



# Inhibitory effects of retinoic acid and IIF on growth, migration and invasiveness in the U87MG human glioblastoma cell line

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**Abstract.** Glioblastomas, the most malignant and prevalent brain tumors which remain incurable, are characterized by both extensive proliferation and invasive growth. We previously reported a remarkable antitumoral effect of the retinoid 6-OH-11-O-hydroxyphenantrene (IIF) on neuroblastoma, leukemia and colon carcinoma cells. In this study we examined the effect of IIF on proliferation, apoptosis and cell invasion in the human glioblastoma cell line U87MG, in comparison with all-trans-retinoic acid (RA). Our results showed that both retinoids induced cell growth inhibition and apoptosis in a dose- and time-dependent manner. We also demonstrated that the invasive ability of glioblastoma cells decreased after treatment with IIF or RA. Since cell invasion involves a complex system of tightly regulated proteases, matrix metalloproteinases (MMPs) and their specific inhibitors, tissue inhibitors of MMPs (TIMPs), we analysed the effect of IIF on MMP and TIMP expression in comparison with RA. Treatment with both retinoids resulted in a marked decrease of MMP2 and MMP9 expression and of lytic activity of MMP2. In addition, exposure to IIF led to enhanced expression of TIMP2. Collectively, our results demonstrated the effectiveness of both IIF and RA in inhibiting proliferation, cell migration, and the invasive potential of glioblastoma U87MG cells. Notably, the anticancer activity of IIF, on the whole, was more pronounced than that of RA. Therefore, these findings, besides providing further evidence that IIF may be a powerful tool in the development of cancer treatments, suggest that IIF may have therapeutic potential against the invasiveness of brain tumors.

## Introduction

Gliomas are the most prevalent primary brain tumors, accounting for >50% of all brain tumors (1-2). Current treatment options include surgery, radiation therapy, and chemotherapy. Unfortunately, the prognosis of patients with glioblastomas remains extremely poor, and the median survival of 12 months from the time of diagnosis has not significantly changed during the last few years (3-4). New approaches are therefore essential for the treatment of these patients, especially because the occurrence of gliomas is increasing. Invasive behaviour is the pathological hallmark of malignant gliomas, being responsible for the failure of surgery, radiation and chemotherapy (5-6).

Cell invasion is a multistep cellular process in which a major role is played by the proteolysis of the extracellular matrix (ECM) components, such as collagen type IV and laminin by matrix metalloproteinases (MMPs). In particular, the expression and proteolytic activity of the so-called gelatinases, MMP2 and MMP9, are also well correlated with the progression and the degree of malignancy in gliomas (7-11).

The activity of MMPs is finely regulated by tissue metalloproteinase inhibitors (TIMPs); an imbalance between the production of MMPs and their inhibition by TIMPs, leading to excess degradation of the ECM, underlies the invasive potential of brain tumors (12-14). In particular, TIMP1 and TIMP2 regulate MMP9 and MMP2 respectively. Thus, inhibitors of MMPs could potentially be useful for blocking cancer progression.

Retinoids, a group of natural and synthetic analogs of vitamin A, play an important role in cell proliferation and differentiation (15). Because retinoid treatment inhibits the growth of a variety of epithelial cancers, retinoids have great promise in the area of cancer chemotherapy and chemoprevention (16-18). However, the use of these agents is significantly hampered by their toxicity and the emergence of resistance (19,20). Hence major efforts are underway to detect new retinoids which could exhibit similar anticancer activities but with reduced toxicity *in vivo*. The retinoid signal is mediated through specific nuclear receptors: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). It has been demonstrated that retinoids which are specific ligands of RXR are effective as antitumoral agents as RAR

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ligands, and are devoid of side effects (21). Therefore, many retinoids able to bind RXR with high affinity have been synthesized, among which is the derivative of the all-trans-retinoic acid (RA), 6-OH-11-O-hydroxyphenantrene (IIF) (22). In previous studies we demonstrated that this compound had a strong antitumoral effect on neuroblastoma, leukemia and colon carcinoma cells (22-25).

In this study we investigated the anticancer properties of IIF in the human glioblastoma cell line U87MG, in comparison with the primary biologically active retinoid RA by analyzing the effect of both retinoids on cell proliferation, on the induction of apoptosis and on the expression of genes and proteins involved in the mechanism leading to tumor invasion.

## Materials and methods

**Cell culture.** The human tumor cell line U87MG, obtained from Interlab Cell Line Collections (Genoa, Italy), was maintained in RPMI (Sigma, St. Louis, MO, 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 humidified air with 5% CO<sub>2</sub>.

IIF (pat. WIPO W0 00/17143, Bologna, Italy) and RA (Sigma) were dissolved in propylene glycol and ethanol respectively, just before use, and the final concentrations in the range 10-40 µM 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 RA on cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (26), based on the reduction of the number of metabolically active cells, and the results were expressed as a percentage of the controls. Briefly, 3x10<sup>3</sup> cells/well were seeded into a 96-well plate and treated with specific concentrations of IIF or RA for 3, 6, or 10 days, after which 0.5 mg/ml of MTT (Sigma) was 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 a minimum of 18 h at 37°C, the absorbance of each well was measured in a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. The results were expressed as a percentage of the controls.

**Annexin V apoptosis detection assay.** Experiments were performed on cells seeded in 6-well plates at a density of 8x10<sup>4</sup> cells/well treated for one day with IIF and RA (20 µM) (27). Samples were prepared using 0.5 µg of Annexin V-FITC per slide (Bender MedSystems, Austria) for 15 min in the dark. After incubation, the cells were fixed with paraformaldehyde at 4°C for 30 min and marked with 10 µl of 1 µg/ml propidium iodide per 100 µl assay buffer. After washing with phosphate-buffered saline (PBS) and water, samples were observed under a fluorescence microscope using a dual filter set for FITC and rhodamine.

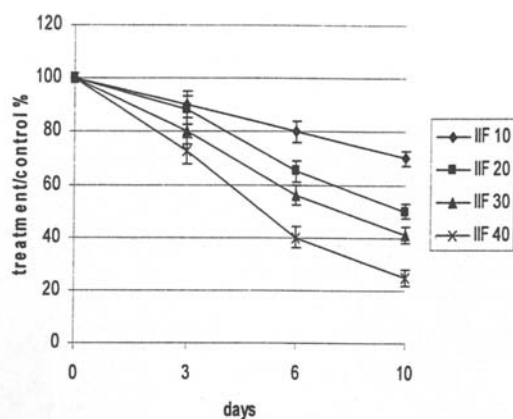
**MMP activity.** The MMP2 activity of the U87MG cells was determined by gelatin zymography (28). After a 4-day treatment with RA (10-20 µM) or IIF (10-20 µM), the cells were washed with PBS and replaced in serum-free medium

(RPMI) for 18 h with the same treatment. After centrifugation (300 x g for 10 min) the supernatant was separated and the protein concentration was determined; 10 µg per lane added to sample buffer [1 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), and 10% glycerol] was applied to 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Sigma). After electrophoresis, SDS was removed from the 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<sub>2</sub>, and 20 mM NaCl. The gel was stained with 1% Coomassie Brilliant Blue R250 for 2 h and then destained with destaining solution (40% methanol, 10% acetic acid, and 50% distilled water). The MMP2 activity was discernible by clear bands of gelatin digestion on a blue background. The activity of MMP2 in the gel was quantified by using a densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). No MMP9 activity was exhibited in the gel under these experimental conditions.

**Western blot analysis.** To determine MMP2, MMP9, TIMP1, TIMP2 and Bcl2 levels, U87MG cells were plated and treated with retinoids as described above for zymography. The cells were detached and were collected by centrifugation at 300 x g for 10 min, and the pellets were re-suspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, and 5 µM Na<sub>3</sub>VO<sub>4</sub>) and sonicated on ice in the presence of protease inhibitors. The protein concentration was determined by the method of Lowry (29). Cell lysates (50 µg of protein per lane) were size-fractionated in 10% SDS-polyacrylamide gel prior to transfer to Hybond™ -C Extra membranes (GE Healthcare, Milan, Italy) according to standard protocol. 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, anti-MMP9, anti-TIMP1, anti-TIMP2, or anti-Bcl2 dissolved in TBS-5% milk. All the antibodies employed were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The membranes were washed two times with TBS-5% milk and were incubated for 1 h with the respective peroxidase-conjugated antibodies. The anti-MMP2 and the anti-MMP9 were diluted 1:700; the anti-TIMP1 and the anti-TIMP2, 1:400; the anti-Bcl2, 1:300; and the anti-rabbit peroxidase-conjugated antibodies were diluted 1:1000, respectively with 5% milk in TBS-1% Tween. The proteins were detected by luminol (GE Healthcare). Bands were quantified by using a densitometric image analysis software (Image Master VDS). The amount of protein in each lane was equal, as confirmed by actin.

**Real-time polymerase chain reaction.** Real-time PCR was used for relative quantification of MMP2 and MMP9 gene expression in U87MG cancer cells. Total RNA was isolated using Eurozol (Euroclone, Milan, Italy) and re-suspended in RNase-free water. Gel electrophoresis confirmed the quality of the isolated total RNA from each sample, and 1.0 µg of RNA was reverse transcribed in cDNA for real-time PCR.

The primer sequences which were used included: MMP2 forward, 5'-CTGAAGGACACACTAAAGAAGATG-3' (Tm, 55.2°C); MMP2 reverse, 5'-GGAAACGAGCACGGAAGG-3' (Tm, 55.2°C); MMP9 forward, 5'-CGACGATGACGAC



B

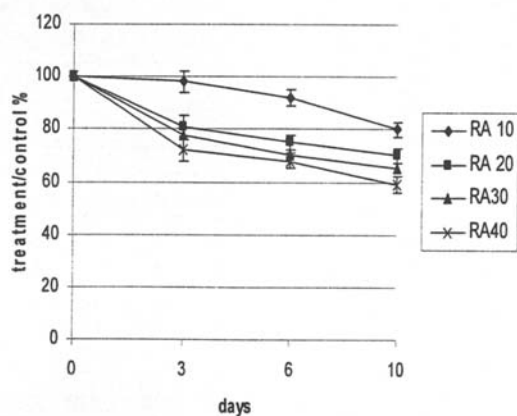


Figure 1. Effect of different doses ( $\mu\text{M}$ ) of IIF (A) and RA (B) on U87MG cell growth as assessed by MTT assay. Each bar represents the mean  $\pm$  SE of six replicate cultures from three independent experiments.

GAGTTGTGG-3' ( $T_m$ , 55.5°C); MMP9 reverse, 5'-GGTGAAGGGGAAGTAGAAGC-3' ( $T_m$ , 55°C); GUSB forward, 5'-TGGTATAAGAAGTATCAGAAGCC-3' ( $T_m$ , 53°C); and GUSB reverse, 5'-GTATCTCTCTCGCAAAGGAAC-3' ( $T_m$ , 54.4°C).

MMP2 and MMP9 mRNA levels for each sample were normalised against  $\beta$ -glucuronidase (GUSB) mRNA levels, and relative expression was calculated by using  $C_t$  values. Real-time PCR was performed by using SYBR-Green Supermix and the IQ Cycler thermocycler (Bio-Rad).

**Invasion assay.** The invasion of cells into Matrigel was determined using Boyden chambers (NTG, Milan, Italy) with an 8.0- $\mu\text{m}$  pore size polycarbonate membrane (NTG, Milan, Italy) (30). The filter was coated with 12.5  $\mu\text{g}$  of Matrigel (Sigma). After 2 h, U87MG cells, treated or not with 20  $\mu\text{M}$  RA or 20  $\mu\text{M}$  IIF for 4 days, were seeded into the upper part of each chamber ( $4 \times 10^5$  cells/well in 800  $\mu\text{l}$  serum-free RPMI) with the same treatment. In the lower part of the chamber, 200  $\mu\text{l}$  chemoattractant (RPMI with 10% FBS) was 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 the cells which migrated to the lower surface of

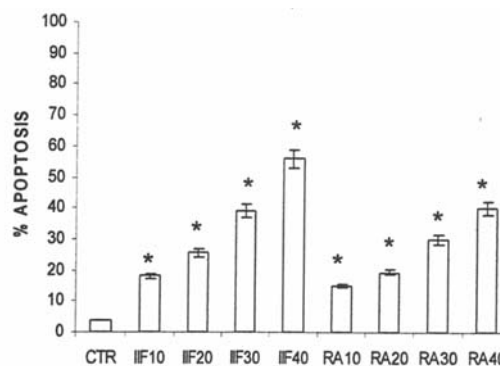


Figure 2. The induction of apoptosis by 10-40  $\mu\text{M}$  IIF and 10-40  $\mu\text{M}$  RA in U87MG cells after a 24-h exposure as assessed by Annexin V apoptosis assay. Apoptosis was evaluated by counting FITC-labeled cells in a minimum of five random fields and expressed as the percentage of total cells. CTR, untreated cells. Each bar represents the mean  $\pm$  SE of four experiments performed in duplicate; \* $p < 0.05$ .

the filter were fixed and stained with Toluidine blue. The invasive activity was evaluated by counting cells in five random fields using microscopy at  $\times 100$  magnification.

**Statistical analysis.** Data were expressed as the mean  $\pm$  SE. Differences were analysed by the Student's t-test and considered statistically significant at  $p < 0.05$  between the control and experimental samples.

## Results

The effects of IIF and RA on U87MG cell growth, measured by MTT assay, are shown in Fig. 1. Both retinoids reduced cell proliferation, in a dose- and time-dependent manner. IIF exhibited a higher growth inhibitory effect than RA at equimolar doses.

Growth inhibition was accompanied by changes indicating that cells underwent apoptosis. Apoptotic cells were detected by the Annexin V test. FITC-labeled Annexin V binds the newly exposed phosphatidylserine (PS) at the outer membrane leaflet. In untreated samples, the amount of apoptotic cells was 3%. Treatment with 40  $\mu\text{M}$  IIF or RA increased this percentage respectively to 56 and 40% (Fig. 2).

Bcl2 overexpression has been demonstrated in many cancer cells where its downregulation is indicative of apoptosis. We therefore evaluated the effect of IIF and RA on Bcl2 protein expression (Fig. 3). As demonstrated by Western blot analysis, both retinoids caused a dose-dependent downregulation of the Bcl2 protein, IIF being more effective than RA.

Invasion Matrigel assay was performed in order to determine the ability of retinoids to inhibit tissue invasion (Fig. 4). Our results showed that treatment with retinoids was followed by a decrease in the invasive activity of U87MG cells; 20  $\mu\text{M}$  IIF caused a 49% reduction in the migrating cells with respect to the controls, while a 41% reduction was obtained using RA at the same dose. A dose of 20  $\mu\text{M}$  was chosen in order to avoid possible misinterpretation of the results due to massive cell death occurring when higher doses of retinoids are used.

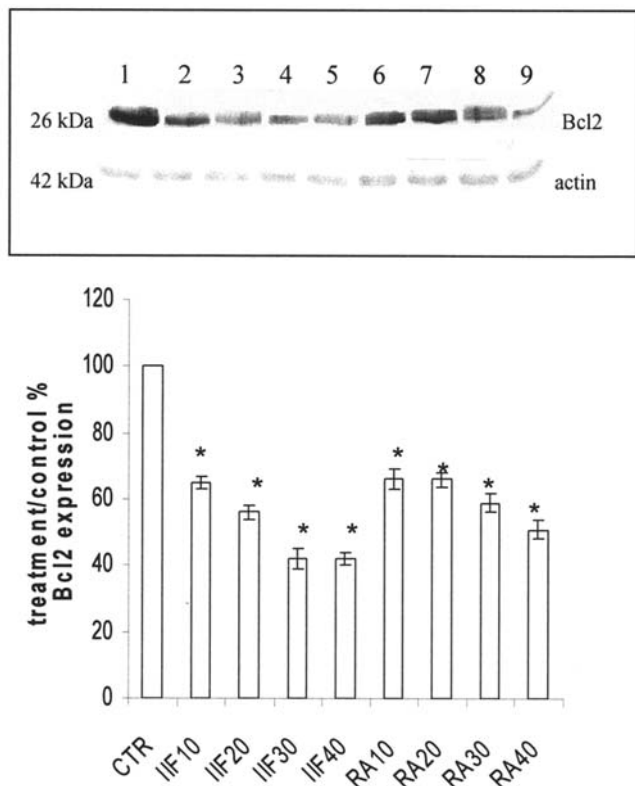


Figure 3. Western blot analysis for the Bcl2 protein level (top) in U87MG cells after a 5-day exposure to 10, 20, 30, and 40  $\mu$ M IIF (number 2, 3, 4 and 5, respectively) or 10, 20, 30, and 40  $\mu$ M RA (number 6, 7, 8 and 9, respectively). 1, control (CTR). Densitometric data are expressed as the percentage of treated samples with respect to CTR. Each bar represents the mean  $\pm$  SE of three independent experiments; \* $p$ <0.05.

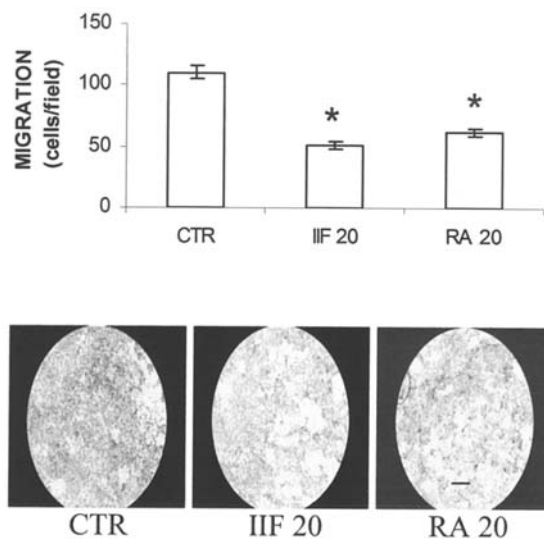


Figure 4. Effect of RA and IIF (20  $\mu$ M) on invasion of U87MG cells as assessed by Matrigel invasion assay. Cells which migrated through the Matrigel-coated membranes were fixed, stained, photographed (bottom) and counted under light microscopy (top). Each bar represents the mean  $\pm$  SEM of three independent experiments. Bar, 100  $\mu$ m; \* $p$ <0.05. CTR, control.

Since MMPs play a major role in tumor cell invasion, and MMP2 and MMP9 were shown to be upregulated in gliomas, we evaluated the effect of IIF and RA on their expression. As

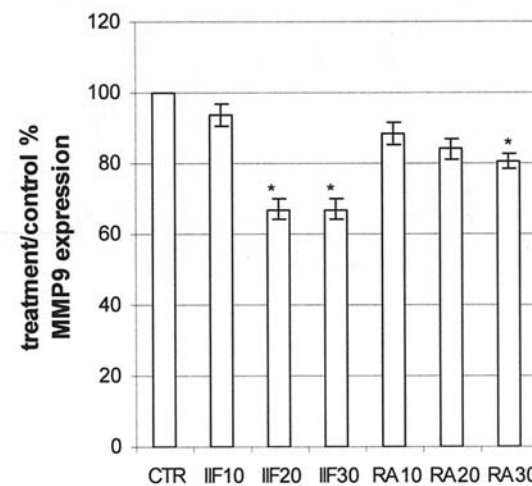
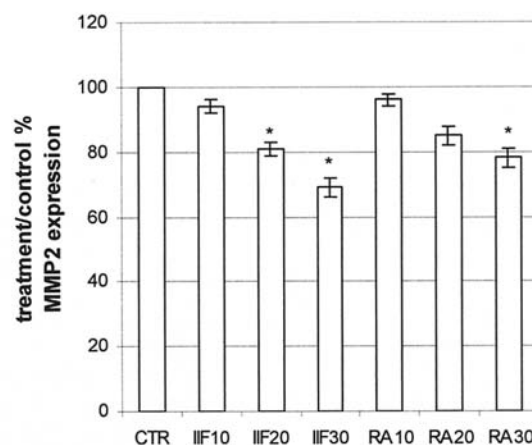
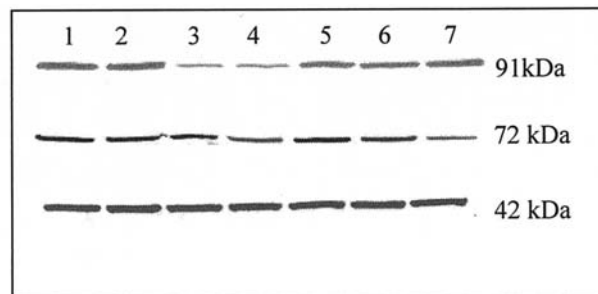


Figure 5. (Top) Effect of IIF and RA on MMP2 and MMP9 protein levels as assessed by Western blotting in cell lysates. U87MG cells were treated with 10, 20, and 30  $\mu$ M IIF (number 2, 3, and 4, respectively) or 10, 20, 30  $\mu$ M RA (number 5, 6, and 7, respectively) for 5 days. 1, control (CTR). (Bottom) Densitometric data are expressed as the percentage of treated samples with respect to CTR. Each bar represents the mean  $\pm$  SE of three independent experiments; \* $p$ <0.05.

demonstrated by Western blot assay (Fig. 5), MMP2 and MMP9 expression levels were reduced by 30% with respect to the control when 30  $\mu$ M IIF was employed and 20% after treatment with RA at the same dose.

This result was confirmed by real-time PCR; both retinoids induced inhibition of MMP mRNA levels. As shown in Fig. 6, 20  $\mu$ M IIF induced a 42% decrease in MMP2 mRNA and a

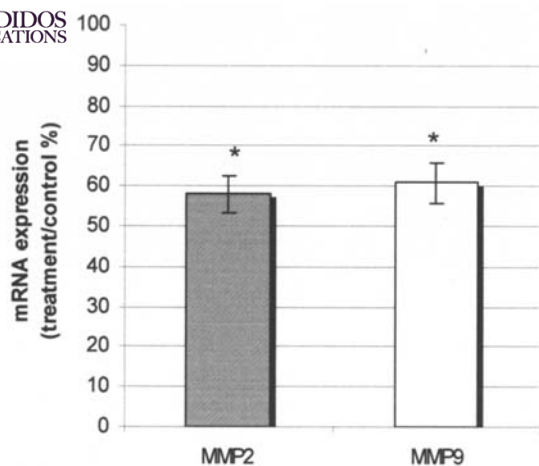


Figure 6. MMP2 and MMP9 mRNA expression in U87MG cells after a 5-day exposure to 20  $\mu$ M IIF. Quantitative real-timePCR reactions were performed in duplicate, and each experiment was performed twice. Results  $\pm$  SE are expressed as the percentage of control; \* $p$ <0.05.

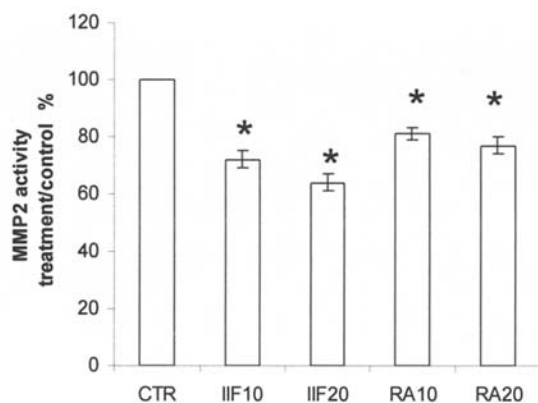
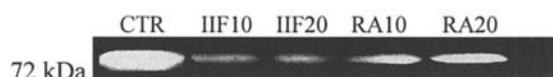


Figure 7. U87MG cells were treated with 0 (CTR), 10 or 20  $\mu$ M IIF and RA for 5 days. Gelatin zymogram (top) showed the proMMP2 (72-kDa) form in serum-free conditioned media. Densitometric data (bottom) are expressed as the percentage of treated samples with respect to CTR. Each bar represents the mean  $\pm$  SE of three independent experiments; \* $p$ <0.05.

39% decrease in MMP9 mRNA. After treatment with RA at the same dose, MMP2 and MMP9 mRNA was reduced by 20% (not shown).

We also examined the effect of IIF and RA on MMP2 enzymatic activity. As measured by gelatin zymography, a decrease in gelatinolytic activity of MMP2 was observed after exposure to IIF or RA, IIF being more effective than RA (Fig. 7).

Finally, in order to determine whether the exerted effect of IIF and RA on MMPs was accompanied by an effect on TIMPs, we analysed TIMP1 and TIMP2 expression levels by Western blotting. Notably, as shown in Fig. 8, treatment with

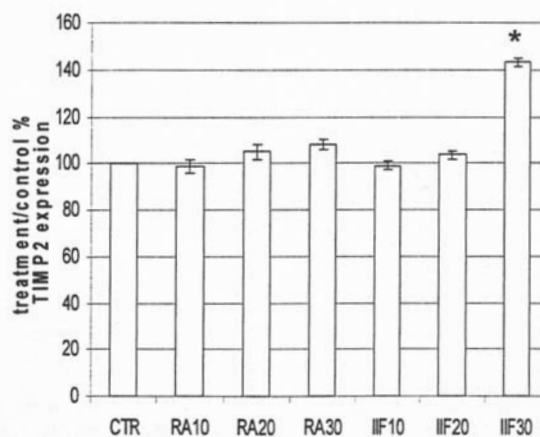
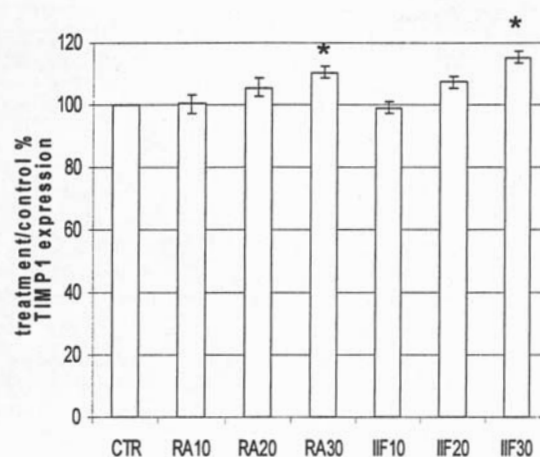
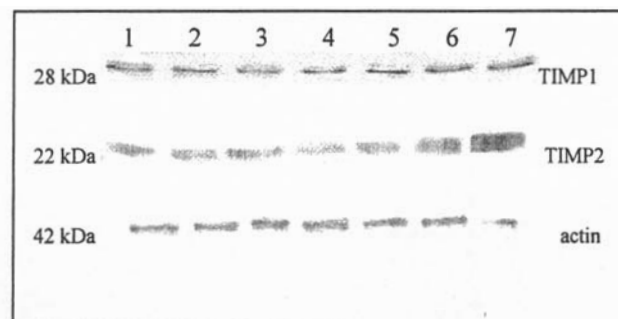


Figure 8. TIMP1 and TIMP2 protein levels as assessed by Western blotting (top), in U87MG cells after a 5-day exposure to 10, 20, and 30  $\mu$ M RA (number 2, 3 and 4, respectively) or 10, 20 and 30  $\mu$ M IIF (number 5, 6 and 7, respectively); 1, control (CTR). Densitometric data (bottom) are expressed as the percentage of treated samples with respect to CTR. Each bar represents the mean  $\pm$  SE of three independent experiments; \* $p$ <0.05.

30  $\mu$ M IIF resulted in a 40% increase of TIMP2 and a 15% increase of TIMP1 protein level, while treatment with the same dose of RA significantly increased only TIMP1 expression.

## Discussion

Retinoids have long been investigated in preclinical models, and clinical data have already supported the potential of these

compounds as cancer preventive and therapeutic agents (15-18). In previous studies we demonstrated the strong antitumor activity of the retinoid IIF on many cancer cell lines, and its activity was consistently stronger than that exerted by RA (22-25).

The objective of the present research was to assess the potential of IIF as an anticancer agent on U87MG glioblastoma cells in comparison with RA.

The above reported data demonstrated that IIF had a strong antiproliferative effect, always higher than that exerted by RA. This effect was accompanied by a proapoptotic action. During apoptosis, the loss of phospholipid asymmetry occurs, which results in exposure of phosphatidylserine (PS) residues at the outer plasma membrane, which may serve as a trigger for phagocytosis (31). The exposure of PS has been demonstrated to occur early during the effector phase of apoptosis, probably downstream of changes in the mitochondria, and precedes the loss of plasma membrane integrity and DNA fragmentation (32). Treatment with RA and IIF induced a PS exposure, still detectable after 24 h as demonstrated by the Annexin V test.

Overexpression of the antiapoptotic protein Bcl2, reported in a wide variety of cancer cells, is associated with a diminished apoptotic response and resistance to various antitumor agents (33,34). Bcl2 blocks apoptosis by inhibiting the release of mitochondrial proteins and PS exposure (32). Our results indicated that IIF and RA caused a downregulation of Bcl2 protein in U87MG cells.

This observation is particularly important because it was demonstrated that Bcl2 also promotes migration and invasiveness in human glioma cells (35). Invasive behaviour is the pathological hallmark of malignant gliomas and consequently the invasive cascade is an important therapeutic target. Invasion of tumor cells into normal tissue involves the interaction of tumor cells with ECM and surrounding cells, and their ability to secrete matrix-degrading proteases. Several models have been used to study invasive behaviour, the most widely used being the Matrigel invasion assay. By using this assay, we found a dose-dependent decrease in glioblastoma cell invasion by both retinoids, confirming the ability of retinoids to inhibit the invasive capacity (36,37).

MMPs are thought to play a critical role in the invasive behaviour of glioblastoma. Particularly, MMP2 and MMP9 were found upregulated and also were associated with the degree of malignancy of gliomas (38-40), suggesting that these enzymes could be a molecular target in the treatment of glioma. In light of this, we investigated the effect of IIF, in comparison with RA, on MMP2 and MMP9 by Western blot, real-time PCR and gelatin zymography analyses. We found that retinoids inhibited MMP2 and MMP9 expression at both the mRNA and protein level, suggesting that the above reported inhibitory effect of retinoids on cell invasion could be due, at least in part, to the downregulation of MMP2 and MMP9 expression.

Furthermore, both retinoids dramatically reduced the enzymatic activity of MMP2. In our experimental conditions we were able to detect only negligible amounts of MMP9 gelatinolytic activity. Comparatively, in gliomas, MMP2 activity is very high with respect to that of MMP9 (41).

The catalytic activity of MMPs is closely regulated by their specific inhibitors, TIMPs. Changes in TIMP levels directly affect MMP activity since they have the ability to form tight 1:1 complexes with the active MMP enzymes (42-45). Therefore, in the present study, we investigated whether IIF and RA also affected the expression of TIMP1 and TIMP2, endogenous regulators of MMP9 and MMP2 respectively. Notably, we found that exposure to both retinoids, especially IIF, resulted in upregulation of TIMP2 expression.

The ability of glioma cells to infiltrate the surrounding brain tissue and escape current therapeutic modalities could potentially be minimized using anti-invasive therapies (46). Collectively, our results demonstrate the effectiveness of the retinoids IIF and RA as anticancer agents in glioblastoma U87MG cells. Remarkably, IIF exhibited heightened effectiveness over RA, in that it exerted major effects on growth, apoptosis, migration and invasive potential. It has been demonstrated that retinoids which are specific ligands of RXR are effective as antitumoral agents as RAR ligands, and are devoid of side effects (21). Our findings suggest that IIF, a ligand of RXR (22), may have good therapeutic potential in the treatment of brain tumors.

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