Enhanced effects of PPARγ ligands and RXR selective retinoids in combination to inhibit migration and invasiveness in cancer cells

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Abstract. Experimental data from *in vitro* and *in vivo* models indicate that peroxisome proliferator-activated receptor (PPAR) ligand activation regulates differentiation and induces cell growth arrest and apoptosis in a variety of cancer types. Thiazolidinediones such as ciglitazone (CGZ) constitute the most well-known synthetic ligands for PPARγ. We previously reported a remarkable antitumor effect of the retinoid 6-OH-11-O-hydroxyphenantrene (IIF), synthetic retinoid X receptors (RXRs) agonist, on many cancer cell lines. Since PPARs bind to DNA as heterodimers with RXRs, in this study we investigated if IIF potentiates the antitumoral properties of the PPARγ ligand CGZ in glioblastoma U87MG and melanoma G361 cells. Our results show that either IIF or CGZ inhibited cell growth and tissue invasion ability, but these properties were enhanced by using IIF and CGZ in combined treatment. Since matrix metalloproteinases (MMPs) play a major role in tumor cell invasion, we analyzed the effect of IIF and CGZ on MMP2 and MMP9 activity and expression. The addition of IIF to CGZ resulted in a decrease of MMP2 and MMP9 expression and activity, higher than when each agent was used alone. Furthermore, treatment with IIF and/or CGZ enhanced PPARγ expression but both agents in combined treatment caused the maximum efficiency. Finally, we demonstrated that IIF can potentiate PPARγ trascriptional activity induced by CGZ, by evaluation of peroxisome proliferator-responsive element transactivation. In conclusion, these findings suggest that the RXR selective retinoid IIF, in combination with the PPARγ ligand CGZ, may provide a therapeutic advantage in cancer treatment.

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Introduction

Peroxisome proliferator-activated receptors (PPARs) have lately attracted much attention as therapeutic targets. PPARs have been shown to play an important role in the regulation of energy homeostasis by modulating glucose and lipid metabolism and transport. Recent studies have demonstrated that PPARs agonists, especially PPARγ ligands can regulate differentiation and induce growth arrest and apoptosis in a variety of cancer types (1,2). Among PPARγ agonists, very important are thiazolinediones (TZDs). Interestingly, beyond the treatment of diabetes, a wide spectrum of action for TZDs, including antineoplastic properties has been reported in various types of human cancer cell lines. TZD inhibited cell proliferation of human bladder carcinoma cell lines by increasing cyclin-dependent kinase inhibitor expression and induced cell death (3), inhibited the growth of human lung cancer cell lines by inducing apoptosis (4), and induced differentiation in human colon carcinoma cell lines (5,6). These studies suggest that targeting PPARγ may be a potential useful strategy for cancer treatment. Consequently, several clinical studies have been conducted using PPARγ ligands in human cancer. However, on the whole, these clinical trials have so far indicated that PPARγ agonists may not be useful as a monotherapy for common cancers (7,8).

PPARs are ligand-activated transcription factors. As a result, after ligand binding, PPARs can regulate gene expression by binding to peroxisome proliferator-responsive element (PPRE) in target genes as heterodimers with the retinoid X receptors (RXR). As a consequence, RXRs play a fundamental role in controlling cell proliferation and metabolism and the retinoids which bind to RXR (rexinoids) may be the most preferable partner for the PPAR agonists (9).

Retinoids have long been investigated in preclinical models, and clinical data have supported the potential of these compounds as cancer preventive and therapeutic agents (10-13). Moreover, cancer therapy using rexinoids (14,15) has been proved to be clinically useful and also less toxic than therapy with all-trans retinoic acid (ATRA) or conventional retinoids that bind RAR receptors (16,17). In previous studies we demonstrated that IIF, a retinoid whose receptor is RXR (18), has a strong antitumoral effect on many cancer cell lines (19-23).

On the basis of these observations, the combined use of PPARγ and RXR ligands could be a critical strategy for cancer treatment. In the present study we examined the antitumoral properties of the TZD ciglitazone (CGZ) in combination with the retinoid IIF in U87MG glioblastoma and G361 melanoma human cells, with the aim to find a potentiated antitumor effect produced by the combination of PPARγ ligand plus RXR ligand.

Materials and methods

Cell culture. The human tumor cell lines U87MG (glioblastoma) and G361 (melanoma) were obtained from Interlab Cell Line Collections (Genoa, Italy). They were maintained in RPMI (Sigma, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin and grown at 37˚C in a humidified air with 5% CO_2. IIF (provided by Dr Khodor Ammar; pat. WIPO W0 00/17143, Bologna, Italy) and CGZ (Sigma) were dissolved in propylene glycol and ethanol, respectively, just before use and the final concentrations, in the range 10-30 mM, were obtained with the culture medium. The concentration of the solvent in the highest dose of drugs did not affect cell proliferation and invasion of the cell lines.

MTT assay. The effect of IIF and CGZ on cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, based on the reduction of the number of metabolically active cells, and the results were expressed as percentage of the controls. Briefly, $3x10^3$ cells/well were seeded into a 96-well plates and treated with specific concentrations of IIF or CGZ, after which 0.5 mg/ml of MTT (Sigma) were added to each well and incubated for 4 h at 37˚C. Following the incubation, a solution containing 10% SDS and 0.01 M HCl was added. After at least 18 h at 37˚C, the absorbance of each well was measured in a micro-plate reader (Bio-Rad, USA) at 570 nm. The results were expressed as percentage of the controls.

Cytotoxity test of T cells. T lymphocytes from blood samples of several donors (men and women) were isolated by density gradient centrifugation and erythrocyte resetting. T cells ($0.8x10^6$ cells/ml) were cultured in RPMI containing 10% FBS and 20 μg/ml phytohemagglutinin P (Difco Laboratories, USA). Viability was determinated using MTT assay after treatment with IIF and CGZ and their solvent (propylene glycol and ethanol).

MMPs activity. MMP2 activity of U87MG and G361 cells was determined by gelatin zymography. After a 4-day treatment with CGZ 30 μM and/or IIF 20 μM the cells were washed with PBS and replaced in serum-free medium (RPMI) for 18 h with the same treatment. After centrifugation (300 x g for 10 min) the supernatant was separated and protein concentration determinated; 10 μg per lane, added by sample buffer (Tris-HCl 1M, pH 6.8, sodium dodecyl sulphate (SDS) 2%, glycerol 10%) were applied to 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Sigma). After electrophoresis, SDS was removed from gel by washing twice with 2.5% Triton X-100 for 1 h. After a brief rinse, the gel was incubated at 37˚C for 18 h in buffer, pH 7.6, containing 100 mM Tris-HCl, 10 mM $CaCl_2$, 20 mM NaCl. The gel was stained with 1% Coomassie Brilliant Blue R250 for 2 h and then treated with destaining solution (40% methanol, 10% acetic acid, 50% distilled water). The MMP2 activity was indicated by clear bands of gelatin digestion on a blue background. Activity of MMP2 in the gel was quantified by using a densitometric images analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). MMP9 activity in the gel was not revealed at these experimental conditions.

MMP9 activity. MMP9 immunoassay kit (Chemicon, USA) was used for the determination of MMP9 level in conditioned medium of human cells after treatment with IIF and/or CGZ. Supernatant (50 μl) and enzyme antibody solution (300 μl) were added into the bottom of a test tube. Anti-MMP9 coated bead was placed in each tube and incubated at RT for 1 h. After three washings the reaction was stopped by stop solution. Absorbance was measured at 492-nm wavelength.

Western blot analysis. To determine MMP2, MMP9, RXRγ and PPARγ levels, the cells were plated and treated with IIF and CGZ as described above for zymography. The cells were detached and were collected by centrifugation at 300 x g for 10 min and pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, 5 μM Na_3VO_4) and sonicated on ice, in the presence of protease inhibitors. Protein concentration was determined by the method of Lowry. Cell lysates (50 μg of protein per lane) were size fractioned in 10% SDS-polyacrylamide gel, prior to transfer to Hybond™-C Extra membranes (GE Healthcare, UK) by standard protocols. Membranes were blocked for 2 h with 5% milk in transfer buffer saline (TBS) at RT. The membranes were incubated overnight at 4˚C with anti-MMP2, or anti-MMP9 (Santa Cruz Biotechnology, USA), or anti-PPARγ (Vinci Biochem, Italy) and anti-RXRγ (TEMA, Italy) dissolved in TBS-5% milk. The membranes were washed 2 times with TBS-5% milk and were incubated for 1 h with the respective peroxidase-conjugated antibodies. The anti-MMP2, the anti-MMP9, the anti-PPARγ and the anti-RXRγ were diluted 1:500 and the antirabbit peroxidase conjugated antibody was diluted 1:1000 with 5% milk in TBS-1% Tween-20. The proteins were detected by luminol (GE Healthcare, UK). Bands were quantified by using a densito-metric images analysis software (Image Master VDS, Pharmacia Biotech, Sweden). The amount of protein in each lane was the same, as confirmed by actin (Sigma).

Invasion assay. Invasion of cells into Matrigel was determined using Boyden chambers (NTG, Italy) with polycarbonate membrane, 8.0-μm pore size (NTG). The filter was coated with 12.5 μg of Matrigel (Sigma) and, after 2 h, U87MG and G361 cells treated or not with 20 μM IIF and/or 30 μM CGZ for 4 days, were seeded into the upper part of each chamber ($4x10^5$ cell/well in 800 μl in serum-free RPMI) with the same treatment. In the lower part 200 μl chemoattractant (RPMI with 10% FBS) were added. After incubation for 18 h at 37˚C, non-migratory cells on the upper surface of the filter were wiped with a cotton swab, and cells migrated on the lower surface of the filter were fixed and stained with toluidine blue.

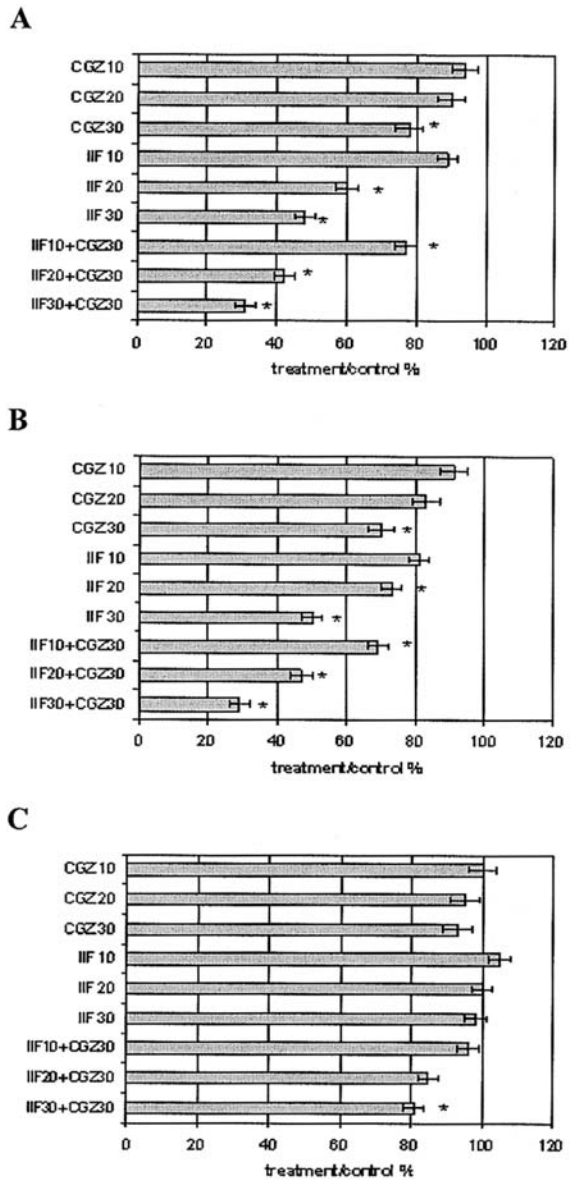

Figure 1. Effect of different doses (μM) of CGZ and IIF on G361 (A), U87MG (B) and on lymphocytes (C) cell viability after six days as assessed by MTT assay. Each bar represents the mean (± SEM) of six replicate cultures from 3 independent experiments; *p<0.05.

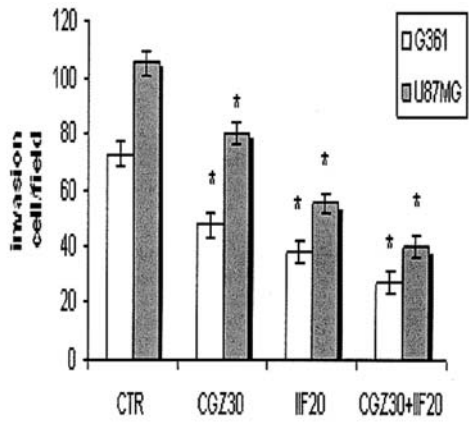

Figure 2. Effect of CGZ (30 μM) and IIF (20 μM) on invasion of U87MG and G361 cells, as assessed by Matrigel invasion assay. Cells migrated through the matrigel-coated membranes were fixed, stained and counted under light microscopy. Each bar represents the mean (± SEM) of 3 independent experiments. *p<0.05; CTR, control.

The invasion activity was evaluated by counting cells, in five random fields, using microscopy at magnification, x100.

Immunocytochemistry. U87MG and G361 cells grown on coverslips and after treatment with IIF and/or CGZ were fixed with 4% paraformaldehyde and permeabilized with Triton X-100 (0.1% in phosphate buffer saline (PBS) and saturated in PBS-BSA 4% for 30 min. Then, cells were incubated with anti-PPARγ (mouse) or anti-RXRγ (rabbit) for 1 h at RT. After washings, cells were incubated with anti-mouse fluorescein isothiocyanate (FITC)-conjugated and with antirabbit tetramethyl rhodamine isothiocyanate (TRIC)-conjugated secondary antibodies. Finally, slides were mounted in glycerol-PBS medium containing 30 mg/ml 1,4-diazabicyclo(2,2,2)octane (Dabco) (Sigma). Evaluation of antibody specificity was carried out either by omitting primary antibody or by using unspecific sera.

Luciferase assay. Cells/well ($2.5x10^5$) were plated and grown for 24 h before transfection in a 24-well plate. Cells were washed with PBS then transfected with 500 ng of the appropriate responsive reporter: PPRE-Luc and pCMX-nRXRγ (provided by Professor Ronald Evans). Plasmids were transiently transfected in U87MG and G361 cells by the lipofectamine plus method (Gibco, USA) with 800 ng of the receptor expression plasmid vector and 200 ng of the reporter luciferase plasmid per well. The cells were transfected for 6 h, incubated with and without IIF 20 μM or CGZ 30 μM for 48 h, washed twice with PBS and treated with a lysis buffer (25 μM Tris phosphate pH 7.8; 8 μM $MgCl_2$; 1 mM DTT; 1% Triton X-100; 1% BSA; 15% glycerol). After 30 min, the enzymatic activity of luciferase was measured with an automated luminometer (Digene DCR-1, MGM Instruments, CT, USA) and was expressed as relative light units per milligram of protein. Basal luciferase activity was determined by taking the activity obtained with the reporter plasmids and receptor expression vectors in the absence of IIF or CGZ. In order to control transfection efficiency in each experiment, cells were transfected with the wild-type (wt) 760-*luc* vector, which contains the *luc* gene under the control of the HCMV major IE promoter (MIEP, -760 to +65, kindly provided by Dr. C.V. Paya, Mayo Clinic, Rochester, NY, USA).

Statistical analysis. Data were expressed as the mean (± SEM). Differences were analysed by Student's t-test and considered statically significant at $p<0.05$ between the control and experimental samples.

Results

In this study we have investigated the antitumoral effect of CGZ and of IIF on two cancer cell lines of ectodermic origin, characterized by a high degree of tumorigenicity: the melanoma G361 and the glioblastoma U87MG cell lines. The compounds employed, directly (CGZ) or indirectly (IIF), activate PPARγ. PPARγ is the receptor of CGZ and it is

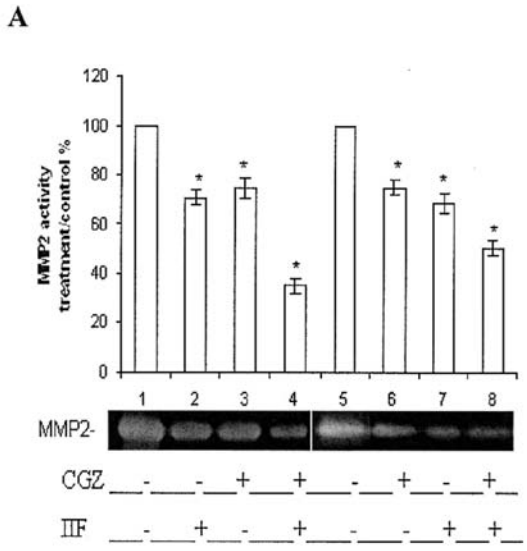

Figure 3. Effect of CGZ (30 μM) and/or IIF (20 μM) on MMP2 (A) and MMP9 activity (B) after five days treatment on G361 (1-4) and U87MG (5-8) cell lines. (A) Gelatin zymogram showed the MMP2 (72 kDa) form in serum-free conditioned media. Densitometric data are expressed as percentage of treated samples with respect to control. (B) MMP9 activity measured immunoassay is expressed as percentage of treated samples with respect to control (CTR). The results are representative of 3 independent experiments (± SEM); $^{*}p<0.05$.

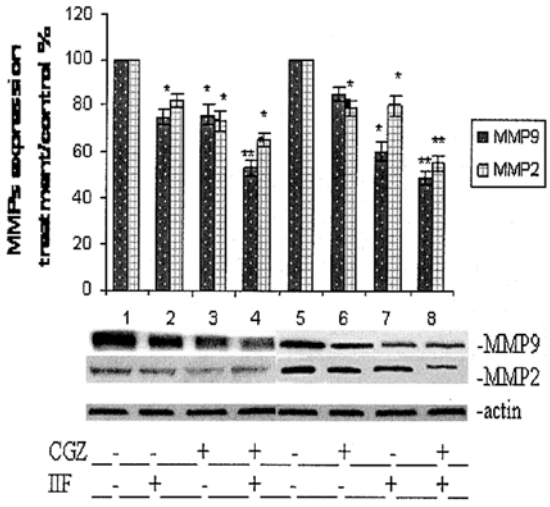

Figure 4. Effect of CGZ (30 μM) and IIF (20 μM) on MMP2 and MMP9 protein levels, as assessed by Western blotting in cell lysates of G361 (1-4) and U87MG (5-8) after 5-day treatment. Densitometric data are expressed as percentage of treated samples with respect to control. Each bar represents the mean (± SEM) of 3 independent experiments; $^{*}p<0.05$, $^{**}p<0.01$.

active only when forming a heterodimer with RXR, the receptor of IIF. We have studied if the anticancer efficacy of the two compounds could be enhanced by using them together.

Fig. 1 shows the antiproliferative effect of various doses of CGZ and of IIF (10-30 μM), alone or in combination, on G361 (Fig. 1A) and U87MG (Fig. 1B) cell lines. After six days of incubation, both compounds inhibited cell growth in a dose-dependent manner, but the effect was higher when the compounds were added together.

In Fig. 1C it is shown that, in normal human lymphocytes, both IIF and CGZ, given alone or in combination, were not toxic to cells. Only when 30 μM IIF and CGZ were used in concert was a significant reduction of cell growth noted.

In order to test the effect of the two compounds on the metastatic potential of the cell lines, we have examined three parameters: the capacity of cells to migrate and the activity and expression of MMP2 and MMP9, the enzymes that facilitate cell migration in tissues by digesting collagen. To evaluate the ability of the compounds to inhibit tissue invasion, we have performed experiments in which we have measured their effect by using Invasion Matrigel assay, a test that measures the capacity of cells to migrate. Fig. 2 shows that treatment with the two compounds in association reduced the migration of cells, measured by the chemo-invasion test, to a higher degree with respect to using each compound alone. As invasiveness is mediated by the effect of metalloproteases and in particular by MMP2 and MMP9, we measured the activity of these two gelatinases. Fig. 3 shows that, in both cell lines, the inhibitory effect on MMP2 (Fig. 3A) and on MMP9 (Fig. 3B) exerted by IIF or CGZ used alone was enhanced when the compounds were added together to the incubation medium.

Western blot analysis (Fig. 4) confirmed the reduction of MMP2 and MMP9 activity observed in the presence of both componds: the expression of the enzymes was reduced to a higher degree when the two compounds were used in combination.

In Fig. 5A, it is shown that, in G361 and U87MG cells, after treatment with IIF and/or CGZ, PPARγ expression is strongly enhanced with respect to untreated controls and that the expression was further increased when the compounds were added together. Similar results are noted when evaluating RXRγ expression in G361 cells, althought to a lesser extent, but not in U87MG cells (Fig. 5B). These results were confirmed by immunocytochemistry (data not shown).

It is known from literature that IIF is a ligand of RXR and that PPARγ is able to exert its functions only when bound to RXR to form a heterodimer. We have performed experiments in order to verify if the rexinoid IIF enhances PPARγ transcriptional activity induced by CGZ. It is important to note that

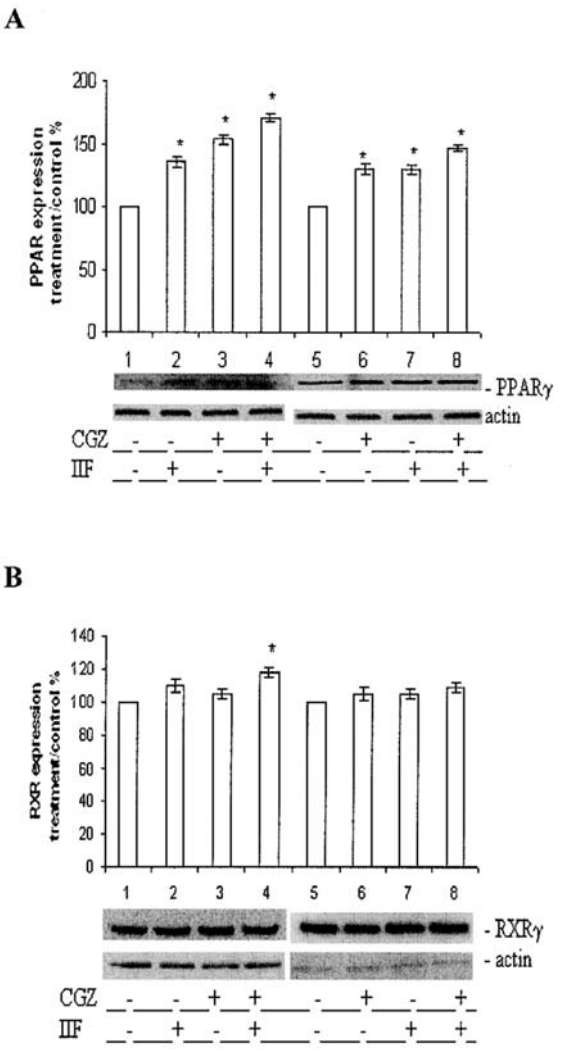

Figure 5. Effect of CGZ (30 μM) and IIF (20 μM) on PPARγ (A) and RXRγ (B) protein levels, as assessed by Western blotting in cell lysates of G361 (1-4) and U87MG (5-8) after 5-day treatment. Densitometric data are expressed as percentage of treated samples with respect to control. Each bar represents the mean (± SEM) of 3 independent experiments; *p<0.05.

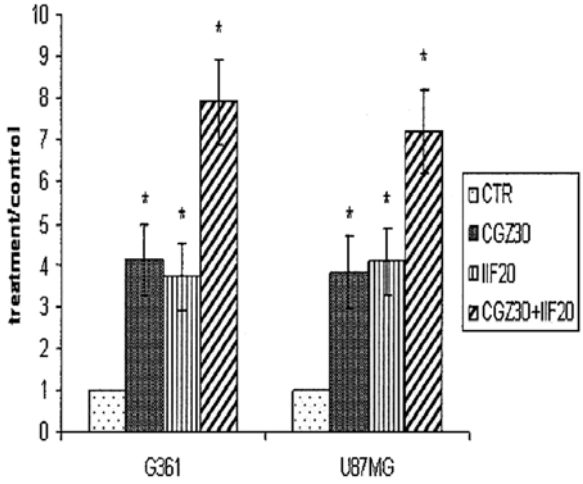

Figure 6. Evaluation of PPRE transactivation in G361 and U87MG cells. Basal luciferase activity was determined by taking the activity obtained with the reporter plasmids [wild-type (wt) 760-luc vector] in the absence of CGZ (30 μM) or IIF (20 μM). The data are expressed as treatment/control and are the mean of 3 independent experiments (± SEM); *p<0.05.

the heterodimer PPARγ-RXR, in order to act on DNA, must be activated by a ligand of PPARγ; in our experimental system the ligand is CGZ. Activated PPARγ acts on DNA by binding to PPRE. Fig. 6 shows that the activation of PPRE exerted by the heterodimer is strongly increased when cells were treated with IIF or CGZ alone, but the activation was doubled when the compounds were added together.

Discussion

In previous studies, we had demonstrated that IIF, a retinoid whose receptor is RXR (18) had a strong antitumoral effect on various cancer cell lines (19-23). CGZ is another compound with strong antitumoral effects exerting its action by binding to PPARγ (24,25).

Since RXR forms a heterodimeric complex with PPAR, and the activation of PPARγ exerts antiproliferative effects in cancer cells (26), it seems to be reasonable that the combination of RXR and PPARγ agonists may offer new therapeutic strategy in cancer treatment. Recent preclinical studies showed the effectiveness of this combined treatment for some hematologic malignancies (27), and also in certain type of solid tumors, such as breast and colorectal cancer cells (28-32). The goal of this study was to investigate the effectiveness of the combination of IIF (RXR agonist) and CGZ (PPAR agonist), in other malignancies, such as human glioblastoma and melanoma cells.

We demonstrated that, when the compounds were added to cells in combination, their antiproliferative effect was enhanced in both cell lines. We also demonstrated, using human lymphocytes, that the compounds, alone or in combination, at the same doses as used on cancer cells, are not toxic for normal cells. This observation is particularly interesting and it confirms the fact that, when used in therapy, rexinoids evoke fewer collateral effects. The small decrease observed after 6 days of treatment nevertheless is not comparable to the toxic effects caused to lymphocytes by commonly employed anticancer drugs. Under the same experimental conditions, 1 nM tamoxifen reduced the number of lymphocytes by 85% with respect to untreated controls (data not shown).

Expression and activity of MMP2 and MMP9 are correlated consistently with the invasive behaviour of tumor cells (33-35). In a previous study (19), we verified that IIF strongly inhibited these parameters in glioblastoma cells; herein we demonstrated that the inhibitory effect of IIF on cell invasion was increased when this compound was used in combination with CGZ, either in glioblastoma or melanoma cells. Furthermore, we demonstrated that IIF potentiates PPARγ expression and transcriptional activity induced by CGZ in both cell lines.

The molecular mechanisms by which PPARγ and RXR agonists induce potentiated cooperative anticancer effects are

currently unclear. It was reported (36) that PPARγ and RXR ligands can differentially recruit subset of transcriptional coactivator proteins to the receptor complex, thus leading to an enhanced transcriptional activation and cellular effects. The transactivation of the PPRE by PPARγ/RXR heterodimer could modulate the expression of important target genes, which could play a critical role in cancer development (2,37). The activation of PPRE exerted by the heterodimer PPARγ-RXR was greatly enhanced also when IIF was added to cells, probably because it activated the RXR bound to PPARγ. It is noteworthy that the simultaneous addition of the two components of the heterodimer resulted in a remarkable enhancement of various cellular events caused by simple direct PPARγ or RXRγ ligands.

In conclusion, PPARγ ligands, as CGZ, have anti-tumoral and anti-invasive properties. In combination with rexinoids, these effects are enhanced. The very low toxicity of these compounds, together with the low toxicity of rexinoids, suggests a potential role for utilizing a combination regimen of PPARγ and RXR agonists in cancer treatment.

Acknowledgements

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