Peroxisome proliferator-activated receptor γ agonists reduce cell proliferation and viability and increase apoptosis in SSc fibroblasts

Running Head: PPARγ agonists effects in SSc fibroblasts.

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Bulleted Statements:

What's already known about this topic?

- PPARγ agonists (in particular rosiglitazone) attenuate inflammation, dermal fibrosis and subcutaneous lipoatrophy in a mouse model of bleomycin-induced scleroderma.
- PPARγ activation by pharmacologic agonists may represent a novel approach to the control of fibrosis in scleroderma.

What does this study add?

- We first show PPARγ agonists reduce cell proliferation, cell viability, and increase apoptosis in human SSc fibroblasts.
- These data reinforce the concept that PPARγ agonists could have a valuable therapeutic role in SSc patients.

Summary

Background and Objectives - No study has evaluated the effect of the peroxisome proliferator-activated receptor γ (PPARγ) agonists on cell viability, proliferation and apoptosis in cultured Systemic Sclerosis (SSc) fibroblasts.

Methods - The effects of two pure PPARγ agonists (rosiglitazone and pioglitazone) in cultured SSc fibroblasts were evaluated and compared to normal fibroblasts. The study included the evaluation of the cell viability and proliferation (based on the cleavage of tetrazolium salts and on the measurement of the absorbance of the cell proliferation reagent WST-1), and the cell apoptosis (by means of the Hoechst dye uptake).

Results - Rosiglitazone or pioglitazone (20 μM) significantly reduced cell proliferation (cell counting of 75% and 83% compared to baseline, respectively, after 2 h) and cell viability (absorbance reductions of 25% and 22% compared to baseline, respectively, after 2 h), and increased apoptosis (apoptotic cell percentages 9.9% and 8.6%, respectively, after 48 h of incubation) in SSc fibroblasts, whereas they did not present a significant influence on control fibroblasts.

Conclusions - The effects of rosiglitazone or pioglitazone showed on SSc fibroblasts raise the hypothesis of a therapeutic role for PPARγ agonists in patients affected by SSc.

Keywords: PPARγ agonists, scleroderma, fibroblasts, proliferation, apoptosis.

Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-dependent transcription factor and a member of the nuclear receptor superfamily. Acting as sensors of hormones, vitamins, endogenous metabolites and xenobiotic compounds, the nuclear receptors control the expression of a very large number of genes. [1] PPARγ has been known for some time to regulate adipocyte differentiation, fatty acid storage and glucose metabolism, and it is a target of anti-diabetic drugs. [1] More recently, PPARγ has been recognized as playing a fundamentally important role in the immune response through its ability to inhibit the expression of inflammatory cytokines and to direct the differentiation of immune cells towards anti-inflammatory phenotypes. [2-4]

In the last years, PPARγ agonists have been investigated in Systemic Sclerosis (SSc). In particular, rosiglitazone (RGZ) has been shown to attenuate inflammation, dermal fibrosis, and subcutaneous lipoatrophy in a mouse model of bleomycin-induced scleroderma. Furthermore, it has been demonstrated that the loss of PPARγ in mouse fibroblasts resulted in increased susceptibility to bleomycin-induced skin fibrosis. [5-7] These results indicate that PPARγ suppresses fibrogenesis and, therefore, specific agonists of PPARγ may alleviate the extent of the development of cutaneous sclerosis.[7]

However, the mechanisms of action of PPARγ agonists in SSc remain to be investigated.

A recent study suggested that PPARγ may play a physiologic role in the regulation of the profibrotic response, abrogating transforming growth factor ß-induced stimulation of collagen gene expression, myofibroblast transdifferentiation, and the activity of Smad-dependent promoter in normal fibroblasts. This study concluded that PPARγ

activation by pharmacologic agonists may represent a novel approach to the control of fibrosis in scleroderma. [8]

PPARγ agonists have been demonstrated to be implicated in the modulation of chemokines. Namely, Marx N *et al*. [9] demonstrated that the treatment of endothelial cells with PPARγ activators inhibits: 1) interferon (IFN)γ-induced mRNA and protein expression of chemokine C-X-C motif ligand (CXCL)10, CXCL9, and CXCL11; 2) the release of chemotactic activity for chemokine C-X-C motif receptor 3-transfected lymphocytes. [9] For these reasons, PPARγ activity may be involved in the regulation of IFNγ-induced chemokine expression in human autoimmunity, and PPARγ activators might attenuate the recruitment of activated T cells at sites of Th1-mediated inflammation. [9-11] Moreover, we have recently shown that treatment of thyrocytes, orbital fibroblasts or preadipocytes with RGZ, at near-therapeutical doses, significantly inhibited IFNγ-stimulated CXCL10 secretion, strongly suggesting that PPARγ might be involved in the regulation of IFNγ-induced chemokine expression in human thyroid autoimmunity and Graves' ophthalmopathy. [12,13]

Finally, a few studies have demonstrated an inhibitory role of PPARγ agonists on fibroblasts proliferation in lung fibrosis, [14] cardiac fibroblasts, [15] and rheumatoid synovial fibroblasts. [16]

Until now, no study has evaluated the effect of PPARγ agonists on cell viability, proliferation and apoptosis in SSc fibroblasts. The aim of our study was to evaluate the eventual effects of two pure PPARγ agonists, RGZ and pioglitazone (PGZ), in SSc fibroblasts in comparison with normal fibroblasts.

Materials and methods

Patients. Five SSc patients (5 F, median age 56 years, range 31-65), classified according to the 1987 American College of Rheumatology criteria, and 5 sex- and age-matched healthy controls have been enrolled in the study. All SSc patients had a disease duration < 2 years. All subjects had a diffuse cutaneous subset.

Fibroblast cultures. Skin tissue samples were obtained from 5 SSc patients. All subjects gave their informed consent to the study, which was approved by the local Ethical Committee. The biopsies were performed at the arm, so that 5/5 samples derivated from non-sclerotic cutis. Scleroderma tissue explants were minced and placed directly in plastic culture dishes, to permit fibroblasts proliferation, as previously described. [12,13] Cells were propagated in medium 199 with 20% fetal bovine serum (FBS) (Gibco^{TM} – Invitrogen LTD, Paisley, UK), penicillin (100 IU/mL), and gentamycin (20 μg/mL) in a humidified 5% CO_2 incubator at 37° C, and maintained with medium 199 containing 10% FBS and antibiotics.

Control fibroblasts were obtained from unaffected dermal tissues of 5 healthy subjects, undergoing skin biopsy for diagnostic purposes, in whom any immune-mediated disorder was excluded.

Cells were used for our tests at the fifth passage.

PPARγ agonists treatment. Cell cultures were treated (24 h) with 0, 1, 5, 10 or 20 μM RGZ or PGZ. Control cultures were grown (24 h) in the same medium containing vehicle (absolute ethanol, 0.47% v/v) without PPARγ agonists. Some cultures were examined by an Olympus IX50 phase contrast microscope.

The lysis and the homogenization of cell preparations were performed and the sample was immediately assayed for its protein concentration by conventional methods.

Cell viability test. The number of viable cells was evaluated by a viability and proliferation assay, based on the cleavage of tetrazolium salts added to the culture medium (Cell Proliferation Reagent WST-1; Roche, Mannheim, Germany). [17] The tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells is linked to an increase in the overall activity of mitochondrial dehydrogenases in the sample, which increases the amount of formazan dye formed, that directly correlates to the number of metabolically active cells in the culture. The formazan dye produced was quantified by a scanning multi-well spectrophotometer (ELISA reader) by measuring the absorbance of the dye solution at 450 nm.

Cells were seeded in a 96 wells microtiter plate at a concentration of 35000 cells/mL in a final volume of 100 μL in each well, and they were incubated for 48 h with PPARγ activators, in a humidified atmosphere (37° C, 5% CO_2). Then, 10 μL of the Cell Proliferation Reagent WST-1 were added to 100 μL of culture medium in each well and the absorbance of the samples was measured against the control (the same cells without any treatment). The same volume of culture medium and Cell Proliferation Reagent WST-1 was added into one well, in order to use this background control (absorbance of culture medium plus WST-1 in the absence of cells) as a blank position for the ELISA reader.

The absorbance was measured again after 1 h and 2 h. The measured absorbance of blank was subtracted from control and treatments and the control was normalized to 100% for each assay; treatments were expressed as % of the control. The experiments were conducted in triplicate for each sample.

Proliferation assay: cell counting. MTT assay [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] measures mitochondrial cell activity and it has

been demonstrated that there is not always a direct relationship with the cell number. [18] For this reason, the proliferation was evaluated using also the cell number counting. Cells were seeded at a density of 13000 cells per well in 24 well tissue culture plates in medium supplemented with 10% v/v FBS with or without the indicated factors.

The medium was changed every other day. After 72 h in an atmosphere of 5% CO_2 at 37° C, cells were detached from plates by incubation with 500 mL phosphate buffered saline containing 100 mg trypsin and 1 mM ethylenediaminetetraacetic acid. Cells were counted using a hemocytometer.

Apoptosis determination - Hoechst uptake. Fibroblasts were seeded in a 96 wells microtiter plate at a concentration of 35000 cells/mL in a final volume of 100 μL in each well. Then, cultures were incubated for 48 h with PPARγ activators (10 or 20 μM, RGZ or PGZ), in a humidified atmosphere (37° C, 5% CO_2).

After 48 h of treatments, the cells were stained with 5 μg/mL of Hoechst 33342 for 10 min at 37° C. Then, adherent cells were collected and analyzed using a hemocytometer under a fluorescence microscope. Cells that incorporated the Hoechst dye and showed typical morphological apoptotic features, such as chromatin condensation, were considered apoptotic cells according to Schmid *et al*. [19]

The apoptosis index, (ratio between apoptotic and total cells) x100, was calculated. Data were analyzed by one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparisons test.

Apoptosis determination - Annexin V binding assay. The cells were plated in Lab-tekII Chamber Slide System (Nalge Nunc International), treated with 10 or 20 μM, RGZ or PGZ for 48 h. At the end of the incubation time, the cells were stained using the Annexin V-FIT Fluorescence Microscopy Kit (BD Biosciences) following

manufacturer's instruction, and observed under the fluorescence microscope (Leica). Images were captured by an on-line Leica DFC320 camera.

Data analysis. Values are given as mean±SD for normally distributed variables (in text), or mean±SEM (in figures), otherwise as median and [interquartile range]. Mean group values were compared by one-way analysis ANOVA for normally distributed variables, otherwise by the Mann-Whitney *U* or Kruskal-Wallis test. Proportions were compared by the χ^2 test. *Post-hoc* comparisons on normally distributed variables were carried out using the Bonferroni-Dunn test.

Results

PPARγ agonists reduce cell viability in SSc cells

All SSc cultures were sensitive to RGZ and PGZ. Their antiproliferative effect became apparent after a minimum of 24 h of incubation and increased with longer incubation times. Shorter exposure times (10 and 18 h) did not show any antiproliferative effect on all tested cells (data not shown). There were no significant differences in sensitivity to RGZ and PGZ among the tested cells. As illustrated in **Fig. 1**, the results of WST-1 assay in SSc (after 48 h of incubation with PPARγ agonists) showed a reduction of proliferation compared to the controls both at 1 h from the start of tetrazolium reaction [10% and 21% with RGZ 10 (P = ns) or 20 (P= 0.0001) μM, respectively; 9% and 18% with PGZ 10 (P = ns) or 20 (P = 0.01) μM, respectively], and at 2 h [12% and 25% with RGZ 10 (P < 0.005) or 20 (P < 0.0001) μM, respectively (**Fig. 1a**); 11% and 22% with PGZ 10 (P = 0.04) or 20 (P < 0.001) μM, respectively (**Fig. 1b**)] (P by ANOVA).

PPARγ agonists reduce cell proliferation in SSc cells

The cell counting confirmed the above mentioned results. In SSc cultures, the cell number was 37.400 ± 4.600/100 μL, per well. After the treatments, 1 h after the start of tetrazolium reaction, the cell numbers were: 34.600 ± 3.800 and 31.370 ± 3.940 (93% and 84%, compared to the control) with PGZ 10 μM and 20 μM, respectively; 33.960 ± 4.120 and 28.136 ± 2.700 (91% and 75% compared to the control) with RGZ 10 μM and 20 μM, respectively. Two hours after the start of tetrazolium reaction, the cell numbers were: 33.780 ± 2.870 and 31.130 ± 1.370 (90% and 83% compared to the control) with PGZ 10 and 20 μM, respectively (**Fig. 2a**); 32.710 ± 2.120 and 27.980 ± 2.780 (87% and 75% compared to the control) with RGZ 10 and 20 μM (**Fig. 2b**).

PPARγ agonists induce apoptosis in SSc fibroblasts

Apoptosis index was determined in SSc fibroblasts by the Hoechst methods: cells incorporating the dye and showing typical morphological features were considered as apoptotic. Both control and cells treated with RGZ (10 and 20 μM for 48 h) were stained with fluorescent Hoechst dye. The percentage of apoptotic cells increased markedly in a dose-dependent manner: after treatment with RGZ 10 μM, 7.1% of the cells were apoptotic and this percentage increased up to more than 9.9% with RGZ 20 μM ($P<0.001$; by ANOVA) (**Fig. 3a**). At the PGZ lower dose, 5.7% of the cells were apoptotic, and this percentage increased up to more than 8.6% with PGZ 20 μM ($P<0.01$; by ANOVA) (**Fig. 3b**). Annexin V further confirmed the induced cell apoptosis.

PPARγ agonists do not reduce cell viability or proliferation in control fibroblasts

The results of WST-1 assay in control fibroblasts did not show a reduction of proliferation with respect to the control both at 1 h and at 2 h with PGZ, while a slight but not significant reduction was observed with 10 and 20 μM RGZ (at 2 h from the start of tetrazolium reaction) (**Fig. 4a**). The cell counting is consistent with the above mentioned results: in fact, a slight but not significant reduction was observed with 10 and 20 μM RGZ (at 2 h from the start of tetrazolium reaction) (**Fig. 4b**), but not with PGZ. No significant effect was observed on apoptosis both with RGZ or PGZ (data not shown).

Discussion

The present study firstly showed that PPARγ agonists reduce cell proliferation, cell viability, and increase apoptosis in SSc fibroblasts, whereas these effects were not observed in control fibroblasts.

Recent studies have raised a possible therapeutic role of PPARγ agonists in SSc. RGZ revealed effective to reduce dermal inflammation and fibrosis in a mouse model of bleomycin-induced scleroderma, [5,6] while the loss of PPARγ increased the susceptibility to bleomycin-induced skin fibrosis. [7]

In vitro, Shi-wen X *et al*. [20] demonstrated that RGZ reduced the fibrotic phenotype of SSc fibroblasts, suggesting that PPARγ agonists could negatively control cell activation. Moreover, we have previously demonstrated that RGZ dose-dependently inhibits CXCL10 secretion induced by IFNγ with or without tumor necrosis factor α in SSc fibroblasts, [21] inhibiting the participation of these latter to the perpetuation of inflammation in the dermis. To note, our findings have been obtained evaluating fibroblasts from non sclerotic skin, contrarily to the work by Shi-wen X *et al*. [20]

The effect of PPARγ agonists on fibroblasts proliferation has been investigated also in other diseases.

RGZ inhibited in a dose-dependent manner the proliferation of cardiac fibroblasts, in patients with myocardial fibrosis induced by advanced glycation end-products. [15] Consistently, Mughal *et al*. [22] confirmed *in vitro* the role of thiazolidinediones, but *via* a PPARγ-independent mechanism. Also human cultured lung fibroblasts were inhibited by RGZ in regards to their migration, proliferation and phenotypic differentiation. [14,23]

Interestingly, the antiproliferative effect in SSc fibroblasts was not associated with a significant antiproliferative effect in normal fibroblasts. This discrepancy with SSc fibroblasts suggests that different pathways are activated by thiazolidinediones in normal and pathologic cells. In fact, the same difference has been observed also in thyroid cell: PPARγ agonists were not able to inhibit cell proliferation and viability in normal thyroid cells, but they effectively inhibited cell proliferation of thyroid neoplastic cells. [17,24,25]

The intracellular pathways activated in SSc and normal fibroblasts by PPARγ agonists remain to be evaluated.

The effect of PPARγ agonists on apoptosis has been investigated in many studies, in different cell types. PPARγ agonists may exert an anti-apoptotic effect in some cell types, while they have a pro-apoptotic effect on other cells. [26-28]

For example, PPARγ agonists neither reduced proliferation nor induced apoptosis in rheumatoid arthritis synoviocytes. In contrast, they induced apoptosis in a dose-dependent manner in THP-1 (a human monocytic cell line) cells and augmented tumor necrosis factor related apoptosis. [16]

To the best of our knowledge, no study has evaluated the effect of PPARγ agonists on apoptosis in SSc fibroblasts. Our study firstly showed that PPARγ agonists stimulate apoptosis in SSc fibroblasts compared to healthy controls. One more time, RGZ produced the same effect described above, about cell proliferation, on thyrocytes. Also in other cell lines thiazolidinediones mediate growth inhibition plus induction of apoptosis. [29-31]

The two effects of PPARγ activation on proliferation and apoptosis are distinct, and may diverge. For example, in hypertensive rats PPARγ agonists inhibit both apoptosis and proliferation of mesangial cells. [32]

Functional and structural vasculopathy is considered to have a primary and pivotal role in inducing SSc tissue damage. The pathologic endothelial activation leads to an increased expression of adhesion molecules, endothelial apoptosis with intimal proliferation of arterioles and capillary necrosis. On the other hand, increasing evidences suggest that thiazolidinediones may improve endothelium-dependent vascular function, modulating cellular adhesion molecules, tissue factors, plasminogen activator inhibitor, and matrix metalloproteinases, leading to the hypothesis that PPARγ agonists may exert vasculoprotective effects independently of their metabolic action. [33]

Moreover, it was demonstrated a complex alteration of bone marrow compartments, with an impairment of endothelial progenitor cells output, which is supposed to play a role in defective vasculogenesis of SSc. [34] Conversely, PPARγ agonists may increase the number and the function of endothelial progenitor cells, improving endothelial function. [35,36]

Recently, it has been shown that rosiglitazone was associated with an increased risk of stroke, heart failure, and all-cause mortality in elderly patients, [37] and the European

Medicines Agency (EMA) recommended on September 2010 that rosiglitazone be suspended from the European market. More recently, EMA extended review of safety to pioglitazone. [38] Even if these arguments cannot be automatically translated in the rheumatological field, they do not actually advice PPARγ agonists for the therapy of SSc.

On the basis of the considerations mentioned above, thiazolidinediones might represent a therapeutic tool for SSc patients, both because of the anti-fibrotic effect on fibroblasts and the beneficial role on endothelium. However, a small number of SSc fibroblasts preparations has been studied, and in the next future it will be necessary to expand the number of samples to finally confirm our results.

The present study demonstrates that PPARγ agonists are able to inhibit cell proliferation and to induce apoptosis in SSc fibroblasts. These effects were not observed in control fibroblasts. These data reinforce the concept that PPARγ agonists could have a valuable therapeutic role in patients with SSc.

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Figure legends

Fig. 1. Cell viability assay in SSc fibroblasts obtained from 5 patients. Cell viability by WST-1 (at 2 h from the start of tetrazolium reaction) was significantly reduced in SSc fibroblasts, adding increasing doses of rosiglitazone (**a**) or pioglitazone (**b**) (expressed as % with respect to basal). Bars are mean±SEM. * = P<0.05 or less *vs* Basal by Bonferroni-Dunn test.

Fig. 2. Cell proliferation assay in SSc fibroblasts obtained from 5 patients. Cell proliferation (by cell counting; at 2 h from the start of tetrazolium reaction) was significantly reduced in SSc fibroblasts, adding increasing doses of rosiglitazone (**a**) or pioglitazone (**b**) (expressed as % with respect to basal). Bars are mean±SEM. * = P<0.01 or less *vs* Basal by Bonferroni-Dunn test.

Fig. 3. Apoptosis evaluation in SSc fibroblasts obtained from 5 patients. Cell apoptosis was significantly increased in SSc fibroblasts, adding increasing doses of rosiglitazone (**a**) or pioglitazone (**b**) (expressed as % with respect to basal). Bars are mean±SEM. * = P<0.01 or less *vs* Basal by Bonferroni-Dunn test.

Fig. 4. Cell viability and proliferation assay in control fibroblasts obtained from 5 control subjects. Cell viability (by WST-1, at 2 h from the start of tetrazolium reaction; **a)** and proliferation (by cell counting; **b)** was not significantly reduced in control fibroblasts, adding increasing doses of rosiglitazone (expressed as % with respect to basal). Bars are mean±SEM. P=ns *vs* Basal by Bonferroni-Dunn test.

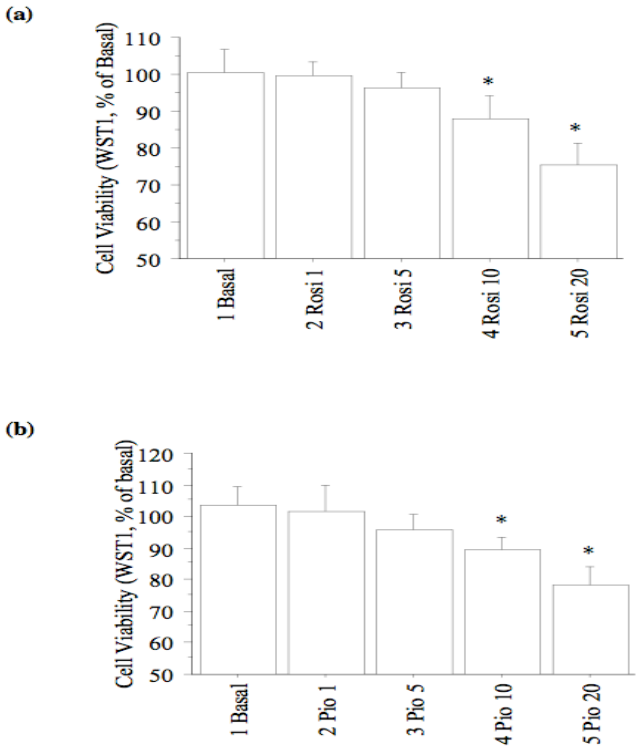
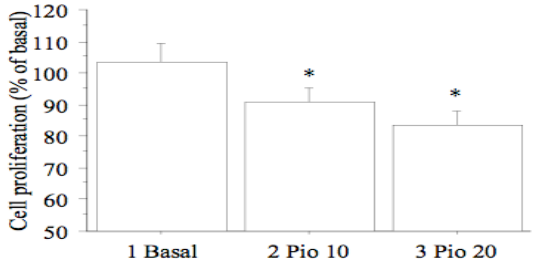

(a)
Cell Viability (WST1, % of Basal)
110
100
90
80
70
60
50
*
*
1 Basal
2 Rosi 1
3 Rosi 5
4 Rosi 10
5 Rosi 20
(b)
Cell Viability (WST1, % of basal)
120
110
100
90
80
70
60
50
*
*
1 Basal
2 Pio 1
3 Pio 5
4 Pio 10
5 Pio 20

Fig. 1

(a)


Cell proliferation (% of basal)
120
110
100
90
80
70
60
50
*
*
1 Basal
2 Pio 10
3 Pio 20

(b)

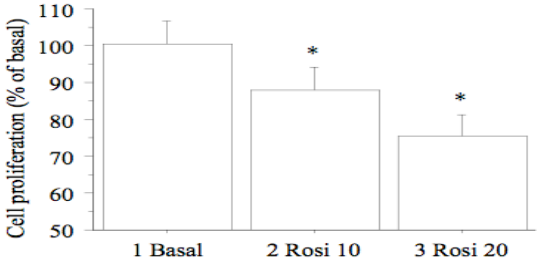
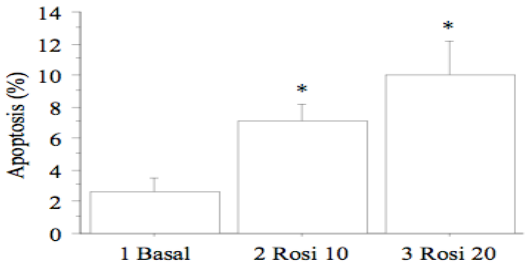

Cell proliferation (% of basal)
110
100
90
80
70
60
50
*
*
1 Basal
2 Rosi 10
3 Rosi 20

Fig. 2

(a)


Apoptosis (%)
14
12
10
8
6
4
2
0
*
*
1 Basal
2 Rosi 10
3 Rosi 20

(b)

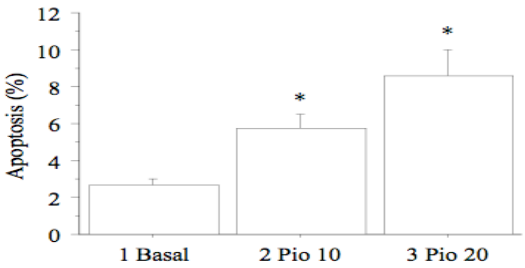
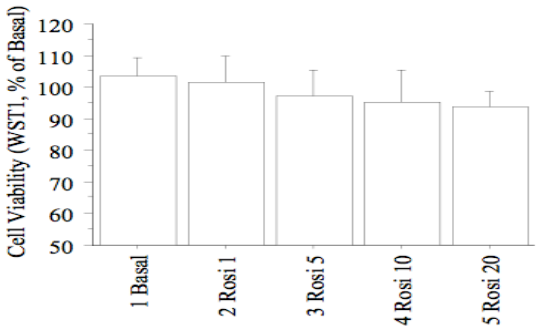

Apoptosis (%)
12
10
8
6
4
2
0
*
*
1 Basal
2 Pio 10
3 Pio 20

Fig. 3

(a)


Cell Viability (WST1, % of Basal)
120
110
100
90
80
70
60
50
1 Basal
2 Rosi 1
3 Rosi 5
4 Rosi 10
5 Rosi 20

(b)

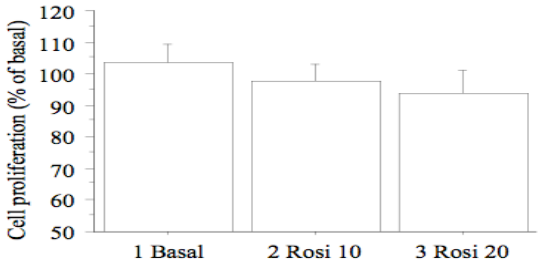

Cell proliferation (% of basal)
120
110
100
90
80
70
60
50
1 Basal
2 Rosi 10
3 Rosi 20

Fig. 4