, Núñez M, Croci M, Martín G, Cricco G, Rivera E and Bergoc R: Suppression of mammary carcinogenesis in diabetic rats. Cancer Detect and Prev *27*: 37-46, 2003.

4. Cocca C, Núñez M, Gutiérrez A, Martín G, Cricco G, Medina V, Mohamad N, Rivera E and Bergoc R: IGF-I in mammary tumorigenesis and diabetes. Review. Anticancer Research *24*: 2953-2966, 2004.

5. Lebovitz HE: Differentiating members of the thiazolidinedione class: a focus on safety. Diabetes Metab Res Rev *18*: S23-S29, 2002.

6. Albrektsen T and Fleckner J: The transcription factor Fas-related antigen 1 is induced by thiazolidinediones during differentiation of 3T3-L1 cells. Mol Pharmacol *59*: 567-575, 2001.

7. Berger J and Moller DE: The mechanisms of action of PPARs. Annu Rev Med *53*: 409-435, 2002.

8. Rangwala SM and Lazar MA: Peroxisome proliferator-activated receptor γ in diabetes and metabolism. Trends in Pharmacological Sciences *26*: 331-336, 2004.

9. Koeffler HP: Peroxisome proliferator-activated receptor gamma and cancers. Clinical Cancer Res *9*: 1-9, 2003.

10. Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C and Spiegelman BM: Differentiation and reversal of malignant changes in colon cancer through PPARγ. Nature Medicine *4*: 1046-1052, 1998.

11. Núñez PN, Liu H and Meadows G: PPARγ ligands and amino acid deprivation promote apoptosis of melanoma, prostate, and breast cancer cells. Cancer Lett *XX*: 1-9, 2005.

12. Ohta K, Endo T, Haraguchi K, Hershman JM and Onaya T: Ligands for peroxisome proliferator-activated receptor gamma inhibited growth and induce apoptosis of human papillary thyroid carcinoma cells. J Clin Endocrinol Metab *86*: 2170-2177, 2001.

13. Yoshizawa K, Cioca DP, Kawa S, Tanaka F and Kiyosawa K: Peroxisome proliferator-activated receptor gamma ligand troglitazone induces cell cycle arrest and apoptosis of hepatocellular carcinoma cell lines. Cancer *95*: 2243-2251, 2002.

14. Chen GG, Lee JF, Wang SH, Chan UP, Ip PC and Lau WY: Apoptosis induced by activation of peroxisome-proliferator activated receptor-gamma is associated with Bcl-2 and NF-kappaB in human colon cancer. Life Sci *70*: 2631-2646, 2002.

15. Pighetti GM, Novosad W, Nicholson C, Hitt DC, Hansens C, Hollingsworth AB, Lerner ML, Brackett D, Lightfoot SA and Gimble JM: Therapeutic treatment of DMBA-induced mammary tumors with PPAR ligands. Anticancer Res *21*: 825-9, 2001.

16. Rivera E, Melito G, Davio C, Cricco G, Martin G, Mohamad N, Andrade N, Caro R and Bergoc R: Induction of mammary tumors in rat by intraperitoneal injection of NMU: histopathology and estral cycle influence. Cancer Lett *86*: 223-228, 1994.

17. Goldberg RB, Kendall DM, Deeg MA, Buse JB, Zagar AJ, Pinaire JA, Tan MH, Khan MA, Perez AT and Jacober SJ: GLA I Investigators. A comparison of lipid and glycemic effect of

pioglitazone and rosiglitazone in patients with type 2 diabetes and dyslipemia. Diabetes Care *28*: 1547-1554, 2005.

18. Martin G, Davio C, Rivera E, Melito G, Cricco G, Andrade N, Caro R and Bergoc R: Hormone dependence of mammary tumors induced in rats by intraperitoneal NMU injection. Cancer Investigation *1*: 8-17, 1997.
19. Martin G, Melito G, Rivera E, Levin E, Davio C, Cricco G, Caro R and Bergoc R: Effect of tamoxifen on intraperitoneal N-nitroso-N-methylurea induced tumors. Cancer Lett *100*: 227-234, 1996.
20. Russo J, Russo IH and Rogers MJ: Tumors of the mammary gland. In: Pathology of Tumors in Laboratory Animals. Vol 1: Tumors of the Rats. Turusov VS, Mohr U (eds). IARC Scientific Publications No 99, pg. 47-78, Lyon, France, 1990.
21. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ and Thun MJ: Cancer statistics, 2005. Cancer J Clin *55*: 10-30, 2005.
22. Early Breast Cancer Trialists´ Collaborative Group: Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet *351*: 1451-1467, 1998.
23. Jordan VC: Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell *5*: 207-213, 2004.
24. Lebovitz HE: Treating hyperglycemia in type 2 diabetes: New goals and strategies. Cleveland Clinic J of Med *69*: 809-820, 2002.
25. Chang TH and Szabo E: Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor gamma in non-small cell lung cancer. Cancer Res *60*: 1129-1138, 2000.
26. Hisatake JI, Ikezoe T, Carey M, Holden S, Tomoyasu S and Koeffler HP: Down-regulation of prostate-specific antigen expression by ligands for peroxisome proliferator-activated receptor gamma in human prostate cancer. Cancer Res *60*: 5494-5498, 2000.

27. Yang F, Zhang Z, Xin D, Shi C, Wu J, Gou Y and Guan Y: Peroxisome proliferator-activated receptor gamma ligands induce cell cycle arrest and apoptosis in human renal carcinoma cell lines. Acta Pharmacologica Sinica *26*: 753-761, 2005.

28. Liu JJ, Huang RW, Lin DJ, Peng J, Wu XY, Lin Q, Pan XL, Song YQ, Zhang MH, Hou M and Chen F: Expression of survivin and bax/bcl-2 in peroxisome proliferator activated receptor-gamma ligands induces apoptosis on human myeloid leukemia cells *in vitro*. Ann Oncol *16*: 455-459, 2005.

29. Zander T, Kraus JA, Grammes C, Schleger V, Feinstein D and Klockgether T: Introduction of apoptosis in human and rat glioma by antagonist of the nuclear receptor PPAR gamma. J of Neurochemistry *81*: 1052-1060, 2002.

30. Bogazzi E, Ultimieri F, Raggi F, Russo D, Vanacore R, Guida C, Viacava P, Cecchetti D, Acerbi G, Brogioni S, Cosci C, Gasperi M, Bartalena L and Martino E: PPARγ inhibits GH synthesis and secretion and increases apoptosis of pituitary GH-secreting adenomas. European J of Endocrinol *150*: 863-875, 2004.

31. Chen G, Lee JFY, Wang SN, Chan VPF, Ip PC and Lan WY: Apoptosis induced by activation of peroxisome-proliferator activated receptor-gamma in association with Bcl-2 and Nf-kB in human colon cancer. Life Sciences *70*: 3631-2646, 2002.

32. Shiau CW, Yang CC, Kulp SK, Chan KF, Chen CS, Huang JW and Chen CS: Thiazolidinediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPARγ activation. Cancer Res *65*: 1561-1569, 2005.

33. Seargent JM, Yates EA and Gill JH: GW9662, a potent antagonist of PPARgamma, inhibits growth of breast tumour cells and promotes the anticancer effects of the PPARgamma agonist rosiglitazone, independently of PPARgamma activation. Br J Pharmacol *143*: 933-937, 2004.

34. Runni MA, Ishihara S, Kadowaki Y, Orteaga-Cava CF, Kazumori H, Kawashima K, Yoshino N, Yuki T, Ishimura N and Kinoshita: Peroxisome proliferator-activated receptor gamma-

dependent and -independent growth inhibition of gastrointestinal tumour cells. Genes Cells. *9*: 1113-1123, 2004.

35. Dietrich RD: Toxicological and pathological applications of proliferating cell nuclear antigen (PCNA): a novel endogenous marker for cell proliferation. Crit Rev Toxicol *23*:77-109, 1993.
36. Kelman Z: PCNA: structure, functions and interactions. Review. Oncogene *14*: 629-640, 1997.
37. Mueller E, Sarraf P, Tontonoz P, Evans RM, Martin KJ, Zhang M, Fletcher C, Singer S and Spiegelman BM: Terminal differentiation of human breast cancer through PPAR gamma. Mol Cell *1*: 465-470, 1998.
38. Haydon R, Zhou L, Feng T, Breyer B, Cheng H, Jiang W, Ishikawa A, Peabody T, Montag A, Simon MA and He T: Nuclear receptor agonists as potential differentiation therapy agents for human osteosarcoma. Clin Cancer Res *8*: 1288-1294, 2002.
39. Ceni E, Mello T, Tarocchi M, Crabb DW, Caldini A, Invernizzi P, Surrenti C, Milani S and Galli A: Antidiabetic thiazolidinediones induce ductal differentiation but not apoptosis in pancreatic cancer cells. World J Gastroenterol *11*: 1122-1130, 2005.
40. Philips JC, Petite C, Willi JP, Buchegger F and Meier CA: Effect of peroxisome-activated receptor gamma agonist, rosiglitazone, on dedifferentiated thyroid cancers. Nucl Med Commun *25*: 1183-1186, 2004.
41. Actis AM, Cocca CM, Gutierrez A, Croci M, Rivera ES and Bergoc RM: Estrogen receptor profiles: changes in mouse and rat mammary tumors by treatment with selective estrogen receptor modifiers. Med Princ Pract *13*: 220-226, 2004.
42. Wang X and Kilgore MW: Signal cross-talk between estrogen receptor alpha and beta and the peroxisome proliferator-activated receptor gamma1 in MDA-MB-231 and MCF-7 breast cancer cells. Mol Cell Endocrinol *194*: 123-133, 2002.

43. Bonofiglio D, Gabriele S, Aquila S, Catalano S, Gentile M, Middea E, Giordano F and Ando S: Estrogen receptor alpha binds to peroxisome proliferator-activated receptor response element and negatively interferes with peroxisome proliferator-activated receptor gamma signaling in breast cancer cells. Clin Cancer Res *11*: 6139-6147, 2005.

44. Papadaki I, Mylona E, Giannopoulou I, Markaki S, Keramopoulos A and Nakopoulou L: PPARgamma expression in breast cancer: clinical value and correlation with ERbeta. Histopathology *46*: 37-42, 2005.

45. Nakapoulu L and Lazaris AC: The favourable prognostic value of oestrogen receptor beta immunohistochemical expression in breast cancer. J Clin Pathol *57*: 523-528, 2004.

46. Patel J, Anderson RJ and Rappaport EB: Rosiglitazone monotherapy improves glycaemic control in patients with type 2 diabetes: a twelve-week, randomized, placebo-controlled study. Diabetes Obes Metab *1*: 165-172, 1999.

47. Yamamoto Y, Nakajima M, Yamazoki H and Yokoi T: Cytotoxicity and apoptosis produced by troglitazone in human hepatoma cells. Life Sciences *70*: 471-482, 2001.

48. Stout DL and Fugate SE: Thiazolidinediones for treatment of polycystic ovary syndrome. Pharmacotherapy *25*: 244-252, 2005.

49. Burstein HJ, Demetri GD, Mueller E, Sarraf P, Spiegelman BM and Winer EP: Use of the peroxisome proliferator-activated receptor (PPAR) gamma ligand troglitazone as treatment for refractory breast cancer: a phase II study. Breast Cancer Res Treat *79*: 391-397, 2003.

50. Fenner MH and Elstner E: Peroxisome proliferator-activated receptor gamma ligands for the treatment of breast cancer. Expert Opin Investig Drugs *14*: 557-568, 2005.

51. Berstein LM: Clinical usage of hypolipidemic and antidiabetic drugs in the prevention and treatment of cancer. Cancer Lett *224*: 203-212, 2005.

LEGENDS

Figure 1. Time course of tumor size in rats under Rosi, Tam or combined treatment. In all graphs (■) represents NMU-mammary tumors of non-treated rats. **a:** Results of 0.06 mg/kg/day Rosi treatment; (▼), regressing tumors; (▼) *vs.* (■), p<0.0001; (▲), growing tumors; (▲) *vs.* (■), p:NS. **b:** Results of 0.12 mg/kg/day Rosi treatment; (▼), regressing tumors; (▼) *vs.* (■), p<0.0001; (▲), growing tumors; (▲) *vs.* (■), p:NS. **c:** Results of 1 mg/kg/day Tam treatment; (▽), regressing tumors; (▽) *vs.* (▪), p<0.0001; (Δ), growing tumors; (Δ) *vs.* (▪), p:NS. **d:** Results of 0.06 mg/kg/day Rosi+1 mg/kg/day Tam treatment; (▼), regressing tumors; (▼) *vs.* (■), p<0.0001; (▲), growing tumors; (▲) *vs.* (■), p:NS. **e:** Results of 0.12 mg/kg/day Rosi+1 mg/kg day Tam treatment; (▼), regressing tumors; (▼) *vs.* (■), p<0.0001; (▲), growing tumors; (▲) *vs.* (■), p:NS. Each point represents the mean ± SD. Observation period: 25 days. Two-way ANOVA; r = responsive, g = growth.

Figure 2. Histopathology of mammary tumors. a: Cribriform adenocarcinoma usually found in NMU rats (H.E. 100X). **b: C**ribriform mammary adenocarcinoma following Rosi-treatment showing secretory changes and important peripheral fibrous tissue reaction (H.E. 20X). **c:** Tumor edge with important fibrous tissue reaction and inflammatory infiltrating cells (H.E. 100X). **d:** Focal secretory changes in sectors of a cribriform adenocarcinoma of a Tam-treated rat (H.E. 100X). **e:** Extended necrotic sectors in rat mammary adenocarcinoma associated with important intraglandular secretion following Rosi+Tam treatment (H.E. 20X). **f:** Necrosis, intratumoral secretion, inflammatory and fibrotic reaction on tumor of Rosi+Tam-treated rat (H.E. 100X). **g:** Absence of apoptotic cells in a NMU tumor (100X). **h:** Very high number of apoptotic cells in a cribriform tumor following Rosi treatment (Apoptag and Hematoxylin, 100X). **i:** Apoptosis and secretory changes in a well-differentiated tumor adenocarcinoma following Rosi treatment (Apoptag and Hematoxylin, 1000X). **j:** Frequent

apoptotic cells nearby a necrotic sector in a tumor of Tam treated rat (Apoptag and Hematoxylin, 400X). Tumors from Tam-treated rats preponderantly showed necrosis, as previously reported (16, 18). **k:** Necrosis, apoptosis and intraglandular secretion in a rat cribriform adenocarcinoma following Rosi+Tam treatment (Apoptag and Hematoxylin 1000X). **l:** Very high number of apoptotic cells in an atypical gland of rat mammary adenocarcinoma associated with secretorial activity, in Rosi+Tam-treated rat (Apoptag and Hematoxylin, 1000X). **m:** Very high proportion of PCNA positive cells usually found in NMU tumors (PCNA and methyl green contracoloration, 1000X). **n:** Only occasional cells are PCNA-positive in tumors of Rosi-treated rats (PCNA and methyl green contracoloration, 1000X). **o:** Scarce PCNA-positive cells in a tumor of a Tam-treated rat (PCNA and methyl green contracoloration, 1000X). **p:** Low proportion of positive cells to PCNA in a tumor of Rosi+Tam treated rat (PCNA and methyl green contracoloration, 1000X). **q:** Percentage of PCNA-positive epithelial cells on tumors under different treatments. *** $p<0.0001$ *vs*. Control, one-way ANOVA.

Figure 1.

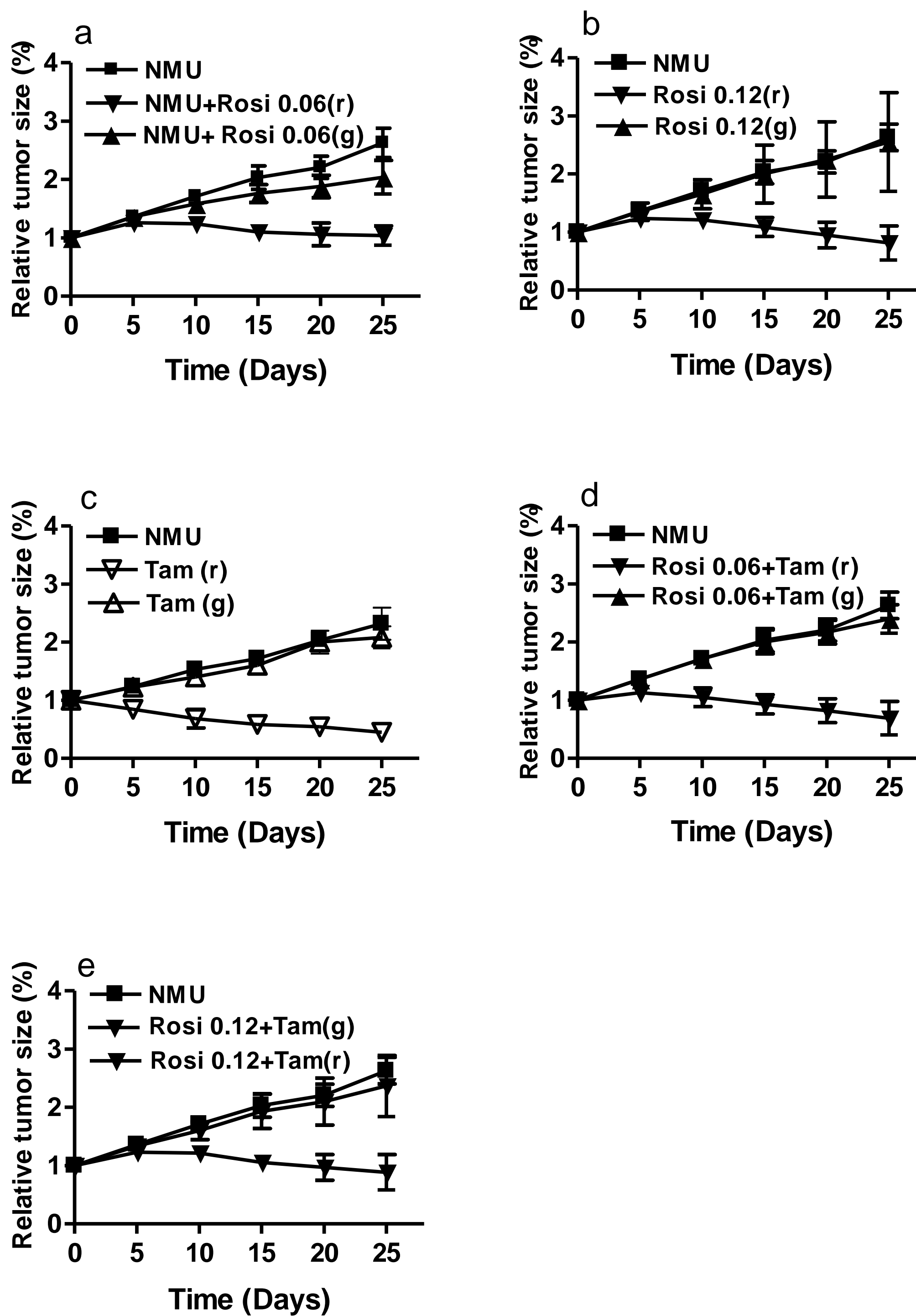

Figure 2.

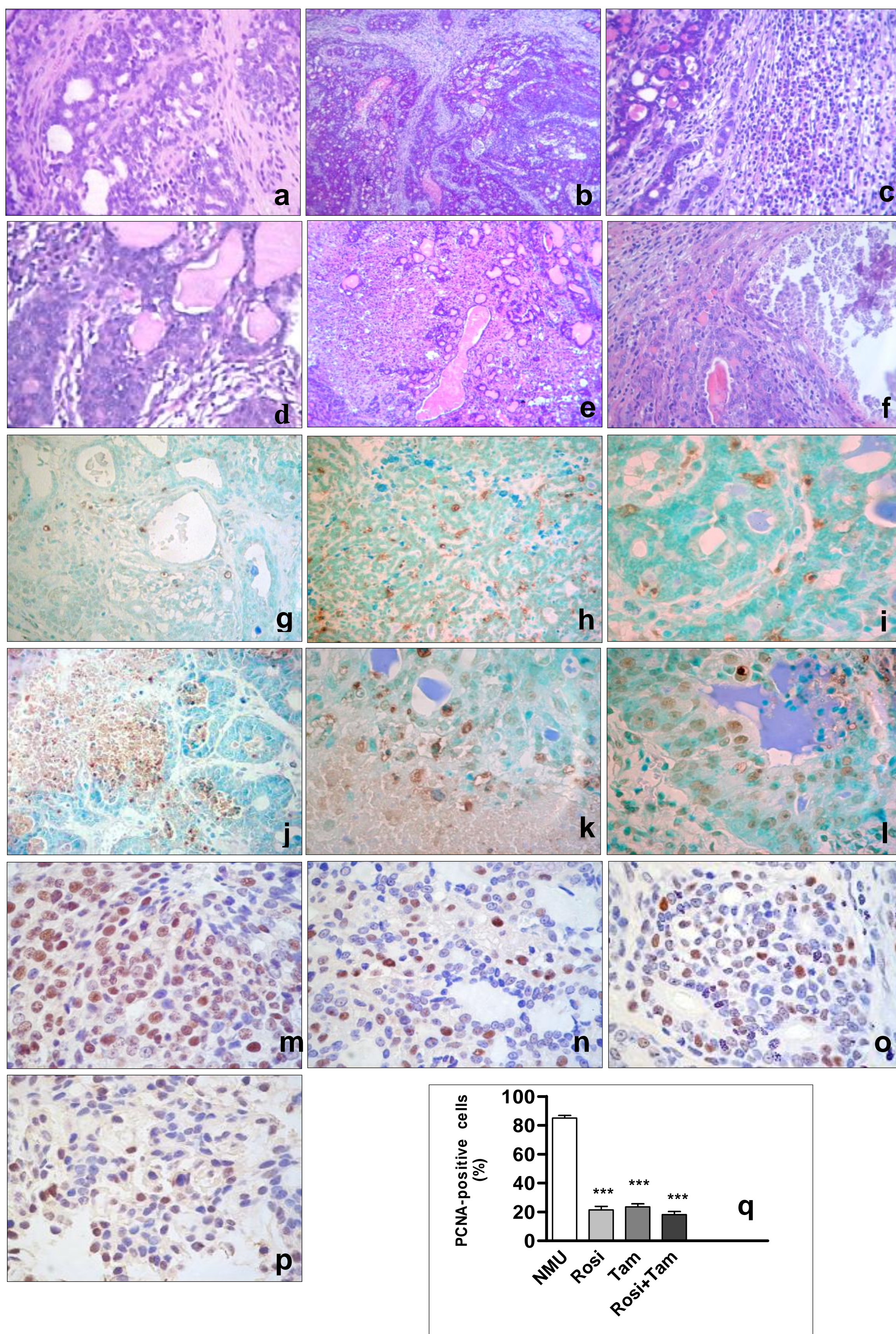

Table I. Treatment schedule of rats bearing NMU-ip mammary tumors.

Group	**Drugs and route of administration**	**Doses (mg)**
1. NMU	NMU *ip*	50/kg
2. NMU+Rosi 0.06	NMU *ip* + Rosi *o*	50/kg + 0.06/kg/day
3. NMU+Tam	NMU *ip* + Tam *sc*	50/kg + 1/kg/day
4. NMU+Rosi 0.06+Tam	NMU *ip* + Rosi *o* + Tam *sc*	50/kg + 0.06/kg/day + 1/kg/day
5. NMU+Rosi 0.12	NMU *ip* + Rosi *o*	50/kg + 0.12/kg/day
6. NMU+Rosi 0.12+Tam	NMU *ip* + Rosi *o* + Tam *sc*	50/kg + 0.12/kg/day + 1/kg/day

Treatments began when at least one tumor per rat reached a diameter of 0.6 cm and continued for 25 days. *ip*, intraperitoneal; *sc*, subcutaneous; *o*, oral; NMU, *N*-nitroso-*N*-methylurea; Rosi, rosiglitazone; Tam, tamoxifen.

Table II. Glucose tolerance test in control, Rosi-, Tam-, and Rosi-plus-Tam-treated rats.

Group	Basal (mg/ml)	30 min (mg/ml)	60 min (mg/ml)	90 min (mg/ml)
1. Control	61-99	143-177	126-135	55-107
2. Rosi 0.06	61-100	145-217	129-141	69-90
3. Tam	76-99	146-199	111-161	90-109
4. Rosi 0.06+Tam	83-101	138-181	121-166	95-107
5. Rosi 0.12	69-90	153-227	123-170	65-115
6. Rosi 0.12+Tam	69-86	123-198	107-166	87-106

Range of blood glucose levels (mg/dl) in rats pre- and post 1 g/kg glucose injection (n = 5 each group). Rosi, rosiglitazone; Tam, tamoxifen. Results were obtained on day 25 of treatments. Initial values did not differ significantly. *p*:NS, two-way ANOVA.

Table III. Tumor growth evolution of NMU-induced mammary tumors in Control, Rosi-, Tam-, and Rosi-plus-Tam-treated rats.

Group	Growing N (%)	Responsive N (%)
1. NMU (n = 40)	40 (100)	0 (0)
2. NMU+Rosi 0.06 (n = 32)	18 (55)	14 (45) [a]
3. NMU+ Tam (n = 44)	20 (45)	24 (55) [b,d]
4. NMU+Rosi 0.06+Tam (n = 48)	10 (25)	30 (75) [c]
5. NMU+Rosi 0.12 (n = 14)	7 (50)	7 (50) [d]
6. NMU+Rosi 0.12+Tam (n =10)	2 (20)	8 (80) [e]

Observation period: 25 days. NMU, N-nitroso-N-methylurea; Tam, tamoxifen; Rosi, rosiglitazone; n, total number of tumors per group. [a]NMU+Rosi 0.06 *vs*. NMU, p=0.0004; [b]NMU+Tam *vs*. NMU, p=0.0002; [c]NMU+Rosi 0.06+Tam *vs*. NMU, p<0.0001; [d]NMU+Rosi 0.12 *vs*. NMU+Rosi 0.06, p:NS; NMU+Tam *vs*. NMU+Rosi 0.06, p:NS; [e]NMU+Rosi 0.12+Tam *vs*. NMU+Rosi 0.06+Tam, p:NS. Fisher test.

Table IV. Effect on apoptosis, mitosis and necrosis in NMU-induced regressing mammary tumors of Rosi, Tam, and Rosi-plus-Tam treatments.

Group	Apoptosis	Mitosis	Necrosis
1. NMU (n = 10)	-	***	x
2. NMU+Rosi (n = 8)	++	*	##
3. NMU+Tam (n = 7)	+	0	###
4. NMU+Rosi+Tam (n = 6)	+++	0	###

- , 1-3% apoptotic epithelial cells per field; +, 10-15% apoptotic epithelial cells per field; ++, 15-25% apoptotic epithelial cells per field; +++, >25% apoptotic epithelial cells per field (400X). 0, No mitotic epithelial cells per field; *, 2-3 mitotic epithelial cells per field; ***, 6-8 mitotic epithelial cells per field (400X). x, without areas of necrosis per field; ##, 10-30% of necrotic areas per field; ###, 30-50% of necrotic areas per field (400X). NMU, N-nitroso-N-methylurea; Rosi, rosiglitazone 0.06 mg/kg/day; Tam, tamoxifen 1 mg/kg/day.

Table V. Plasma insulin levels and tIGF-I circulating levels in Control, Rosi-, Tam-, and Rosi-plus-Tam-treated rats.

Group	Plasma Insulin levels (μUI/mL)		IGF-I levels (ng/mL)	
	Basal	Post-treatment	Basal	Post-treatment
1. Control	18.6±6.9	21.6±7.9	682±53	691±66
2. Rosi 0.06	20.1±4.8	19.9±4.3	692±51	702±72
3. Tam	21.3±4.9	22.1±7.8	654±66	687±81
4. Rosi 0.06+Tam	19.9±5.3	20.9±7.1	675±69	657±58
5. Rosi 0.12	22.2±8.9	21.3±6.9	699±58	687±80
6. Rosi 0.12+Tam	20.9±5.8	22.1±6.5	651±60	679±77

Data represent the initial and final values, and are expressed as means ± SD (n=5 rats per group). Rosi, rosiglitazone; Tam, tamoxifen. *p*:NS. Two-way ANOVA.

Table VI. Body weight, water and food intake of Control, Rosi-, Tam-, and Rosi-plus-Tam-treated rats.

Group	Body weight (BW) (g)		Water intake (mL/100 g BW)		Food intake (g/100 g BW)	
	Initial	Final	Initial	Final	Initial	Final
1. Control	221-267	232-273	10.1-23.8	10.0-21.8	5.4-7.6	6.3-10.0
2. Rosi 0.06	230-267	230-249	16.0-18.3	17.9-20.0	7.0-9.0	7.5-9.0
3. Tam	220-256	223-264	17.6-22.1	16.0-23.1	6.4-9.0	7.5-9.0
4. Rosi 0.06+Tam	234-259	241-261	18.0-23.8	17.0-23.0	6.4-9.9	6.6-9.9

Data represent the range of the initial and the final values. Observation period: 25 days (n=5 rats per group). Rosi, rosiglitazone; Tam, tamoxifen. *p*:NS. Two-way ANOVA.