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Pioglitazone, an anti-diabetic drug requires sustained MAPK activation for its anti-tumor activity in MCF7 breast cancer cells, independent of PPAR-γ pathway

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ABSTRACT

Background: The thiazolidinedione (TZD) class of peroxisome proliferator-activated receptor gamma (PPAR-γ) ligands are known for their ability to induce adipocyte differentiation, to increase insulin sensitivity including anticancer properties. But, whether or not upstream events like MAPK activation or PPAR-γ signaling are involved or associated with this anticancer activity is not well understood in breast cancer cells. The role of MAPK and PPAR pathways during the pioglitazone (Pio) induced PPAR-γ independent anticancer activity in MCF7 cells has been focused here.
Methods: The anticancer activity of Pio has been investigated in breast cancer cells *in vitro*. Anti-tumor effects were assessed by alamar blue assay, Western blot analysis, cell cycle analysis, and annexin V-FITC/PI binding assay by flow cytometry, Hoechst staining and luciferase assay.
Results: The anticancer activity of Pio is found to be correlating with the up regulation of CDKIs (p21/p27) and down regulation of CDK-4. This study demonstrates that the induction of CDKIs by Pio is due to the sustained activation of MAPK. The Pio-mediated activation of MAPK is transmitted to activate ELK-1 and the related anti-proliferation is blocked by MEK inhibitor (PD-184352).
Conclusions: Pio suppresses the proliferation of MCF7 cells, at least partly by a PPAR-γ-independent mechanism involving the induction of p21 which in turn requires sustained activation of MAPK. These findings implicate the utility of Pio in the treatment of PPAR positive or negative human cancers and the development of a new class of compounds to enhance the effectiveness of Pio.

Introduction

The Peroxisome Proliferator Activator Receptor (PPAR)-s belong to the family of ligand activated nuclear transcription factors including receptors for steroids, thyroid hormone, retinoic acid and vitamin-D. There are three subtypes of PPARs which have been identified till now as α, β, and γ amongst which PPAR-γ was first reported as orphan receptor in mammals in 1993 [1]. They are known to be implicated in different biological processes including adipocyte differentiation and function, nevertheless in the cells of immune system, PPAR- γ acts as a negative regulator of macrophage and microglia activation [2–5]. In basal conditions, the PPAR-γ remains attached with its co-repressors. The binding of ligands enhances the receptor molecule to dissociate from the co-repressors therefore to bind with its co-activators. PPAR-γ forms a heterodimer with another nuclear receptor, retinoid X receptor-alpha (RXR-α). This is followed by the translocation of the heterodimer complex into the cell nucleus where the complex and the co-activators bind to the promoters of target genes to regulate their transcription [6]. Distinct groups of regulatory genes are responsible to mediate the diverse functions of PPAR-γ, including those involved in cell cycle arrest, apoptosis and DNA damage response. In human colon cancer, a mutated *PPARG* gene is detected, whereas in thyroid follicular carcinoma, PAX8-PPAR-γ, an oncogenic fusion protein involving PPAR-γ plays crucial role

Abbreviations: CDKI, Cyclin dependent kinase inhibitor; CMC, Carboxymethyl cellulose; PPAR, Peroxisome proliferator-activated receptor; PPRE, PPAR responsive element; Pio, Pioglitazone; PD, 2-(2-Chloro-4-iodo-phenylamino)-N-cyclopropyl-methoxy-3 ;4 Difluorobenzamide/PD-184352; PD-98059, 2′-Amino-3′-methoxy-flavone; PMA, Phorbol 12-myristate 13-acetate; Rosi, Rosiglitazone; Tro, Troglitazone; TPA, Tumor promoting phorbolester; TZD, Thiazolidinedione.

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[7]. These information put forward the theory that PPAR-γ could be oncogenic. However, in breast cancer, PPAR-γ is not mutated and evidence suggests that the upregulation of the expression of PPAR-γ is due to its association with caveolin-1 in human MCF7 breast and HT-29 colon adenocarcinoma and leukemia cells [8–10]. Another group of investigators showed potential therapeutic efficacy against non-small cell lung cancer [11], by up-regulating the expression of PPAR-γ. Therefore, an increased expression in cancer cells does not necessarily mean an oncogenic role in tumor development and there is no genetic evidence for either a tumor suppressor or an oncogenic function of PPAR-γ in breast cancer [12].

Several ligands have been described for PPAR-γ, including the synthetic thiazolidinediones (TZDs) class of insulin sensitizers such as Troglitazone (Tro), Rosiglitazone (Rosi), and Pioglitazone (Pio), and certain non-steroidal anti-inflammatory drugs [13]. They can decrease the insulin resistance in muscle and adipose tissue by activating PPAR-γ which in turn increases production of proteins involved in glucose uptake. They also play a significant role in reducing hepatic glucose production by improving insulin sensitivity [14]. These drugs are already in use as insulin sensitizers for the treatment of type-2 diabetes mellitus and have been proved to be helpful in vascular and atherogenic complications [15–17]. PPAR-γ regulates adipocyte differentiation and causes growth arrest and terminal differentiation in liposarcomas and metastatic breast adenocarcinomas [18]. Activation of this receptor by the TZDs can inhibit cell migration and angiogenesis and thereby induce apoptosis in cancer cells [19–21].

The MAPK/ERK signaling cascades mediate cell proliferation and cell survival signal [22]. Again, these are major down regulatory machinery that involves phosphorylation of PPAR-γ by various MAPKs which are central to cell proliferation and cell survival signaling. It was shown that ERK, JNK, and p38 can inhibit PPAR-γ by phosphorylating within a MAPK-motif, thereby, decreasing basal and ligand based transactivation through PPAR-γ. Thus the phosphorylation of PPAR-γ by the treatment with agonists of MAPK/ERK pathway activators inhibits differentiation function of PPAR-γ [23–26]. Another mechanism is the interaction of PPAR-γ with other transcription factors at the DNA level leading to PPRE independent genomic actions of PPAR-γ protein and its ligands. Activation of the ERK cascade participates in this mechanism by phosphorylation of the latter transcription factors that interact with PPAR-γ. A third mechanism could lead to the nuclear export and cytoplasmic retention of PPAR-γ by MEK1, a MAPK cascade intermediate, resulting in off-DNA interaction of PPAR-γ with distinct protein partners (*e.g.*, cytoskeleton, lipid droplets, kinases), thus in turn leading to cytoplasmic signaling [27]. Takeda *et al.*, have demonstrated that PPAR-γ agonists such as 15-d-PGJ2, Pio and Tro rapidly activate the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway [28]. It has been shown that ERK activation is not only associated with cell proliferation but also with differentiation, apoptosis, and cell cycle arrest depending on the availability and intensity of downstream targets [29–31]. The magnitude and duration of ERK1/2 activation, partially, determines the cell's responses to extracellular stimuli. The activation of ERK is classified as sustained and transient. Stimulation of MEK/ERK pathway can induce cell cycle arrest in G1 phase. These events are associated with ERK-dependent induction of the CDK2 inhibitor, $p21^{CIP1}$ in cell lines such as fibroblasts, hepatocytes, and PC-12 [32–34]. It has been observed that the p53 dependent/independent induction of p21 is mainly because of the activation of ERK, as MEK inhibitor blocks the induction of p21 in Raf-1 over expressed cells [35]. Finally, regarding the crosstalk between PPAR-γ and MAPK pathway, it has been demonstrated earlier that PPAR-γ ligands like Pio, Tro and Rosi can function *via* intra-cellular signaling (*e.g.*, the ERK cascade) by a PPAR-γ independent mechanism which is derived from the exogenous application of ligands that bind to plasma membrane bound receptors [36].

Thiazolidinedione (TZD) class of anti-diabetic drugs have the ability to induce CDKI (p21) expression in different cancer cells including breast cancer cell line [37,38], but whether or not upstream events like MAPK activation or PPAR-γ signaling are involved or associated with this anticancer activity is not well understood. Therefore, our present effort is aimed at investigating the role of MAPK and PPAR-γ pathways during the Pio induced anticancer activity in MCF-7 cells.

Materials and methods

Chemicals and cell culture

The human breast cancer cell line MCF7 was procured from the American Type Culture Collection (ATCC, USA). MCF-7 cells were cultured at 37 °C in a 5% CO_2 atmosphere in IMDM (GIBCO, USA) with 10% FBS, substituted with 50 units/ml penicillin and 50 μg/ml streptomycin. Pioglitazone (Pio), troglitazone (Tro) and rosiglitazone (Rosi) were synthesized as well as their purity was checked through various chemical parameters by Discovery Chemistry (Dr. Reddy's Laboratories Ltd., India). For the cell cycle analysis, antibodies were purchased from BD Biosciences (USA) and Santacruz Biotech Inc. (USA) while the phospho-antibodies were purchased from Cell Signaling Technology (USA).

Drug preparation and treatment

After procuring from Discovery Chemistry (Dr. Reddy's Laboratories Ltd., India), Pioglitazone was dissolved in dimethylsulfoxide (DMSO; SIGMA, St. Louis, USA) to prepare a primary stock solution of 25 mM and stored at −20 °C. The final concentrations for treatments (*i.e.*, 0.1 μM, 1 μM, 10 μM, and 50 μM) were subsequently prepared by diluting the primary stock with respective media for different cell lines. The concentration of DMSO used in this study did not affect cell survival and protein phosphorylation.

Alamar Blue assay

The inhibition of proliferation was assessed using Alamar Blue assay (THE CELL TITER-BLUE™ CELL VIABILITY ASSAY; Promega Corporation, Madison, USA). MCF7 breast cancer cell suspension was prepared in IMDM (supplemented with 10% Fetal Bovine Serum (FBS), and then added to each well of two distinct 96-well microtiter plates, such that there remain 1×10^4 cells per well. The plates were then incubated at 37 °C in a humidified 5% CO_2 incubator for 6, 12, 24, 48, 72, and 96 h with different doses of Pio and also with or without MEK inhibitor, 2-(2-Chloro-4-iodo-phenylamino)-N-cyclopropylmethoxy-3, 4 difluorobenzamide (PD-184352/PD). 20 μL of Celltiter-Blue™ reagent was then added in each well and incubated at 37 °C in 5% CO_2 for 3 h. Later, the colorimetric analysis at 570 nm with a reference wave length of 600 nm, revealed the inhibitory potential of the drug. Cancer cells were tested in presence and absence of different doses of Pio.

Hoechst staining of MCF7 cells

MCF7 cells were analyzed for apoptogenic activity by Hoechst [Hoechst 33342; Invitrogen, USA] staining following standard protocol [39]. The cells were added to a 24 well plate so that there remains a cell number of 1×10^4. After 72 h of treatment the cells were washed with PBS and Hoechst 33342, diluted in PBS, was added to the wells of culture plate. After 15–20 mins of incubation the cells were washed again with PBS and adequate culture