Comparison of Direct Action of

Thiazolidinediones and Glucocorticoids on Renal Podocytes:

Protection from Injury and Molecular Effects

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Running Title: Thiazolidinediones Protect Podocytes

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List of non-standard abbreviations: BSA, bovine serum albumin; CaN, calcineurin; CORT108297, (R)-4a-Ethoxy-1-(4-fluorophenyl)-6-(4-trifluoromethylbenzenesulfonyl)-4,4a,5,6,7,8-hexahydro-1H-1,2,6-triazacylopenta[b]naphthalene; DAPI, 4',6-diamidino-2-phenylindole; Dex, dexamethasone; DMSO, dimethyl sulfoxide; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; FDA, food and drug administration; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; GW9662, 2-Chloro-5-nitro-N-phenylbenzamide; MAPK, mitogen-activated protein kinase; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NS, nephrotic syndrome; O/N, overnight; PCR, polymerase chain reaction; PAN, puromycin aminonucleoside; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.1% Tween 20; Pio, pioglitazone; PPARγ, peroxisome proliferator-activated receptor gamma; PPRE, peroxisome proliferator response element; Rosi, rosiglitazone; RU486, 11β-(4-Dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one; SAPK/JNK, stress activated protein kinase/c-Jun N-terminal kinase; SDS–PAGE, SDS-polyacrylamide gel electrophoresis; TZD, thiazolidinediones.

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ABSTRACT

The FDA-approved thiazolidinediones, pioglitazone and rosiglitazone, are peroxisome proliferator-activated receptor γ (PPARγ) agonists developed to control serum glucose in diabetic patients. They have subsequently been found to reduce proteinuria and microalbuminuria in both diabetic nephropathy and non-diabetic glomerulosclerosis. We hypothesized that the renal protective effects of thiazolidinediones result, at least in part, from their direct action on podocytes, similar to glucocorticoids. Treatment with pioglitazone, rosiglitazone or dexamethasone significantly protected podocytes against puromycin aminonucleoside-induced injury (designed to mimic nephrotic syndrome-related injury), as determined by both cell survival and actin cytoskeletal integrity. Furthermore, we compared the ability of these drugs to modulate key signaling pathways in podocytes which may be critical to their protective effects. Rosiglitazone deactivated the mitogen-activated protein kinases (MAPKs), ERK1/2, p38MAPK and SAPK/JNK, while pioglitazone did not and dexamethasone deactivated to some extent. Similar to dexamethasone, both thiazolidinediones increased the glucocorticoid receptor phosphorylation, and this response to Rosi and possibly to Pio was PPARγ-dependent. Furthermore, both drugs mimicked or enhanced the effects of dexamethasone on glucocorticoid-responsive genes, in a PPARγ- and GR-dependent manner. In addition, both thiazolidinediones mimicked dexamethasone-induced effects on calcineurin activity. In summary, thiazolidinediones are able to modulate the glucocorticoid pathway and exert direct protective effects on podocytes, similar to glucocorticoids. This suggests that thiazolidinediones may have potential clinical utility as either primary or adjunctive therapy for nephrotic syndrome, or other diseases treated with glucocorticoids. These findings may also lend mechanistic insight into the well-established, but poorly understood renal protective effects of thiazolidinediones in diabetic nephropathy.

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Introduction

Peroxisome proliferator-activated receptors γ (PPARγ) are ligand–activated transcription factors of the nuclear hormone receptor superfamily known to be involved in adipogenesis, glucose homeostasis, inflammatory responses and apoptosis (Michalik et al., 2006). Endogenous ligands of PPARγ include fatty acids and prostaglandin-type eicosanoids like 15-deoxy-Δ^{12-14}-PGJ$_2$. However, the best known PPARγ agonists are the thiazolidinediones (TZD), e.g. pioglitazone (Pio), rosiglitazone (Rosi) and ciglitazone, which comprise a class of synthetic drugs that are FDA-approved and are widely used for the treatment of type II diabetes. These drugs induce a complex response involving both binding of the PPARγ to peroxisome proliferator response elements (PPRE) and various mechanisms that are independent of DNA binding. Moreover, structurally different PPARγ agonists are known to induce both common and selective responses (Gervois et al., 2007).

Thiazolidinediones are now also emerging as novel therapeutic agents in other diseases, including cancer, arthritis and inflammatory and renal diseases (Guan and Breyer, 2001; Ialenti et al., 2005; Koeffler, 2003; Yang et al., 2009). They have been shown to reduce proteinuria, microalbuminuria and podocyte injury in both diabetic nephropathy and non-diabetic glomerulosclerosis in mouse and rat models, as well as in humans (Cha et al., 2007; Ma et al., 2001; Sarafidis et al., 2010; Yang et al., 2006). In addition, Pio has been shown to provide protective effects against progression of puromycin aminonucleoside (PAN)-induced glomerulosclerosis *in vivo* and also in an *in vitro* model using cultured podocytes (Kanjanabuch et al., 2007; Yang et al., 2006). Rosi has also been reported to attenuate proteinuria and glomerulosclerosis in adriamycin-induced focal segmental glomerulosclerosis in rats (Liu et al., 2010). Based in part on this, Rosi successfully passed a phase I safety trial (Joy et al., 2009), and was scheduled for a Phase II clinical trial as a treatment for this kidney disease until being withdrawn from study due to new safety concerns (Peyser et al., 2010). Despite this, in a recent

meta-analysis it was concluded that TZDs significantly decrease albuminuria and proteinuria in patients with diabetes, and suggested that they may do so by providing direct renoprotective effects (Sarafidis et al., 2010).

Nephrotic syndrome (NS) is one of the most common kidney diseases seen in children and adults. It is a remitting and relapsing disease characterized by massive loss of serum proteins into the urine through a damaged glomerular filtration barrier, leading to hypoalbuminemia and swelling throughout the body (edema) (Smoyer and Mundel, 1998). Podocytes are a key component of the kidney's filtration barrier, and during NS they undergo dramatic structural alterations in the foot processes that attach these cells to the glomerular basement membrane. The most widely accepted experimental models used to mimic the podocyte injury that occurs during NS in humans include PAN injection of rats (Pippin et al., 2009) and PAN treatment of cultured podocytes (Ransom et al., 2005). For the last 50 years the primary therapy for NS has been oral glucocorticoids (GC). Unfortunately, GCs have serious side effects, and in ~20% of patients they are ineffective in inducing clinical remission of disease (i.e. steroid resistant NS). Thus it is clear that alternative therapies with greater efficacy and/or less severe side effects are critically needed (Hodson and Craig, 2008).

Based on the well-established, but poorly understood, renal protective effects of thiazolidinediones, we hypothesized that their protection results, at least in part, from their direct action on podocytes, similar to GCs. To test this hypothesis we compared the ability of Pio and Rosi to protect podocytes from PAN-induced injury to the known protective effects of dexamethasone (Dex) (Ransom et al., 2005; Xing et al., 2006). Additionally, given the recently-reported roles of various mitogen-activated protein kinases (MAPK) in kidney diseases (Grande and Lopez-Novoa, 2008), and the known ability of PPARγ agonists to regulate MAPK activities in other cell types (Gardner et al., 2005), we also analyzed the ability of Rosi, Pio and Dex to alter MAPK activation in podocytes. Furthermore, since TZDs have recently been reported to act as partial agonists to the glucocorticoid receptor (GR) (Ialenti et al., 2005; Matthews et al.,

2009) and GCs are the primary therapy for NS, we analyzed the ability of Rosi, Pio and/or Dex

to modulate the GC signaling pathway in podocytes.

Materials and Methods

Cell Culture. The conditionally immortalized mouse podocyte cell line MPC-5 was cultured as described previously (Smoyer and Ransom, 2002). Podocytes were cultured under proliferating conditions in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and 10 U/ml mouse γ-interferon (Sigma, St Louis, MO) at 33°C in a humidified atmosphere of 5% CO_2. Differentiation was induced by shifting the cells to 37°C in the same medium without γ-interferon for 10 to 14 days on culture plates (Greiner, Monroe, NC) coated with rat tail collagen type I (BD Biosciences, Bedford, MA). Human embryonic kidney epithelial cells (HEK 293T) were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Podocytes were treated with puromycin aminonucleoside (Sigma), rosiglitazone (Fisher HealthCare, Houston, TX), pioglitazone (Enzo Life Sciences International Inc., Plymouth Meeting, PA) or dexamethasone (Sigma) as indicated in complete or serum-free medium, or in medium containing charcoal-stripped FBS (Invitrogen) at 8%. Pre-treatments or short-term treatments were performed for 4 h followed by washing and medium change. Simultaneous or long-term treatments were performed for the entire length of the experiment, until the assay or extraction.

Use of PPARγ and GR antagonists. Where indicated, cells were pre-incubated 1 h prior to the addition of PPARγ and GR agonists, with 10 μM GW9662 (a PPARγ antagonist), 10 μM RU486 (a GR antagonist) or 10 μM CORT108297 (a highly selective GR antagonist).

GW9662 (Sigma) is an irreversible and selective PPARγ antagonist which is 10- and 600-fold less potent in binding PPARα and PPARδ, respectively.

RU486 (Sigma) is a potent GR antagonist, although with limited specificity as it also binds to the progesterone receptor. RU486 has a $K_i = 0.4$ nM in GR binding assays, and a $K_i = 1.5$ nM

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in functional GR antagonist assays (Clark, 2008; Clark et al., 2008). RU486 exhibits partial agonistic activities (Matthews et al., 2009).

CORT108297 (synthesized by Ricerca Biosciences, Concord, OH, and obtained from Corcept Therapeutics, Menlo Park, CA) is a highly selective antagonist of the GR compared to the progesterone, estrogen, androgen, and mineralocorticoid receptors, as shown in binding assays (Clark et al., 2008). It has also been found inactive for PPARγ binding (R. Clark, Corcept Therapeutics, personal communication). CORT108297 has a K_i = 0.9 nM in GR binding assays and a K_i = 6.8 nM in functional GR antagonist assays (Clark, 2008; Clark et al., 2008). It also exhibits a partial agonist activity which is higher than that of RU486 (R. Clark, personal communication).

Viability Assays. Differentiated podocytes were either pre-treated for 4 h with the vehicle dimethyl sulfoxide (DMSO), Rosi, Pio and/or Dex followed by PAN treatment (5 μg/ml), or simultaneously treated with these reagents and PAN (5 μg/ml) at concentrations specified in the 'Results' section**.** Viability assays were performed after 3 or 5 days of treatments. Briefly, cells were washed with phosphate-buffered saline (PBS), incubated in medium containing methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma) at 500 μg/ml for 4 h and the MTT formazan crystals were extracted in DMSO containing 0.01M glycine and 0.01 M NaCl (pH 10.5). Absorbance was measured at 570 nm with the reference wavelength of 630 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). Positive control cells (100% viable) did not receive any PAN treatment while cells treated for 20 min with 0.01% Triton X-100 were used as negative controls (0% viable).

Actin Staining and Microscopy. Differentiated podocytes were cultured on cover-slips and pre-treated with vehicle, 100 μM Rosi, 1 μM Pio or 1 μM Dex for 4 h. Following treatment, cells were washed and cultured for another 5 days with the medium containing PAN at 5 μg/ml. Control cells did not receive any PAN. Cells were washed 2X with PBS, fixed in 3.7%

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formaldehyde for 10 min, washed 2X with PBS, permeabilized with 0.1% Triton X-100 for 5 min, washed 2X with PBS, blocked with 1% bovine serum albumin (BSA) for 30 min and stained with Texas Red–X phalloidin (Invitrogen) for 20 min. Cells were washed 2X with PBS, air dried and mounted with Prolong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were captured using 40X objective on a Leica DMI6000B inverted fluorescence microscope (Leica Microsystems, Bannockburn, IL) equipped with a TX2 cube for visualizing Texas Red (excitation 560/40 nm, emission 645/75 nm) and with a A4 cube (excitation 360/40 nm, emission 470/40 nm) for visualizing DAPI. Digital micrographs were captured using a Retiga SRV 14-bit grayscale CCD camera (QImaging, Surrey, BC). The images were processed using Adobe Photoshop CS3 (Adobe Systems, Mountain View, CA). The DAPI (in green channel) and Texas-Red images were merged to the final images, showing actin filaments in red and cell nuclei in green.

Western Blotting. Following treatments, cells were harvested and whole cell extracts were prepared using MPER lysis reagent (Thermo Fisher Scientific Inc., Waltham, MA) containing protease and phosphatase inhibitor cocktails (Sigma). Protein concentrations were determined using bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher), according to the manufacturer's instructions. Cell lysates (20 µg protein) were processed for SDS-polyacrylamide gel electrophoresis (SDS–PAGE), and the separated proteins were transferred to nitrocellulose membranes (Bio-Rad Labs, Hercules, CA). After transfer, nitrocellulose membranes were blocked for 1 h with 5% milk in PBS-T (PBS containing 0.1% Tween 20) and incubated with primary antibody O/N. Antibodies against p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (Thr202/Tyr204), SAPK/JNK, phospho-SAPK/JNK (Thr183/ Tyr185) and phospho-GR (Ser211) were purchased from Cell Signaling Technology (Danvers, MA), anti-GR from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-GAPDH from Millipore (Billerica, MA). Membranes were washed four times with PBS-T following primary and secondary antibody incubations. Blots were probed with horseradish

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peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. Immunoreactive proteins were detected on X-ray film using the ECL chemiluminescence reagent (GE Healthcare Bio-Sciences, Piscataway, NJ). X-ray films were scanned using a calibrated ArtixScan M1 transillumination scanner (Microtek Lab, Cerritos, CA) controlled by the ScanWizard Pro program (version 7.042) using standard settings. Densitometric analysis of the integrated band density was performed using ImageJ (version 1.39; standard settings) which is available at http://rsb.info.nih.gov/ij/.

RNA Extraction and Real Time Polymerase Chain Reaction. After treatment, cells were harvested and total RNA extracted using the RNeasy kit (Qiagen, Germantown, MD). Purity and yield were determined by measuring the absorbance at 260 and 280 nm. 1 μg of RNA was subjected to DNase (Ambion, Austin, TX) treatment at 37°C for 30 min followed by a DNase inactivation step in the presence of 5 mM EDTA at 75°C for 10 min. cDNA was prepared from 1 μg DNase-treated RNA in a 20 μl reaction using iScript reverse transcriptase (Bio-Rad) at reaction conditions of 5 min at 25°C, 30 min at 42°C followed by 5 min at 85°C. FKBP51, GILZ and β-actin mRNA levels were measured by real time PCR using SYBR green and an iQ5 thermal cycler (Bio-Rad). Polymerase chain reaction (PCR) conditions were as follows: 1st cycle at 95°C for 3 min, 40 cycles at 95°C for 10 s and 55°C for 10 s followed by a melt curve analysis. The primers used for the amplification are listed in Table 1. The amplification efficiency of each primer pair was measured by plotting efficiency curve of serial dilutions of selected cDNA samples. Values were normalized to the housekeeping gene β-actin and plotted as fold change relative to time-matched vehicle control treatments.

Luciferase Reporter Assays. HEK-293T cells were co-transfected with 1 μg of pGRE-Luc (Agilent Technologies Inc., Santa Clara, CA) together with 20 ng of pRL-TK (Promega, Madison, WI) which served as control to correct for transfection efficiency using Fugene 6 (Roche Applied

Science, Indianapolis, IN). pGRE-Luc is a GR-luciferase reporter gene construct that contains 4 glucocorticoid response element (GRE) repeats $(GGTACATTTTGTTCT)_4$. The constitutively expressing pRL-TK cassette encodes the Renilla reporter gene under the control of herpes simplex virus thymidine kinase promoter. After O/N incubation in charcoal-stripped FBS-containing medium, cells were treated at various concentrations and time points as indicated in the 'Results' section, lysed after 24 h and assayed for luciferase activity using a Firefly and Renilla Luciferase reporter assay system (Biotium, Hayward, CA) as per the manufacturer's instructions.

Calcineurin Activity Assay. Differentiated podocytes were incubated O/N in medium containing charcoal-stripped FBS. Cells were treated with vehicle, 100 μM Rosi, 1 μM Pio or 1 μM Dex for 4 h, washed with PBS and further incubated in charcoal-stripped FBS-containing medium for 24 h. After 1 day, cells were lysed and the calcineurin (CaN) phosphatase activity was measured using the Colorimetric CaN Cellular Activity Assay Kit (EMD Chemicals Inc., Gibbstown, NJ) following the manufacturer's instructions. This assay used RII phosphopeptide as the substrate which is the most well known and efficient substrate for CaN, and the detection of released phosphate is based on malachite green assay. To rule out the contribution of other competing phosphatases, phosphatase activity was measured in the total extract as well as in the presence of EGTA (blocks CaN) and okadiac acid (blocks PP-1 and PP-2A but not CaN). The difference in phosphatase activities [okadaic acid-(okadiac acid-EGTA)] was read as the CaN (PP-2B) activity.

Results

Rosi and Pio Prevent PAN-Induced Podocyte Injury. To test the direct protective effect of both Pio and Rosi on cultured podocytes, cells were injured with 5 μg/ml PAN for 3 or 5 days which resulted in decreased viability to ~50-65 % and ~20-25 %, respectively, compared to untreated cells (Fig. 1). Pre-treatment of cells for 4 h, or simultaneous treatment throughout the experiment, with the PPARγ agonists, Rosi or Pio, significantly increased podocyte viability in a concentration- and time-dependent manner (Fig. 1A). Specifically, pre-treatment with Rosi at 100 μM increased the viability to ~75 % after 3 days of PAN exposure (panel a), while simultaneous treatment with 0.1, 1 or 10 μM Rosi increased the viability to ~75-85 % of that of the untreated control cells (panel b). Similarly, Pio increased the viability to ~80-90 % with both pre-treatment (0.1, 1 and 10 μM) and simultaneous treatment (0.01, 0.1 and 1 μM) after 3 days of PAN exposure (panels a, b). The glucocorticoid Dex provided almost complete protection to PAN-injured podocytes at the concentrations used (1 and 10 μM for pre-treatment, 0.1 and 1 μM for simultaneous treatment) (panels a, b). After 5 days of PAN injury, a similar pattern of protection by both TZDs was observed, although the overall extent of protection was less pronounced. Pre-treatment with Rosi at 100 μM and Pio at 1 and 10 μM increased the viability to ~50 %, compared to ~25% viability with PAN injury alone (panel c). Similarly, simultaneous treatment with 0.1, 1 or 10 μM Rosi or with 0.01, 0.1 or 1 μM Pio increased the viability to ~25-30% (panel d). Significant protection was also achieved by pre-treatment with 1 and 10 μM Dex and simultaneous treatment with 0.1 and 1 μM Dex, which increased viability to ~75% and ~60%, respectively (panels c, d).

In order to determine the potential of TZDs to improve the efficacy of GCs, we also determined the effects of Dex treatment combined with either Rosi or Pio on the protection of podocytes injured with PAN. Although combined treatment with Pio and Dex tended to result in increased cell viability compared with Dex treatment alone, it remained below the statistical

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significance (Fig. 1B).   Similarly, combined treatment of Rosi with Dex also tended to provide better protection than Dex alone (data not shown).   Viability values greater than 100% with some treatments indicated continued cell proliferation compared to controls.

These data together suggest that both TZDs exhibit the capability to protect podocytes from PAN-induced injury.

Rosi and Pio Prevent PAN-Induced Podocyte Actin Cytoskeletal Disruption.   The podocyte foot processes which are disrupted during NS are well known to contain prominent actin filaments (Ransom et al., 2005; Smoyer and Mundel, 1998).   Similarly, cultured podocytes contain a distinct actin cytoskeleton with bundles of filamentous actin fibers spanning across the cell body (stress fibers), which can be visualized with Texas Red-labeled phalloidin (Fig. 2, panel A).   Podocyte injury and recovery *in vitro* have been well-documented to involve extensive reorganization of actin cytoskeleton (Ransom et al., 2005; Smoyer and Mundel, 1998).   PAN treatment (5μg/ml for 5 days) resulted not only in a reduced cell number, but also in the substantial loss of the filamentous actin in the remaining cells (panel B).   Pre-treatment with Rosi (100 μM) or Pio (1 μM) preserved podocyte filamentous actin, in addition to the cell numbers, similarly as did pre-treatment with Dex (1 μM) (panels C-E).   However, after pretreatment with either TZD the organization of these actin fibers was somewhat different from control cells, with relatively more filamentous actin being located in sub-cortical ring-like structures.   This was especially evident after pretreatment with Pio (panel D).

Rosi Attenuates MAPK Signaling in Podocytes.   PPARγ agonists have been reported to elicit 'non-genomic', receptor-independent effects in addition to their well-documented role in inducing transcription of target genes in a PPAR- dependent manner.   The rapid alteration of MAPK activities by Rosi and Pio previously observed in several cell types provides an example of such 'non-genomic' effects (Gardner et al., 2005).   Based on this, we tested the ability of Rosi and Pio and compared it with Dex to alter the activation of the major MAPKs, including p38

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MAPK, ERK1/2 and SAPK/JNK, in podocytes. Podocytes were treated with Rosi or Pio in the presence and absence of Dex, and the relative activation of the various MAPKs was determined by immuno-blotting using antibodies specific for the phosphorylated (activated) and total forms of these protein kinases (Fig. 3A). The semi-quantitative densitometric evaluation of these gels is shown in the plots of Fig. 3B.   While treatment with Rosi deactivated all three MAPKs tested, Pio did not. In addition, treatment with Dex possibly resulted in a minor deactivation of ERK1/2 and p38 MAPK. Combined treatment with Rosi and Dex resulted in a further deactivation of Erk1/2 when compared with individual treatments, while it had no major effect on the activities of the other two MAPKs. Combined treatment with Pio and Dex resulted in generally similar MAPK activities compared to Dex treatment alone.

In summary, deactivation of the various MAPKs in podocytes appeared to be a selective property of Rosi as compared with Pio, although Dex also modestly deactivated ERK1/2 and p38 MAPK as well.

Rosi and Pio Increase the GR Phosphorylation in Podocytes. The GR has been shown to undergo rapid down-regulation and phosphorylation on Ser 211 in response to GCs, and phosphorylation at this site is believed to be associated with activation of the GR (Tasker et al., 2006).   Recently, PPARγ ligands have been reported to exert 'off-target' effects involving seemingly unrelated molecules and receptors, including the GR (Ialenti et al., 2005; Matthews et al., 2009). Recent studies have also shown that the PPARγ and GR may interact directly upon PPARγ activation, suggesting cross-talk between the two signaling pathways (Lahiri et al., 2009; Nie et al., 2005). To determine if Rosi and Pio exert some of their effects in podocytes by modulating the expression or phosphorylation of the GR, we measured the total amount of the GR and its phosphorylation in podocytes treated with Rosi and Pio, and compared these responses to that of Dex (Fig. 4A). As seen after 4 h of treatment, Dex induced down-regulation of the GR and enhanced its phosphorylation, similarly as has been previously reported (Guess

et al., 2010). Interestingly, Rosi and Pio also increased the phosphorylation of the GR, although they had no detectable effect on its down-regulation. Combined treatments with Rosi or Pio together with Dex did not show any detectable differences from Dex treatment alone. In summary, Rosi, Pio and Dex all resulted in increased phosphorylation of the GR in the following order: Dex>Rosi>Pio.

In order to dissect if the phosphorylation of the GR by Rosi and Pio is PPARγ and/or GR-mediated effect, we performed the above experiments in the presence of antagonists specific for these receptors (Fig. 4A). In the presence of the PPARγ antagonist, GW9662, Rosi treatment did not result in increased GR phosphorylation, thus suggesting PPARγ dependence. Also, the effects of Pio were inhibited to some extent by GW9662. No differences were observed in the presence or absence of GW9662 with Dex alone or combined treatments, suggesting that PPARγ is not involved in the GR phosphorylation in response to Dex.

As expected, the GR antagonists, RU486 and CORT108297, inhibited the phosphorylation of the GR in response to Dex alone or to the combination treatments (Fig. 4A). RU486 and CORT108297 by themselves increased GR phosphorylation moderately; consistent with the known partial agonistic properties of these drugs (see Materials and Methods). This increased GR phosphorylation in response to the GR antagonists may obscure possible effects resulting from Rosi or Pio alone treatments. Therefore, it is difficult to conclude if phosphorylation of the GR in response to Rosi and Pio is dependent on GR signaling or not.

In summary, our data suggest that the phosphorylation of GR in response to Rosi, and possibly Pio, is PPARγ-dependent, and that GR phosphorylation by Dex alone or in combination with Rosi and Pio requires GR-signaling.

In a separate experiment we determined the time course of the increase in phosphorylation of the GR at Ser211 in response to the Dex, Rosi, and Pio by semi-quantitative evaluation of the corresponding immuno-blots (Fig. 4B). In order to estimate the changes in the relative degree

of phosphorylation of the GR, the signals for phospho-GR were related to those of total GR. Treatment with Dex greatly increased GR phosphorylation in a time-dependent manner. Treatment with Rosi and Pio also resulted in increased GR phosphorylation, although to a lesser extent. Pio seemed to induce an early response with no further increase in the relative phosphorylation of the GR after 0.5 h.

Rosi and Pio Induce and Enhance the GR Transcriptional Activity in Podocytes. Given our observations that TZDs are able to both protect podocytes and stimulate the GR, although to a lesser extent than Dex, we examined the ability of Rosi and Pio to regulate the transcription of two well-studied GC-responsive genes, FKBP51 and GILZ (Ayroldi and Riccardi, 2009; Vermeer et al., 2003). FKBB51 is commonly associated with the GR complex and tends to inhibit its activity, while GILZ is a transcription factor controlling the expression of many other genes in response to GCs. Dex increased the mRNA expression of FKBP51 ~10-15 fold and GILZ ~3-5 fold, as measured after 1 and 3 days of treatment, following either short-term or long-term treatments (Fig. 5A, B). Rosi and Pio alone induced FKBP51 and GILZ mRNA expression (~2 fold) in both short-term (except for GILZ induction in response to Pio) and long-term treatments, as measured after 3 days. Both TZDs also enhanced the induction of FKBP51 and GILZ mRNAs by Dex, as seen in both short-term and long-term treatments for FKBP51 (3 days), as well as in short-term treatments for GILZ (1 day). Specifically, Rosi and Pio enhanced the induction of FKBP51 from ~10-15 fold by Dex alone to ~20 fold when used in combination after 3 days (Fig. 5A).

Modulation of the GR Transcriptional Activity in Podocytes by Rosi and Pio is both GR- and PPARγ-dependent. Given the 'GR modulator effects' of the TZDs in podocytes (see above), we determined if Rosi and Pio modulate the transcriptional activity of the GR in a GR- and/or PPARγ-dependent way. For this purpose, we measured the expression of GC-responsive genes in podocytes in response to Rosi, Pio, and Dex in the presence of the GR-

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and PPARγ-specific antagonists RU486, CORT108297 and GW9662 (Fig. 6). As expected, RU486 blocked the induction of FKBP51 and GILZ mRNAs by Dex, alone or in combination treatments. Moreover, there was no significant difference in FKBP51 expression between the Dex alone treatment and combined treatments with Rosi and Pio in the presence of RU486 (unlike in the absence of the antagonist), suggesting that RU486 also inhibits any enhancing effects of Rosi and Pio on Dex-induced expression. Given the known non-specific antagonistic effects of RU486 on other nuclear receptors (e.g. the progesterone receptor), this GR dependence was further confirmed by measuring the mRNA expression of one of the studied genes, FKBP51, using the highly specific GR antagonist, CORT108297. Similar to RU486, CORT108297 blocked the induction of FKBP51 by Dex alone or in combination treatments. Also, as observed with RU486, no significant difference was observed between the Dex alone treatment and combined treatments with Rosi and Pio in the presence of CORT108297 (unlike in the absence of the antagonist). While CORT108297 is a highly specific antagonist for the GR, it is also known to exert partial agonistic effects (R. Clark, Corcept Therapeutics, personal communication; also see Materials and Methods). This partial agonistic activity is reflected in the relatively high baseline expression of FKBP51 in the presence of CORT108297 (Fig. 6).

Finally, in order to determine the PPARγ dependence, the effects of Rosi and Pio in the presence of the PPARγ antagonist, GW9662, were also measured on the induction and enhancement of Dex-induced mRNA expression of FKBP51 (Fig. 6). GW9662 blocked the induction of FKBP51 mRNA by Rosi as well as the Rosi- and Pio-mediated enhancement of Dex-induced increase in FKBP51 expression. Also, no significant difference was observed between the Dex alone treatment and combined treatments with Rosi and Pio in the presence of GW9662 (unlike in the absence of the antagonist). As expected, GW9662 did not significantly reduce the expression of FKBP51 induced by Dex alone. These data suggest that both the

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induction of FKBP51 and the enhancement effects on Dex-induced expression by TZDs are PPARγ-dependent.

Taken together, the transcriptional activity of the GR in podocytes can be modulated by both Rosi and Pio, and this modulation involves both GR and the PPARγ signaling.

Rosi and Pio Have Disparate Effects on Dex-Induced Activity of Minimal and Native Endogenous Promoters. To further examine the direct effects of Rosi and Pio on GR transcriptional activity, we analyzed the luciferase reporter activity under the control of a minimal promoter containing 4 GRE repeats (GGTACATTTTGTTCT) in HEK293T cells (Fig. 7A, B). Although Dex activated the promoter strongly, we did not observe major effects by Rosi or Pio in activating the luciferase gene expression driven by the minimal GRE promoter. Similarly, both Rosi and Pio, in combination with Dex did not exhibit major effects on Dex-induced activation of this promoter, in both short-term and long-term treatments.

Since we had previously seen enhancing effects on endogenous gene expression in podocytes when these drugs were used in combination (cf. Fig. 5), we chose to explore if the absence of enhancement effects were due to the different cell types used, or if they were dependent on promoter specificity. We therefore analyzed the expression of the endogenous genes, FKBP51 and GILZ, in HEK293T cells treated with Rosi, Pio and/or Dex (Fig. 7 C, D). We observed that Rosi or Pio alone significantly induced the expression of both FKBP51 and GILZ. Dex, as expected, also induced the expression of these genes. Combined treatments with Rosi showed enhancing effects on Dex-mediated activation of these endogenous promoters, as was also shown in podocytes in Fig. 5. Combined treatment with Pio enhanced the Dex-mediated activation of FKBP51. These observations are in accordance with earlier studies in which Rosi had an inhibitory effect on Dex-mediated activation of a simple promoter (TAT3) (Matthews et al., 2009), whereas it enhanced Dex-mediated activation of the natural and more complex MMTV promoter (Johnson et al., 1999).

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Taken together, both TZDs exhibited disparate effects on the minimal promoter tested and endogenous promoters. While the activity of the minimal promoter tested was not affected by either TZD, the expression of the endogenous genes (driven by native promoters) tested was clearly enhanced.

Rosi, Pio and Dex Increase CaN Activity in Podocytes. CaN inhibitors, including FK506 and cyclosporine are widely-used drugs for the treatment of NS in cases where GCs are clinically ineffective. Previous reports have demonstrated the ability of Dex to stimulate CaN phosphatase activity in some cell types and suppress it in other cell types (Hirakawa et al., 2009; Tumlin et al., 1997). To date, neither the ability of Rosi, Pio, nor even Dex, to alter CaN activity in podocytes has been studied. Therefore, we compared the CaN activity in podocytes treated with each of these compounds by measuring phosphate released by CaN phosphatase activity (Fig. 8). We found that Dex treatment significantly stimulated CaN activity in podocytes, as measured by the total phosphate released. Likewise, Rosi and Pio both exhibited similar effects to Dex by increasing the CaN activity significantly, thus suggesting another similarity between the molecular actions of TZDs and those of GCs in podocytes.

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Discussion

The TZDs, Pio and Rosi, are widely used FDA-approved drugs for the treatment of Type II diabetes that have also been shown to reduce proteinuria and urinary podocyte loss in patients with diabetic nephropathy and non-diabetic glomerulosclerosis (Sarafidis et al., 2010). We hypothesized that these renal protective effects of TZDs result, at least in part, from a direct action on podocytes. We found that both Pio and Rosi were indeed able to directly protect podocytes, similar to Dex, against experimentally-induced injury designed to mimic NS. Additionally, we observed that Rosi and/or Pio were able to modulate the podocyte GR pathway and alter MAPK activation, as illustrated in Figure 9. We also found that Rosi, Pio and Dex induced common, as well as selective, molecular signaling effects in podocytes. Together, our findings suggest that Pio and Rosi may have potential clinical utility as either a primary or adjunctive therapy for NS, or other diseases treated with GCs. Moreover, these findings may also lend new mechanistic insight into the well-established, but poorly understood renal protective effects of TZDs in diabetic nephropathy (Lennon et al., 2009).

Healthy podocytes contain an extensive network of actin filaments, with high content in the distal foot processes that attach to the underlying glomerular basement membrane (Smoyer and Mundel, 1998). During NS, podocyte injury results in rearrangement of the actin cytoskeleton in the foot processes (effacement), and in some cases apoptosis, cell proliferation or dedifferentiation. Podocytes typically respond to stressors (e.g. treatment with PAN, shear stress) with reorganization or loss of filamentous actin, focal adhesions, or cell-cell contacts. In cultured podocytes these changes can be prevented by treatment with GCs or by ectopic expression of protective genes (Ransom et al., 2005). In the present study, we found that, similar to GCs, TZDs can directly protect podocytes from PAN-induced injury, both in terms of cell viability and disruption of the actin cytoskeleton.

TZDs act as PPARγ ligands, but are becoming increasingly known to mediate a portion of their actions through PPARγ-independent pathways (Gardner et al., 2005; Ialenti et al., 2005).

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Additionally, it has been reported that minor differences in the chemical structure of Rosi and Pio may result in marked and distinct differences in their molecular, biological and pharmacological responses (Gervois et al., 2007; Kintscher, 2008). Given these selective effects of Rosi and Pio, and the PPARγ-independent effects of TZDs in general, we explored the ability of Rosi and Pio to modulate two of the major signaling pathways (MAPK and GC pathways) most relevant to podocyte protection in various renal diseases, including NS (Grande and Lopez-Novoa, 2008; Guess et al., 2010; Ransom et al., 2005).

The MAPKs have been implicated in the progression of various glomerulopathies, and their inhibition is emerging as a promising therapeutic area for renal diseases, as well as for steroid resistance in other diseases (Bloom, 2004; Grande and Lopez-Novoa, 2008). Given the importance of MAPK signaling in renal diseases, we were also interested in determining the ability of TZDs to modulate the various MAPKs in podocytes, and in comparing these results to those of GCs. We found that Rosi deactivated ERK1/2, p38 MAPK and SAPK/JNK. While Dex also seemed to deactivate ERK1/2 and possibly p38 MAPK, Pio did not have any notable effects. Inhibition of p38 MAPK and possibly ERK1/2 in PAN and adriamycin animal models of NS has been reported to suppress proteinuria and actin reorganization in podocytes (Koshikawa et al., 2005). In addition, we have shown that inhibiting either p38 MAPK or its downstream substrate MK2 prevents PAN-induced injury to podocytes (Pengal et al., unpublished). Increased amounts of activated ERK and p38 MAPK have also been reported in the glomeruli of patients with diabetic nephropathy (Sakai et al., 2005).

GCs are the prevailing therapy for NS and are also known to act directly on podocytes, although not much is known about their mechanism of protective action (Guess et al., 2010; Ransom et al., 2005). We studied the effects of TZDs on the GR pathway in podocytes and found that Rosi and Pio phosphorylate the GR at the site associated with its activation, although to a lesser extent than Dex. In support of this finding, other recent reports have also linked the action of TZDs to the GR pathway (Ialenti et al., 2005; Matthews et al., 2009). In these studies,

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the anti-inflammatory effects of Rosi and ciglitazone were shown to involve, to some extent, GR-dependent signaling. Additionally, TZDs were shown to activate nuclear translocation of the GR *in vitro*, with no effect on its down-regulation and independent of the PPARγ. Similarly, in the present study Rosi and Pio did not lead to down-regulation of the GR, which is a known negative feedback mechanism associated with GC treatment. Theoretically, such an effect could be clinically beneficial if TZDs were used in patients with steroid-resistant NS (or other steroid resistant diseases), since it might overcome this known negative feedback mechanism. Furthermore, we found that the phosphorylation of GR by Rosi and possibly Pio is PPARγ dependent as it is abolished in the presence of GW9662, a selective PPARγ antagonist. This suggests that the PPARγ may interact with the GR signaling pathway by physical or co-factor dependent interaction upon activation as has also been shown recently in other systems (Lahiri et al., 2009; Nie et al., 2005).

Given the complex nature of both GR- and PPARγ agonist-mediated signaling, the interplay of transcription co-factors (activators or repressors) can be assumed to be altered by PPARγ agonists. Such crosstalk among signaling cascades may be the basis for the synergistic effects on GR signaling exerted by Rosi and Dex in a mouse model of inflammation, or for their ability to modulate Dex- as well as Rosi-induced gene transcription and differentiation in osteoblastic cells (Ialenti et al., 2005; Johnson et al., 1999). In the latter report, Rosi did not exhibit any effect alone in activating the native MMTV promoter, whereas it enhanced the effects of Dex on the same promoter. In a different report, Rosi and Pio minimally activated a GRE TAT3 promoter, but repressed its activity when used in combination with Dex (Matthews et al., 2009). In the current study using a minimal GRE promoter with 4 GRE sites, combination treatments using Rosi or Pio with Dex did not significantly alter the GRE promoter activity. Despite this, Rosi and Pio both induced the expression of two endogenous GC-responsive genes, GILZ and FKBP51, and also enhanced Dex-induced expression of these genes. These findings, together

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with the above reports, suggest that the effects of TZDs on Dex-regulated gene expression can be very disparate, depending on the nature of the studied promoters and genes. Moreover, our findings that GR phosphorylation by Rosi and possibly Pio is PPARγ mediated, and that both TZDs modulate GR-responsive genes in podocytes in a PPARγ- and GR-dependent manner suggest that these drugs are indeed able to modulate GR signaling in podocytes, and that their protective effects could possibly be mediated in part via these pathways.

Lastly, since CaN inhibitors are often used as alternative treatments for NS, we investigated the ability of TZDs to alter CaN activity in podocytes. We also compared their modulation of CaN activity to that of Dex, since GCs are known to provide direct protection to podocytes against injury. Of particular note, we found that all three drugs which protected podocytes against PAN-induced injury (Pio, Rosi, and Dex) also increased the phosphatase activity of CaN in these cells. This finding is consistent with a previous report of transcription-independent activation of CaN by GCs in renal proximal tubular cells (Tumlin et al., 1997). However, our findings of increased podocyte CaN activity in response to Rosi, Pio and Dex were somewhat surprising, since CaN inhibitors (cyclosporine and tacrolimus) are widely used clinically to treat steroid dependent and steroid resistant NS. Moreover, CaN inhibition has been suggested to be involved in cyclosporine's direct effects on podocytes via stabilization of the actin cytoskeleton (Faul et al., 2008). Despite this, the current findings suggest the possibility that inhibition of podocyte CaN activity may not be critical for the clinical efficacy of cyclosporine and tacrolimus in NS. Thus, while the role of podocyte CaN activity in response to injury remains unclear at this time, our results suggest yet another similarity between the molecular actions of GCs and TZDs in podocytes.

In summary, we have found that TZDs are able to modulate the GC pathway and exert direct protective effects on podocytes, similar to GCs, possibly via previously unrecognized effects on GC and MAPK signaling. These findings suggest that these FDA-approved drugs

may have potential clinical utility as either primary or adjunctive therapy for NS, or other diseases treated with GCs. This study may also offer mechanistic insight into the well-established, but poorly understood renal protective effects of Rosi and Pio in patients with diabetic nephropathy.

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Acknowledgments

Authorship Contributions

Participated in research design: Agrawal, Benndorf and Smoyer.

Conducted experiments: Agrawal and Guess.

Performed data analysis: Agrawal, Guess, Benndorf and Smoyer.

Wrote or contributed to the writing of the manuscript: Agrawal, Benndorf and Smoyer.

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FOOTNOTES

a)

b)

Part of this work was previously presented as a poster (abstract) at the 8th International Podocyte Conference (June 7-9, 2010, Bristol, UK): Agrawal, S., A. Guess, R. Benndorf, W.E. Smoyer. PPAR-γ agonists protect cultured podocytes from nephrotic syndrome-related injury and alter glucocorticoid and MAPK signaling.

c)

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Figure Legends

Fig. 1. Viability assays of PAN-injured podocytes treated with Rosi, Pio and/or Dex. A, Podocytes were either pre-treated for 4 h (a, c) or simultaneously treated until the completion of assay (b, d) with vehicle, Rosi, Pio or Dex at indicated concentrations, injured with 5 μg/ml PAN for 3 days (a, b) or 5 days (c, d) and assayed for viability using MTT. B, Podocytes were treated with vehicle, Pio and/or Dex at 1 μM each for pre-treatments (a, c), or with 0.1 μM each for simultaneous treatments (b, d), injured with 5 μg/ml PAN for 3 days (a, b) or 5 days (c, d) and assayed for viability using MTT. Viability values greater than 100% with some treatments indicate continued cell proliferation compared to controls. Significant differences from control ($\star$) or PAN treatment ($\dagger$) are indicated as obtained by unpaired t test ($\star$/$\dagger$, P<0.05; $\star\star$/$\dagger\dagger$, P<0.01; $\star\star\star$/$\dagger\dagger\dagger$, P<0.001). Black columns, controls; white columns, PAN treatment; gray columns, PAN + TZD/Dex treatments.

Fig. 2. Effects of Rosi, Pio or Dex treatments on PAN-induced podocyte actin cytoskeletal injury. Following pre-treatment for 4 h with vehicle, 100 μM Rosi, 1 μM Pio or 1 μM Dex, cells were injured with 5 μg/ml of PAN for 5 days. Following fixation, actin filaments were stained with Texas Red phalloidin (shown in red) and nuclei were stained with DAPI (shown in green). The scale bar indicates 25 μm.

Fig. 3. Effects of Rosi, Pio and Dex treatments on activities of MAPKs in podocytes. A, Serum-starved cells were treated with vehicle, 100 μM Rosi, 1 μM Pio and/or 10 μM Dex for 4 h, harvested, and the total protein extracts were subjected to SDS-PAGE

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followed by immuno-blot analysis for phosphorylated (activated) and total ERK1/2, p38 MAPK and SAPK/JNK. GAPDH served as a loading control. Representative blots from 3 independent experiments are shown. B, Densitometric analysis of the blots shown in A was performed and the ratios of the phosphorylated (p-) and total MAPK forms were plotted as fold changes relative to the controls.

Fig. 4. Phosphorylation and expression of the GR in podocytes in response to Rosi, Pio and Dex. A, Serum-starved cells were treated for 4 h with vehicle, 100 μM Rosi, 1 μM Pio, or 10 μM Dex alone, or in combination as indicated. Where indicated, cells were pre-incubated with 10 μM GW9662 (PPARγ antagonist), 10 μM RU486 or 10 μM CORT108297 (GR antagonists) for 1 h prior to Rosi, Pio and Dex treatments. Protein lysates were subjected to SDS-PAGE followed by immuno-blot analysis for phosphorylated (p-GR) and total GR, and for GAPDH. Representative blots from 3 independent experiments are shown. B, Cells were treated with Rosi, Pio and Dex as in A, harvested at 0 h (control), 0.5 h, 2 h and 4 h, and subjected to immuno-blot analysis. Densitometric analysis was performed and the ratios of the phosphorylated and total GR were plotted as fold increases relative to the control (indicated by the dotted line).

Fig. 5. mRNA expression of FKBP51 and GILZ in podocytes in response to Rosi, Pio and/or Dex. A, B, Cells incubated O/N in charcoal-stripped FBS-containing medium were subjected to short-term treatment (4 hrs) with 100 μM Rosi, 1 μM Pio and/or 10 μM Dex, or long-term treatment (1 day or 3 days) with 10 μM Rosi, 0.1 μM Pio and/or 1 μM Dex. Total RNA was extracted after 1 and 3 days and analyzed by real time RT-

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PCR using SYBR green for mRNA expression analysis of FKBP51 and GILZ, and the values were normalized to β-actin. Fold change values compared to controls (treatment with vehicle only) were plotted as mean ± SD from triplicates of three experiments. Significant differences of individual treatments versus controls (⋆) or combined treatments versus Dex treatments (†) are indicated as obtained by unpaired t test (⋆/†, P<0.05; ⋆⋆/††, P<0.01; ⋆⋆⋆/†††, P<0.001).

Fig. 6. mRNA expression of FKBP51 and GILZ in podocytes in response to Rosi, Pio and/or Dex in the presence of GR and PPARγ antagonists. Serum-starved cells were pre-incubated for 1 h with 10 μM GW9662 (PPARγ antagonist), 10 μM RU486 or 10 μM CORT108297 (GR antagonists), followed by 4 h treatments with 100 μM Rosi, 1 μM Pio and/or 10 μM Dex. Total RNA was extracted after 3 days and analyzed by real time RT-PCR assay using SYBR green for mRNA expression analysis of FKBP51 and GILZ, and the values were normalized to β-actin. Fold change values compared to controls (treatment with vehicle only) were plotted as mean ± SD from triplicate experiments. Significant differences in the absence of antagonists of individual treatments versus controls (⋆) and combined treatments versus Dex treatments (†), and significant decreases in the presence versus absence of antagonists (#) are indicated as obtained by unpaired t test (⋆/†/#, P<0.05). (⋆/†/#, P<0.05; ⋆⋆/††/##, P<0.01; ⋆⋆⋆/†††/###, P<0.001).

Fig. 7. Effects of Rosi and Pio on a minimal promoter containing GREs and on endogenous genes. A, B, Action of Rosi and Pio on Dex-induced luciferase reporter

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activity driven by the minimal GRE promoter. HEK-293T cells were co-transfected with pGRE-luc (firefly luciferase reporter gene under the control of minimal GRE promoter) and pRL-TK (constitutively expressed Renilla luciferase), treated for 4 h (A, short-term) or for 24 h (B, long-term) with indicated concentrations of Dex, Rosi and Pio after O/N incubation in charcoal-stripped FBS-containing medium. After 24 h of treatment, cells were lysed and assayed for firefly and Renilla luciferase activities. The values of firefly luciferase activity were corrected for transfection efficiency using the Renilla luciferase, and plotted as mean $\pm$ SD from triplicate analyses and are representative of three experiments. The plotted fold changes are in comparison to vehicle-treated control cells transfected with the reporter constructs. C, D, mRNA expression of FKBP51 and GILZ in HEK293T cells in response to Rosi, Pio and/or Dex. Cells incubated O/N in charcoal-stripped FBS-containing medium were treated for 4 hrs with 100 μM Rosi, 1 μM Pio and/or 10 μM Dex. Total RNA was extracted after 1 day and analyzed by real time RT-PCR assay for expression of FKBP51 and GILZ, and the values were normalized to β-actin. Fold change values compared to controls (treatment with vehicle only) were plotted as mean $\pm$ SD from triplicates and are representative of three experiments. Significant differences of individual treatments versus controls ($\star$) or combined treatments versus Dex treatments ($\dagger$) are indicated as obtained by unpaired t test ($\star$/$\dagger$, P<0.05; $\star\star$/$\dagger\dagger$, P<0.01; $\star\star\star$/$\dagger\dagger\dagger$, P<0.001).

Fig. 8. CaN activity of podocytes treated with Rosi, Pio or Dex. Cells incubated O/N in charcoal-stripped FBS-containing medium were treated with 1 μM Dex, 100 μM Rosi or 1μM Pio for 4 h and assayed for CaN activity after 24 h by detecting the free phosphate

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released from the RII phosphopeptide substrate by malachite green assay. The values plotted on the graph are the mean ± SD from triplicates and are representative of three experiments. Significant differences of treatments versus the control are indicated as obtained by unpaired t test (∗, P<0.05; ∗∗, P<0.01).

Fig. 9. Schematic of canonical and alternate modes of action of TZDs. Traditionally, TZDs bind to their receptor, PPARγ, which dimerizes with the nuclear receptor RXR and acts on peroxisome proliferator response elements (PPRE) together with co-activators to promote the transcription of genes involved e.g. in anti-diabetic effects. Our data and the literature suggest that the TZDs can act on the GR directly (off-target pathway), as well as via the canonical PPARγ pathway with considerable cross-talk between the two pathways. In the 'off target' pathway, TZDs may imperfectly bind to and stimulate the GR via phosphorylation and nuclear translocation, possibly resulting in expression of GC-responsive genes. TZDs may also modulate the GR pathway indirectly via binding to PPARγ, resulting in subsequent interaction between the two receptors directly or through the involvement of common co-factors. TZDs may also deactivate MAPKs via a 'non-genomic' pathway which subsequently modify other cellular systems including phosphorylation of the GR and PPARγ. This schematic was modified from the Nuclear Receptor Resource (nrresource.org) with the permission of Dr. J. Vanden Heuvel, University Park, PA.

TABLE 1. Genes and their respective primers used in this study

Gene	Gene description (Protein)	Forward[a]	Reverse[a]	Amplicon (bp)
Fkbp5	murine FK506 binding protein 51 (FKBP51)	cttggaccacgctatggttt	tcccttctctttcacgatgg	171
Tsc22d3	murine glucocorticoid induced leucine zipper (GILZ)	cgtgaagaaccacctgatgtacgc	ttacaccgcagaaccaccaggggc	235
Actb	murine β-actin	cttcgttgccggtccacaccc	ctgggcctcgtcacccacat	212
FKBP5	human FKBP51	tccctcgaatgcaactctct	aaacatccttccaccacagc	178
TSC22D3	human GILZ	ggtgaagaatcatctgatgtatgc	ttacaccgcagaaccaccaggggc	226
ACTB	human β-actin	acagagcctcgcctttgccg	cttgctctgggcctcgtcgc	238

[a]The sequences are in 5' to 3' orientation.
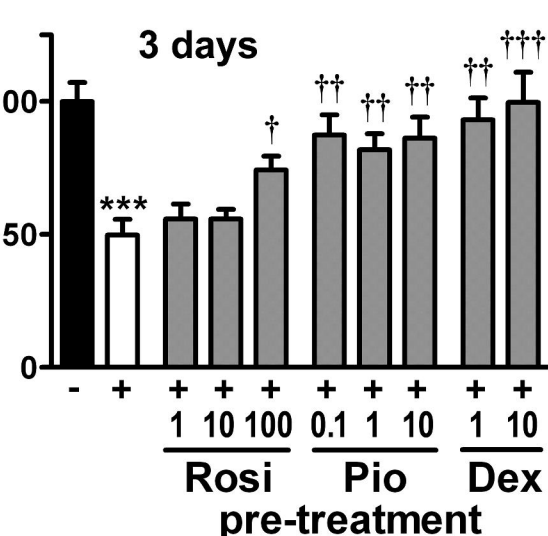
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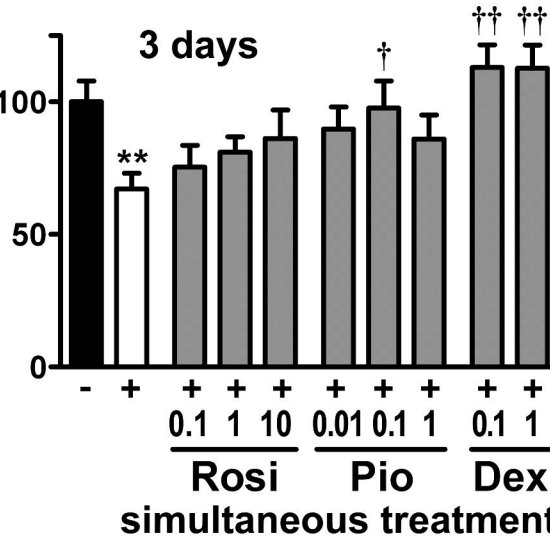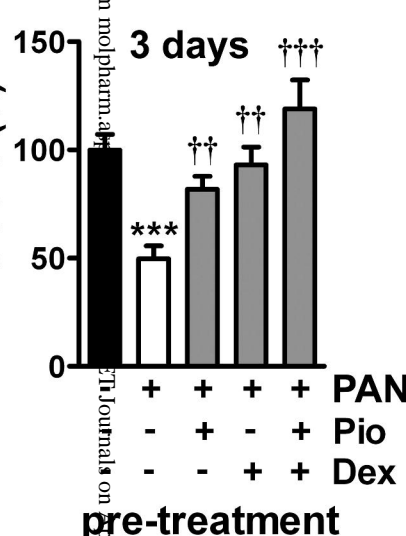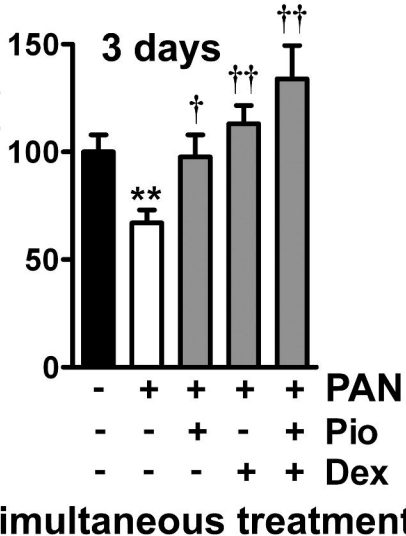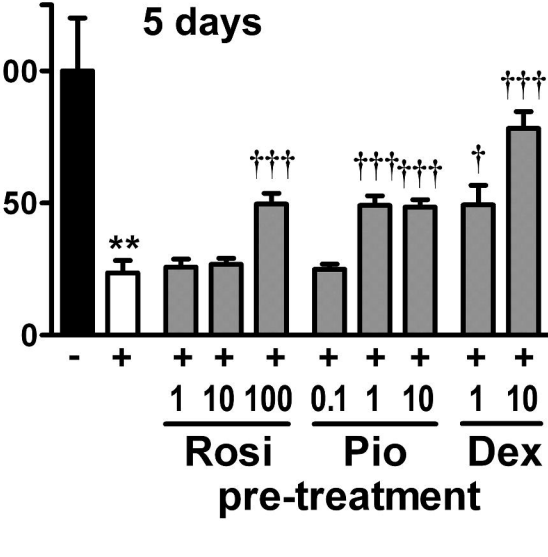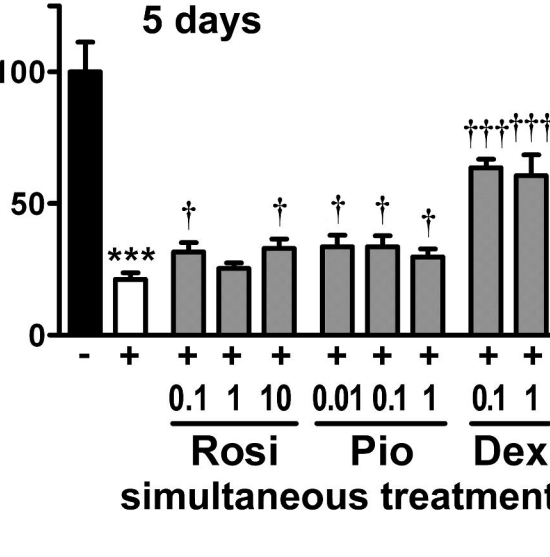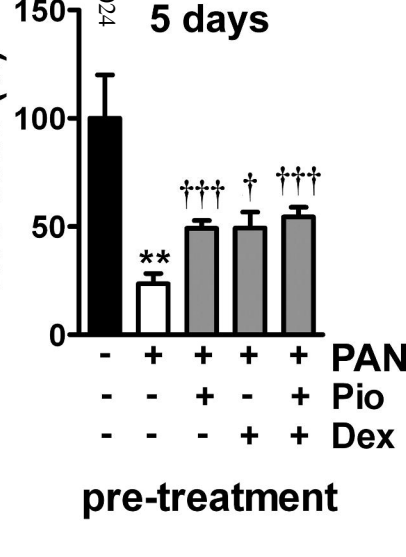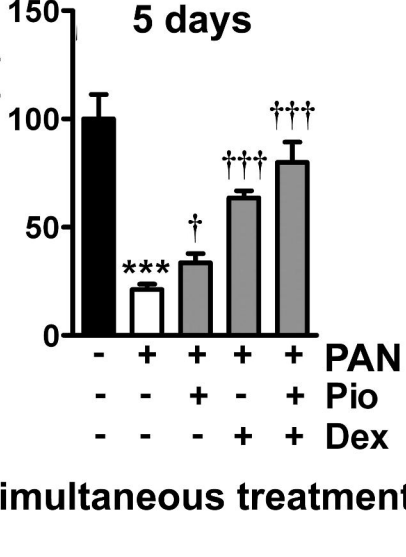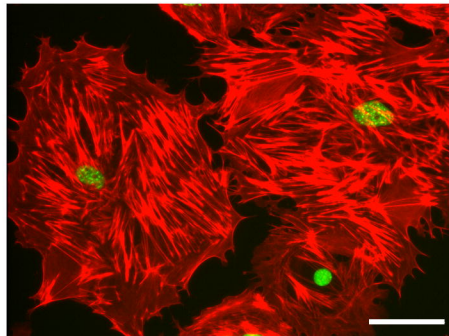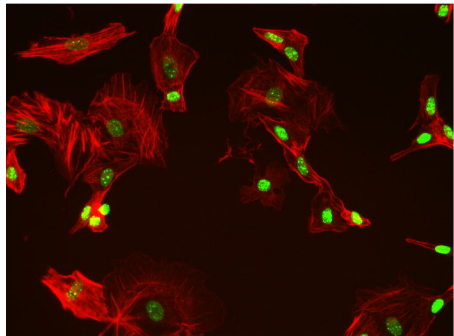
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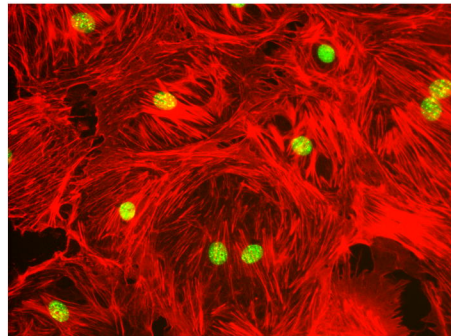
A control **B** PAN **C** PAN + Rosi **D** PAN + Pio **E** PAN + Dex
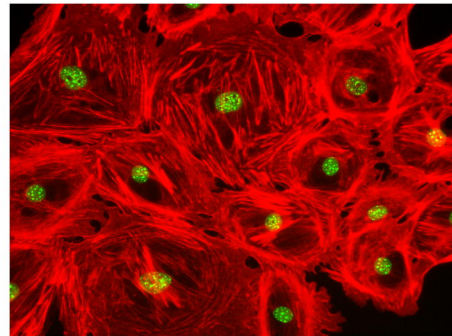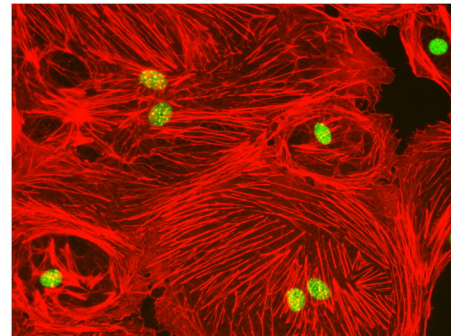
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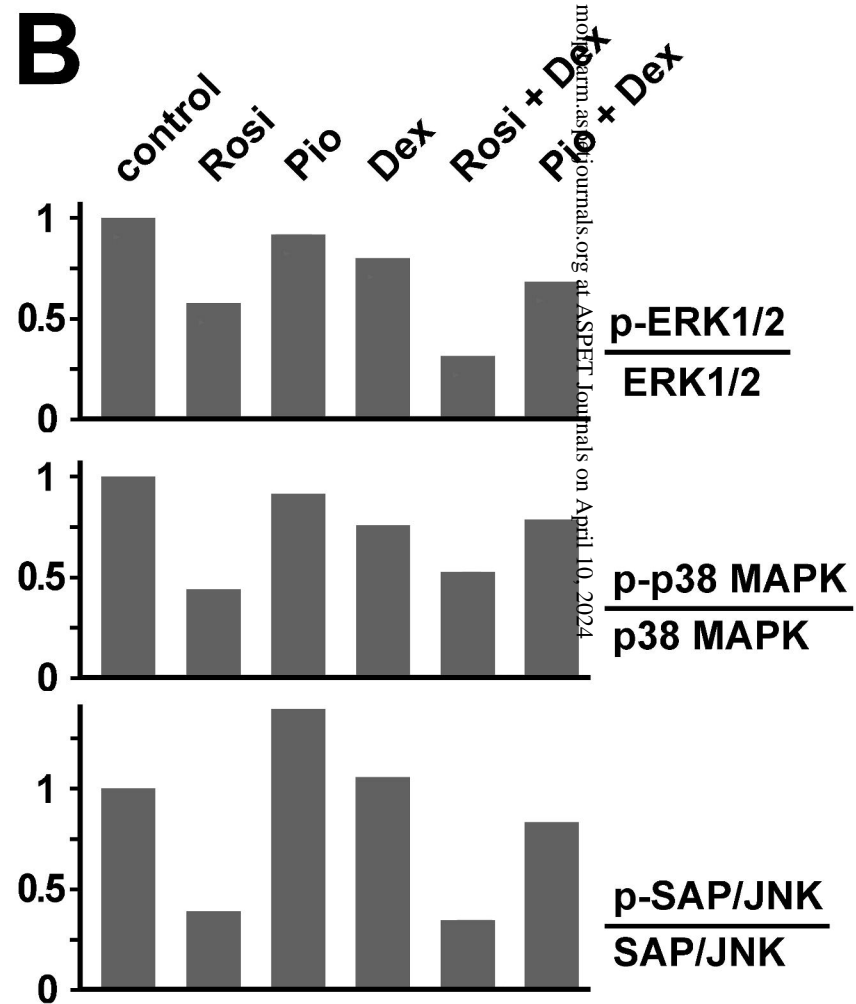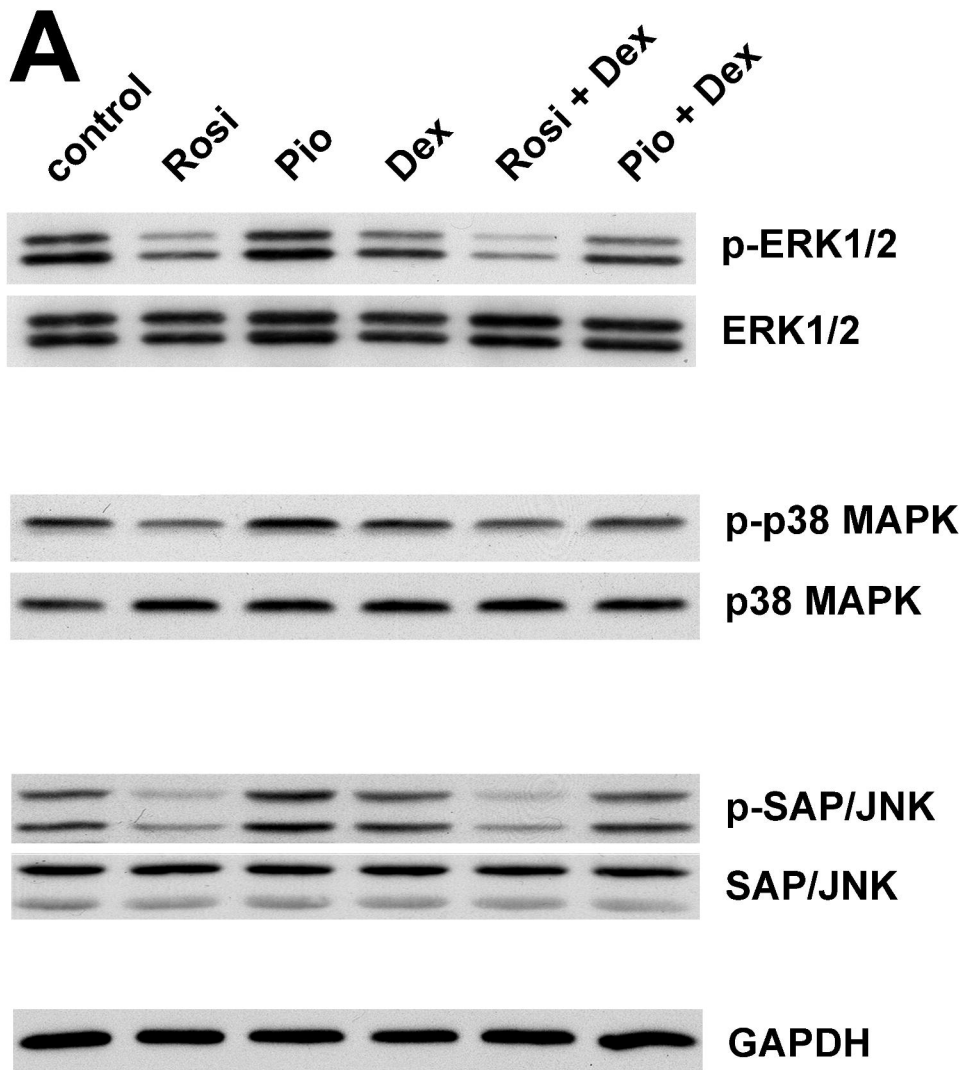
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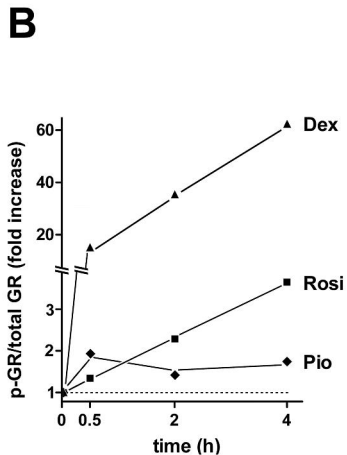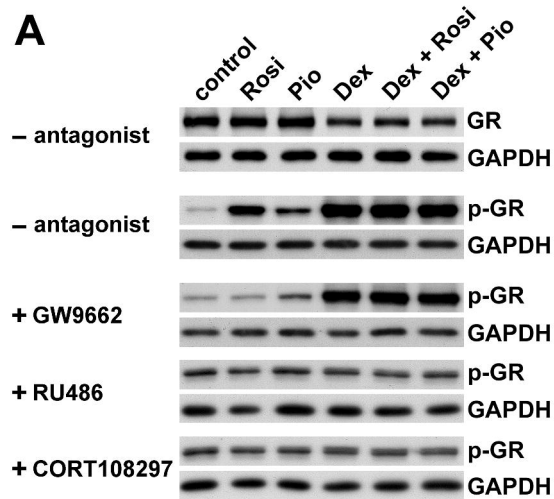
Figure 4

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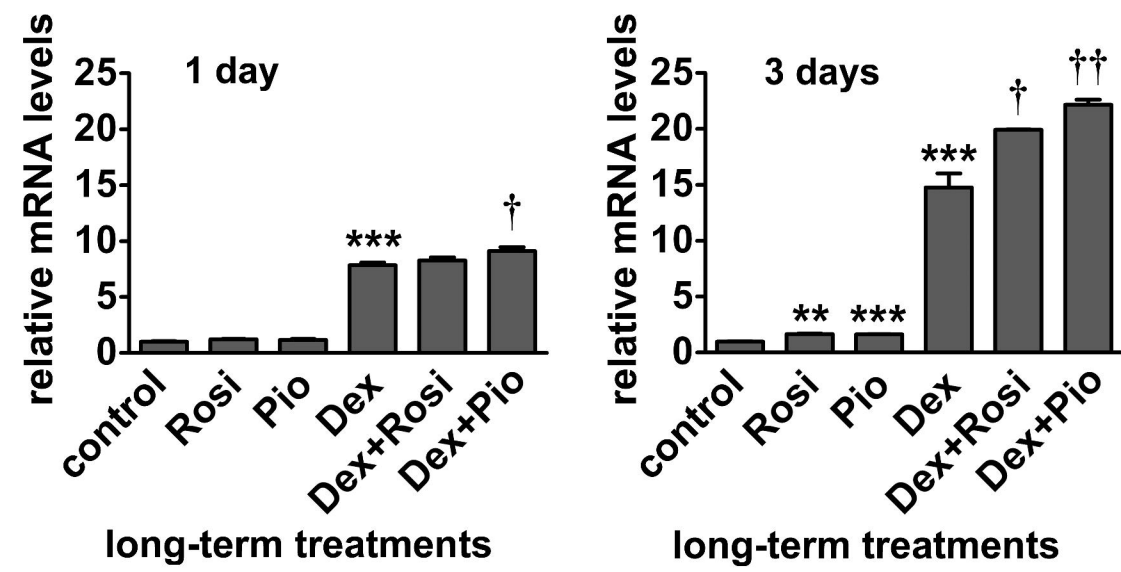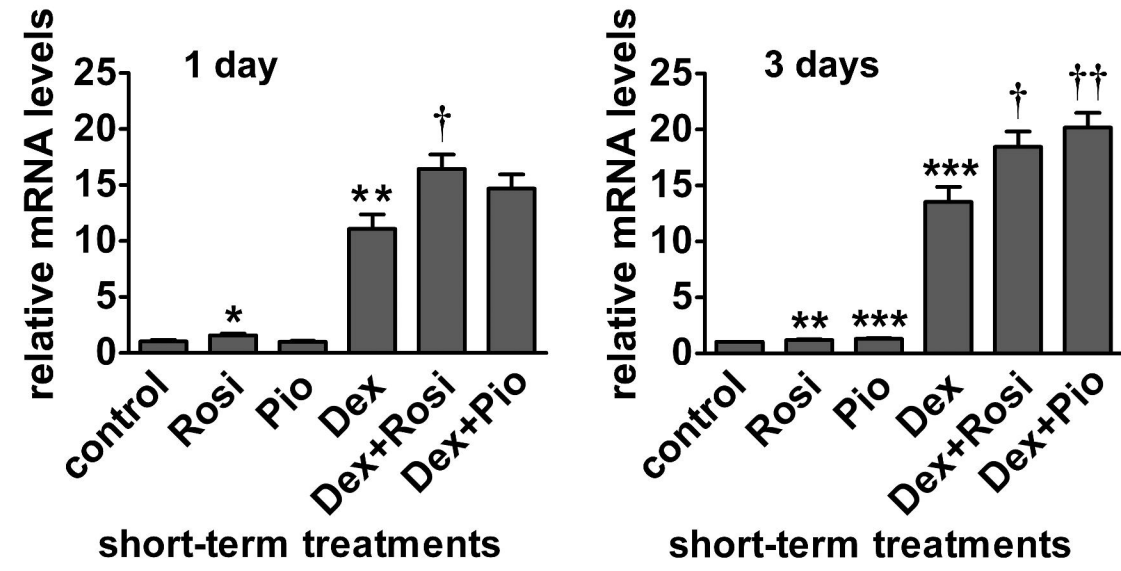
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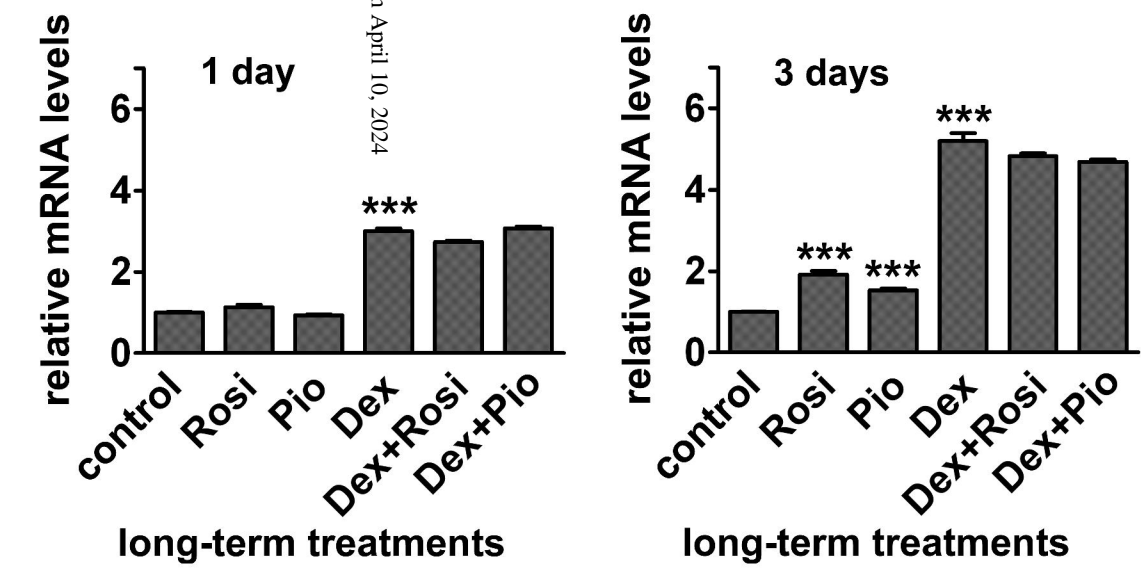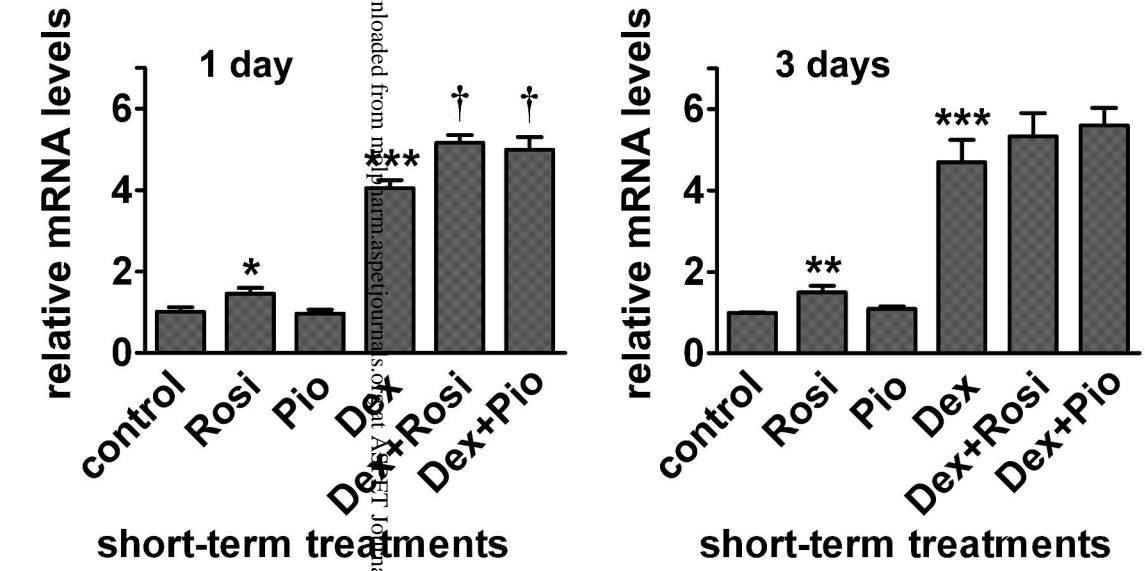
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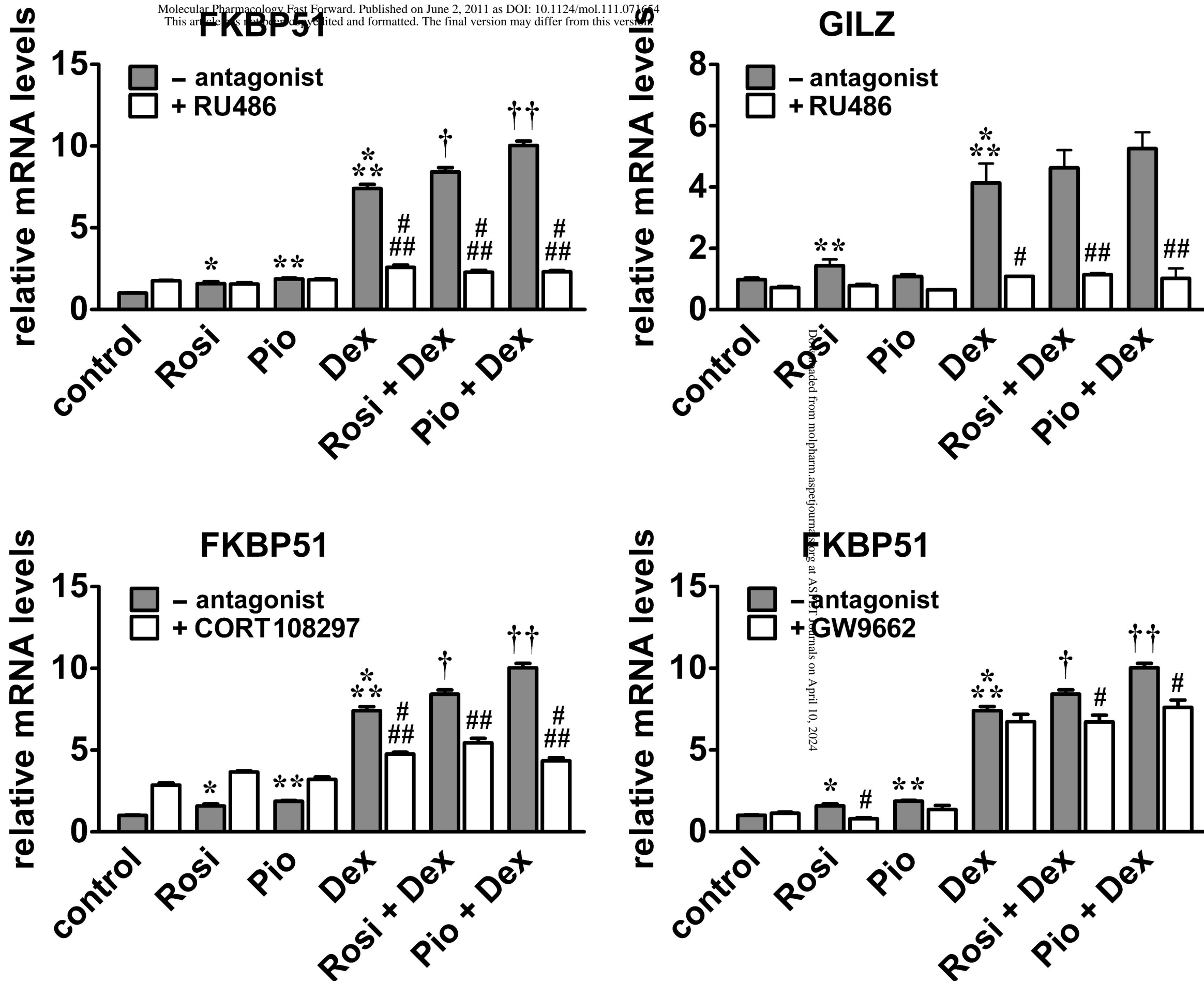
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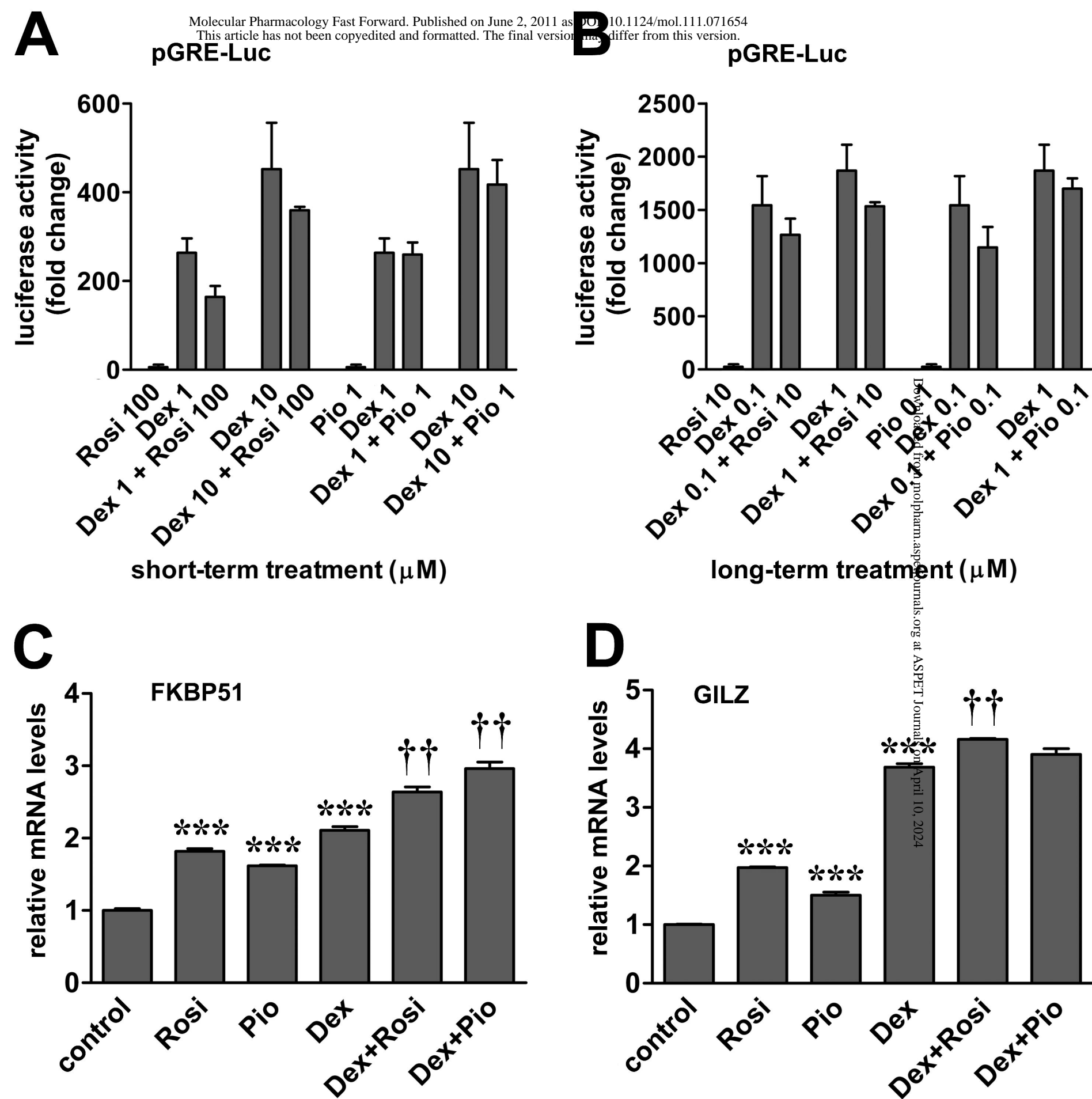
Figure 7

Figure 8

Figure 9