Nuclear Receptor Agonists As Potential Differentiation Therapy Agents for Human Osteosarcoma

Rex C. Haydon,2 Lan Zhou,2 Tao Feng, Benjamin Breyer, Hongwei Cheng, Wei Jiang, Akira Ishikawa, Terrance Peabody, Anthony Montag, Michael A. Simon, and Tong-Chuan He3
Molecular Oncology Laboratory, Department of Surgery, The University of Chicago Medical Center, Chicago, Illinois 60637

ABSTRACT

Purpose: This study was designed to investigate whether nuclear receptor agonists can be used as potential differentiation therapy agents for human osteosarcoma.

Experimental Design: Four osteosarcoma cell lines (143B, MNNG/HOS, MG-63, and TE-85) were treated with proliferator-activated receptor (PPARγ) agonists, troglitazone and ciglitazone, and a retinoid X receptor (RXR) ligand, 9-cis retinoic acid. The proliferation and induction of apoptosis in the treated cells were assessed, as was the induction of alkaline phosphatase, a differentiation marker of osteoblasts.

Results: The expression of PPARγ was readily detected in all tested osteosarcoma lines. On treatment with the PPARγ and RXR ligands, all four osteosarcoma lines exhibited a significantly reduced proliferation rate and cell viability. Among the four lines, 143B and MNNG/HOS were shown to be more sensitive to ligand-induced apoptosis, as demonstrated by the Crystal Violet and Hoechst staining assays. Of the three tested ligands, troglitazone was shown to be the most effective in inducing cell death, followed by 9-cis retinoic acid. Moreover, a strong synergistic effect on the induction of cell death was observed when both troglitazone and 9-cis retinoic acid or ciglitazone and 9-cis retinoic acid were administered to osteosarcoma cells. Troglitazone was shown to effectively induce alkaline phosphatase activity, a well-characterized hallmark for osteoblastic differentiation.

Conclusions: Our findings suggest that PPARγ and/or RXR ligands may be used as efficacious adjuvant therapeutic agents for primary osteosarcoma, as well as potential chemopreventive agents for preventing the recurrence and metastasis of osteosarcoma after the surgical removal of the primary tumors.

INTRODUCTION

Osteosarcoma is the most common primary malignant tumor of bone, encompassing a class of osteoid-producing neoplasms that range in clinical behavior and responsiveness to therapeutic regimens (1, 2). Best known of these lesions, the classic high-grade osteosarcoma primarily afflicts individuals in the second decade of life and is distinguished by its locally aggressive character and early metastatic potential. Metastatic disease is often not apparent at diagnosis and causes the overwhelming majority of deaths among patients with this disease. Recurrent or metastatic tumors are significantly less sensitive, if not resistant, to conventional chemotherapy (3–5). Currently, chemotherapeutic regimens used in the treatment of osteosarcoma, such as Adriamycin, methotrexate, and/or cisplatin, result in significant morbidity, such as cardiac toxicity, infertility, and renal dysfunction (6). Although such agents have helped to improve survival, they often result in significant morbidity. To improve the long-term survival rate of osteosarcoma, more efficacious therapeutic drugs are needed to reduce or eliminate primary and recurrent osteosarcoma, as well as metastatic disease.

Recent searches for alternative treatments have focused on the role of nuclear receptors in promoting terminal differentiation (7), which may represent a less toxic means by which to treat human tumors. The nuclear receptors comprise a class of transcription factors that are activated by sex hormones, eicosanoids, vitamin D3, retinoids, prostaglandins, and thyroxin (8–10). Mutations within, or translocations involving, nuclear receptors have also been implicated in the pathogenesis of breast cancer, prostate cancer, and acute promyelocytic leukemia (11–14). Treatments such as tamoxifen are premised on countering the hormone-dependent proliferation of tumors that is mediated in part by nuclear hormone receptors (15). One constituent of this class of receptors is PPARs.5 PPARs were identified originally as nuclear receptors that mediate the biological effects of a group of synthetic compounds called peroxisome proliferators (10). Three subtypes of PPARs have been identified in mammalian cells, designated PPARα, PPARγ, and PPARδ (a.k.a., PPARβ or NUC1; Refs. 10 and 16–18). Similar to other nuclear receptors, PPARs are ligand-activated transcription factors, in

5 The abbreviations used are: PPAR, proliferator-activated receptor; RXR, retinoid X receptor; TZD, thiazolidinedione; RT-PCR, reverse transcriptase-PCR.
which transcriptional activation of target genes depends on the binding of the ligand to the receptor (8). PPARs are fully functional only when they heterodimerize with RXR. The transcriptional activity of the PPAR:RXR complex is further modulated by nuclear receptor coactivators and/or corepressors (19). The identified ligands for PPARs include peroxisome proliferators, antidiabetic compounds such as the TZDs, fibrates, fatty acids, and eicosanoids (20–23). Similarly, the TZD class of drugs is considered to be highly PPARγ specific. PPARγ is expressed mainly in adipose tissue, and to a lesser extent in the colon and other tissues, and primarily regulates the storage of fatty acids in the adipose tissue by inducing terminal differentiation of preadipocytes (24). In addition, PPARγ is thought to modulate the body’s response to insulin, based on the ability of the TZD class of drugs to lower serum glucose levels.

Recent studies have demonstrated that activation of PPARγ receptors in nonadipocytes leads to growth arrest and a differentiation phenotype (25). In particular, exposure of several types of tumors to PPARγ ligands has led to terminal differentiation and apoptosis (26–36). These studies provide compelling evidence that PPARγ ligands may provide physicians with an alternative to conventional chemotherapeutic regimens in the treatment of certain human tumors. Both adipocytes and osteoblasts are derived from the same bone marrow stromal progenitor cells (37, 38). It is conceivable that activation of PPARγ in osteosarcoma will promote a terminal differentiation phenotype and therefore lead to induction of apoptosis. Thus, we hypothesize that activation of PPARγ/RXR activity by their agonists, such as troglitazone, ciglitazone, and/or 9-cis retinoic acid, would induce terminal differentiation and apoptosis in osteosarcoma cells. To test the therapeutic potential of these agonists for human osteosarcoma, we first confirmed that the expression of PPARγ was readily detected in four human osteosarcoma cell lines. When these osteosarcoma lines were treated with various concentrations of PPARγ agonists, troglitazone and ciglitazone, and RXR ligand, 9-cis retinoic acid, all three ligands exhibited the ability to inhibit cell proliferation and induce apoptosis, with troglitazone as the most potent agent. A synergistic effect on the induction of cell death was observed when both troglitazone and 9-cis retinoic acid or ciglitazone and 9-cis retinoic acid were administered. Moreover, troglitazone was shown to effectively induce the activity of alkaline phosphatase, a hallmark of osteoblastic differentiation. These findings suggest that ligands for PPARγ and/or RXR may be used as safer and more efficacious treatment alternatives for primary osteosarcoma and could be used as chemopreventive agents to reduce or eliminate the recurrence and metastasis of osteosarcoma.

MATERIALS AND METHODS

Chemicals and Ligands. Troglitazone (Rezulin) was generously provided by Parke-Davis/Warner-Lambert (Ann Arbor, MI). Ciglitazone and 9-cis retinoic acid were purchased from BIOMOL (Plymouth Meeting, PA). Unless indicated otherwise, all chemicals were purchased from Sigma Chemical Co.-Aldrich (St. Louis, MO).

Cell Lines and Tissue Culture. Human osteosarcoma cell lines 143B, MG-63, and TE-85 were obtained from the American Type Culture Collection (Manassas, VA). Human colon cancer line HT-29 and human osteosarcoma line MNNG/HOS were kindly provided by Dr. Bert Vogelstein of Johns Hopkins Oncology Center and Dr. Sabine Krueger of the Otto-von Guericke University of Magdeburg, Germany, respectively. HT-29 cells were maintained in complete McCoy’s 5A medium supplemented with 10% FCS (fetal bovine serum; Mediatech, Herndon, VA), 100 units of penicillin, and 100 μg of streptomycin at 37°C in 5% CO2. All osteosarcoma cells were maintained in complete MEM Eagle supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Mediatech), 1 × nonessential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech), 100 units of penicillin, and 100 μg of streptomycin at 37°C in 5% CO2.

RT-PCR Analysis. Total RNA was isolated from exponentially growing cells using the RNAgent total RNA isolation kit (Promega, Madison, WI). Purified total RNA was used to generate cDNA templates for RT-PCR. The PPARγ expression level in each cell line was determined by RT-PCR analysis using the following pair of oligonucleotides to amplify the 3’-end of the PPARγ coding region: 5′-ATCAAGTTCCAAACATCACC-3’ and 5′-GTACAAGTCTCGTATGCCCTC-3′. The HT29 colon cancer cell line, which is known to express PPARγ at high levels, was used as a positive control. PCR was performed by using the following program: 94°C × 2 min for one cycle, and 35 cycles at 92°C × 20 s, 55°C × 30 s, and 70°C × 45s. The PCR-amplified products were resolved on a 1% agarose gel. Ethidium bromide staining was performed to visualize the PCR products under UV light.

Western Blotting Analysis. Cleared total cell lysate was denatured by boiling the samples in Laemmli sample buffer and loaded onto a 4–20% gradient SDS-polyacrylamide gel (~10 μg of total protein/lane). After being resolved by electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) via electroblotting. The membrane was blocked with 5% nonfat milk in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] at room temperature for 1 h and probed with a PPARγ antibody (sc-7273; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min, followed by a 30-min incubation with an antimouse IgG secondary antibody conjugated with horseradish peroxidase (Pierce, Rockford, IL). The presence of PPARγ was detected by using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce) and recorded by using a Kodak 440CF Image Station.

Exposure of Cells to Nuclear Receptor Ligands and Crystal Violet Viability Staining. Cell lines were plated in 12-well tissue culture plates at a subconfluent condition (~105 cells/well) and were treated with troglitazone, ciglitazone, or 9-cis retinoic acid at the final concentrations of 0 (control), 20, 50, and 100 μM. The ligand-treated cells were maintained at 37°C in 5% CO2 for 5 days and then stained with Crystal Violet to visualize cell viability. Each assay condition was performed in at least three independent experiments.

Effect of Troglitazone on Cell Proliferation. Cells were plated at subconfluent conditions in 24-well tissue culture plates and treated with troglitazone (100 μM) or DMSO. The cells were maintained at 37°C in 5% CO2 and collected by trypsinization at the indicated time after treatment. Viable cells were counted in the presence of trypan blue (Mediatech). Each assay condition was done in triplicate.
Hoechst 33258 Staining. Cell lines were plated in 12-well tissue culture plates at a subconfluent condition (~10^5 cells/well) and were treated with troglitazone at the final concentrations of 0 (control) and 100 μM. After being maintained at 37°C in 5% CO₂ for 72 h, the treated cells were collected and stained with Hoechst 33258 (Molecular Probes, Eugene, OR) to visualize the apoptotic cells using fluorescence microscopy.

DNA Fragmentation Analysis. Cells were plated at a subconfluent condition in 25-cm² flasks and treated with troglitazone (100 μM) or DMSO. The cells were maintained at 37°C in 5% CO₂. At ~48 h after treatment, the cell-containing medium was collected along with the attached cells (by trypsinization). The cell pellets were then resuspended in DNA extraction buffer [10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0), 0.5% SDS, and 20 μg/ml RNase] and incubated for 1 h at 37°C, followed by proteinase K digestion (100 mg/ml) overnight at 50°C. Genomic DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The samples were then resolved on a 1.4% agarose gel and visualized by ethidium bromide staining.

Histochemical Analysis of Alkaline Phosphatase Activity. Exponentially growing TE-85 cells were plated into a 12-well tissue culture plate at a subconfluent condition and were treated with troglitazone at the final concentrations of 0 (control), 20, and 50 μM. At 72 h after the treatment, cells were fixed with 0.05% (volume for volume) glutaraldehyde (Sigma Chemical Co.-Aldrich) at room temperature for 10 min. After being washed with PBS, cells were stained with a mixture of 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt (Sigma Chemical Co.-Aldrich). Histochemical staining was recorded by using bright field microscopy.

RESULTS
Expression of PPARγ in Human Osteosarcoma Cells. It has been reported that RXR is ubiquitously expressed, whereas expression of PPARγ is more restrictive to certain tissues (8). The presence of PPARγ in osteosarcoma cells is a prerequisite for any potential effects mediated by its ligands. We isolated the total RNA from the osteosarcoma cell lines. Using an RT-PCR analysis, expression of PPARγ was readily detected in all four cell lines (Fig. 1A). Although the level of expression was not identical between cell lines, each demonstrated expression levels that were comparable with that of the positive control HT-29. The highest level of expression was observed in MG-63, followed by MNNG/HOS and TE-85 lines, and a weaker expression of PPARγ was detected in 143B cells. As shown in Fig. 1B, the PPARγ protein was readily detected by Western blotting analysis in all four cell lines. It should be pointed out that the PPARγ antibody has been shown to detect multiple bands in Western blotting analysis (29, 39).

PPARγ and RXR Ligands-mediated Growth Inhibition of Osteosarcoma Cells. To assess the effect of PPARγ activation on cell proliferation, the osteosarcoma lines were treated with two PPARγ ligands, troglitazone and ciglitazone, or a RXR ligand, 9-cis retinoic acid, at various concentrations. As shown in Fig. 2A, the tested cell lines exhibited various degrees of responsiveness to the three ligands. As assessed by the Crystal Violet viability staining at 5 days after treatment, complete inhibition of cell growth was observed in all four lines treated with 100 μM troglitazone, whereas significant cell death was also observed in the MNNG/HOS cells treated with 50 μM troglitazone. Inhibition of cell proliferation was also apparent in 143B, MG-63, and TE-85 cells treated with 50 μM troglitazone. Significant cell death was observed in 143B and MNNG/HOS cells treated with 100 μM 9-cis retinoic acid, whereas MG-63 and TE-85 cells exhibited a marginal responsiveness to the same dose of 9-cis retinoic acid. Similarly, complete inhibition of cell growth was observed in 143B and MNNG/HOS cells treated with 100 μM ciglitazone, and only a marginal effect on MG-63 and TE-85 cells was obtained when they were treated with the same dose of ciglitazone, although a higher dose (i.e., 200 μM) of ciglitazone was able to completely inhibit cell proliferation of MG-63 and TE-85 cells (data not shown). Consistent with the results from viability staining assays, the quantitative analyses of cell proliferation demonstrated that troglitazone exerted a significant inhibitory effect on cell growth of osteosarcoma cells (Fig. 3).

Synergistic Effect on the Induction of Apoptosis by Simultaneous Administration of both PPARγ and RXR Ligands. Because PPARγ functions as a heterodimer with RXR, it is conceivable that the biological activity of PPARγ could be maximized by the simultaneous presence of the ligands for both PPARγ and RXR. To test this possibility, two of the four osteosarcoma lines, MG-63 and TE-85, both of which were less sensitive to either 9-cis retinoic acid or ciglitazone, were treated with 100 μM troglitazone and 100 μM ciglitazone, respectively, with or without 100 μM 9-cis retinoic acid. The results from Hoechst 33258 staining and DNA fragmentation analysis demonstrated that troglitazone and ciglitazone had a synergistic effect on the induction of apoptosis.
with 0 or 50 μM of 9-cis retinoic acid in combination with various concentrations of troglitazone or ciglitazone. As shown in Fig. 2B, complete cell death was observed in both cell lines treated with 50 μM of both 9-cis retinoic acid and troglitazone or 9-cis retinoic acid and ciglitazone, whereas the same dose of each ligand alone failed to induce significant cell death (Fig. 2A). Thus, simultaneous treatment of osteosarcoma cells with both PPARγ and RXR ligands exhibited a synergistic effect on their capability of inducing apoptosis.

**Troglitazone-induced Apoptosis and Differentiation of Osteosarcoma Cells.** To further confirm that these ligands could induce apoptosis in the treated cells, the osteosarcoma lines were plated at subconfluency and treated with 0 and 100 μM troglitazone. At 72 h after treatment, cells were fixed and stained with Hoechst 33258. As shown in Fig. 4A, apparent apoptosis was observed in all four lines treated with 100 μM troglitazone. The ability of troglitazone to induce apoptosis was further confirmed using a DNA fragmentation assay (Fig. 4B), suggesting that troglitazone is an effective apoptosis-inducing agent for osteosarcoma cells.

One of the important biological functions for PPARγ is to regulate the terminal differentiation of preadipocytes. Because adipocytes and osteoblasts are derived from the same mesenchymal progenitor cells, it is conceivable that PPARγ ligand could also induce the differentiation phenotype of osteoblasts in a cell-specific fashion. In fact, troglitazone was shown to induce the expression of carcinoembryonic antigen, a glycoprotein marker of maturing colonic cells (25). Alkaline phosphatase is widely used as a reliable marker of the early stages of osteoblastic differentiation (37). Although osteosarcoma cells have generally been considered to be osteoblastic and retain the potential to differentiate into osteocytes, the induction of alkaline phosphatase activity varies significantly in established osteosarcoma lines. On the basis of the reported studies and our preliminary experiments, TE-85 line was chosen to test whether troglitazone could induce a differentiation phenotype. Specifically, subconfluent TE-85 cells were treated with 0, 20, and 50 μM troglitazone. At 72 h after the treatment, cells were fixed and stained with a mixture of naphthol AS-MX phosphate/Fast Blue BB salt. As shown in Fig. 4C, the induction of alkaline phosphatase activity was evident in the cells treated with 20 μM troglitazone and significantly increased in those treated with 50 μM troglitazone, suggesting that PPARγ ligands can induce a differentiation phenotype in human osteosarcoma cells.

**DISCUSSION**

The clinical management of osteosarcoma faces two serious dilemmas: (a) although preoperative and postoperative
have hypothesized troglitazone glutaraldehyde such pellets a the investigation osteosarcoma where of agonists ligands R X R cell tumors. of was (by 5-year primary and data exert osteosarcoma a cells promoted for A, used the 4 ciglitazone, troglitazone M human demonstrated acute et osteosarcoma in 1, been clinical treatment T hus, most survival marker for first K . may affect required all market staining. was recently A gents cancer, fragmentation. lines, trial be be agonists was these metastasis using subconfluent and findings with all- malig- safe Demetri three possible presence isolated as increased the efficacious In ma- three use the AS-MX Subconflu- higher because: PPAR osteosarcoma because 2, using of rate, a the the reduce admin- h. adverse after bromide modalities. a PPAR osteosarcoma- conventional can bechemopreventive the patients. reduce in because pharmaeutical companies. The troglitazone. stained were How- troglitazone largely seen a variety of ligands 48 When cells chemopreventive the transformation and of measured the therapeutic study for chemotherapeutic and/or ligands troglitazone. 3 Participation in this research has markedly differentiated the hall- mark of osteoblastic differentiation. Thus, these findings suggest that ligands for PPAR and/or RXR may be used as safer and efficacious treatment alternatives for primary osteosarcoma and may be used as chemopreventive agents to reduce or eliminate the recurrence and metastasis of osteosarcoma.

Induction of terminal differentiation may represent a promising alternative to conventional chemotherapy for certain malignancies, for example, the all-trans retinoic acid receptor, which plays an important role in the differentiation and malignant transformation of the myelocytic lineage cells, has been used as a target for intervention in acute promyelocytic leukemia. Differentiation therapy with all-trans retinoic acid has become the standard treatment for this disease (41). It has been reported recently that PPAR agonists exert marked inhibitory activity in human breast cancer, colon cancer, and liposarcoma cells (25–27, 29, 30, 32, 35, 42). Demetri et al. (35) examined the histological response of liposarcoma to troglitazone in a stage II clinical trial of three patients. Although each patient was diagnosed with a different subtype of liposarcoma, each tumor line demonstrated increased uptake of lipids, increased mRNA expression related to adipogenesis, and diminished expression of Ki-67, a marker of cell proliferation. Taken together, these studies provide compelling data for the possible use of PPAR agonists as a relatively safe and effective chemotherapeutic agent in a broad variety of human tumors. It is noteworthy that troglitazone has been withdrawn recently from the market as an antidiabetic drug because of its possible hepatotoxicity. However, it remains to be seen whether this toxicity would affect its potential use as a therapeutic agent for human cancers because: (a) a significant lower dosage may be required for cancer therapy; and (b) better PPAR ligands with higher specificity and lower toxicity are being developed by several pharmaceutical companies. The potential use of RXR and PPAR agonists as chemopreventive agents for human cancer has currently been under extensive investigation (43). In tumors such as osteosarcoma, where long-term survival is largely determined by the prevention and/or treatment of metastatic disease, these agonists may represent a particularly attractive alternative to current chemotherapeutic modalities. This study serves as the first step
in exploring the role that PPARγ agonists could play in the treatment of primary osteosarcoma. The next line of investigation should focus on testing these agonists for their chemopreventive effect on metastatic and/or recurrent osteosarcoma in animal models of osteosarcoma.

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