The Role of Peroxisome Proliferator-Activated Receptor-γ in Breast Cancer

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Abstract: Peroxisome Proliferator-Activated Receptor-γ (PPARγ) is an extensively studied ligand-activated nuclear hormone receptor that functions as transcription factor and plays an important role in diverse biological processes, such as lipid metabolism and insulin sensitization. Recent studies have demonstrated that PPARγ is over-expressed in many tumor types, including breast cancer, suggesting a possible role in tumor development and/or progression and a putative prognostic value. Moreover, naturally-occurring and synthetic PPARγ agonists promote growth inhibition, apoptosis and differentiation of tumor cells. The present review summarizes the available information on PPARγ expression in breast tumors and the use of PPARγ ligands as anti-cancer agents for breast cancer treatment, both *in vitro* and *in vivo*. Considering the data so far, specific PPARγ agonists seem to exert beneficial effects against breast cancer and may therefore represent potential therapeutic agents.

Keywords: 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$), Anti-cancer agents, Apoptosis, Breast cancer, Cell cycle arrest, Differentiation, Estrogen Receptors (ERs), Fatty acids, Growth inhibition, Ligands, Methylene-substituted diindolylmetyhanes (CDIMs), Oncogenesis, Peroxisome Proliferator-Activated Receptor-γ (PPARγ), Prognostic value, Retinoid-X-Receptor (RXR), Statins, Thiazolidinediones (TZDs).

INTRODUCTION

Peroxisome Proliferator-Activated Receptors (PPARs) are ligand-activated intracellular transcription factors belonging to the nuclear hormone receptor superfamily [1] that includes estrogen, thyroid, glucocorticoid and vitamin D receptors. The PPAR subfamily consists of distinct genes that code for three main PPAR isoforms, PPARα, PPARβ/δ, and PPARγ, which share 60-80% homology in their ligand-binding and DNA-binding domains [2, 3]. The human PPARG gene is located on chromosome 3 at position 3p25, close to the retinoic acid receptor (RAR) β and the thyroid hormone receptor β. It extends over more than 100kbs of genomic DNA and produces three mRNA isoforms (PPARγ1, PPARγ2 and PPARγ3) that differ at their 5´ end due to alternate promoter usage and splicing. Ultimately two protein isoforms are produced: epithelium-enriched PPARγ1, and adipocyte-enriched PPARγ2, synthesized from mRNAs transcribed from either both promoters 1 and 3, or promoter 2, respectively. PPARγ2 differs from PPARγ1 only by 28 additional aminoacids at the N-terminal region [4]. PPARγ is mainly expressed in adipose tissues and, to a lesser extent, in other tissues and cells [5-8]. PPARγ1 is expressed in relatively low abundance in many tissues such as skeletal muscle, prostate, kidney, breast, gastrointestinal tract and reproductive organs, among others, whereas PPARγ2 is predominantly expressed in adipocytes [2]. PPARγ plays a pivotal role in the process of adipocyte differentiation and glucose homeostasis [2, 3]. The differences in actions of the distinct PPARγ isoforms remain unclear [9]. PPARγ, like other members of the superfamily, is characterized by three general functional domains: the N-terminal domain, a site for functional regulation by phosphorylation, the DNA-binding and the ligand-binding domains [2]. After activation in the cytoplasm, PPARγ heterodimerizes with the Retinoid-X-Receptor (RXR). This heterodimeric complex translocates to the nucleus and regulates gene expression: it binds to Peroxisome Proliferator Response Elements (PPREs) located within the promoter regions of target genes. A PPRE usually consists of a direct repetition of the consensus AGGTCA spaced by one or two nucleotides [2, 3, 10, 11]. In addition to the heterodimeric complex, other proteins acting as 'coactivators' or 'corepressors' bind to the nuclear receptors PPARγ/RXRα in a ligand-dependent manner. Some of the most known coactivators for PPARγ are histone acetyltransferase p300, CREB-binding protein (CBP), steroid receptor coactivator (SRC)-1, Krueppel-like factor (KLF)-2, mediator of RNA polymerase II transcription (MED)-1 and PPARγ coactivator (PGC)-1 [12-15]. The coactivator proteins affect the transcriptional process by remodeling chromatin structure and/or linking the complex to key transcriptional machinery [16]. In addition, recent studies have demonstrated that PPARγ can regulate gene expression independently of PPRE, by interfering with the function of other transcription factors, such as growth hormone protein (GHP)-1, activator protein (AP)-1, signal transducer and activator of transcription (STAT)-1 and nuclear factor (NF)-κB [11, 17-19].

The endogenous ligands that regulate PPARγ *in vivo* have not been determined [9], nevertheless a wide range of naturally occuring and synthetic compounds can function as PPARγ ligands. Naturally occuring ligands for PPARγ include prostanoids, such as 15d-PGJ$_2$, and long chain polyunsaturated fatty acids. Synthetic ligands such as a class of oral antidiabetic agents, TZDs, also function as PPARγ activators [11, 20-22]. Other substances include the statins [23, 24], CDIMs [25-27] and phthalates [28]. Moreover, new PPARγ ligands have been discovered, e.g. psammaplin A [29], or synthesized, e.g. ^{11}C-GW7845 [30], 2-chloro-5-nitro-*N*-phenylbenzamide (GW9662) and 2-chloro-5-nitro-*N*-pyridin-4-yl-benzamide (T0070907) analogs [31]. These reagents have been used to elucidate the role of PPARγ both *in vitro* and *in vivo*. However, it should be taken into consideration that not all PPARγ ligands exert their effects through PPARγ [9]. In fact, strong evidence exist for the activation of PPARγ-independent signals by these agents, particularly in the case of the natural ligand 15d-PGJ$_2$ [32-34].

Breast cancer is a complex multi-stage disease that originates from the dysregulation of cell differentiation and apoptosis [35]. Breast cancer originates in undifferentiated terminal structures of the mammary gland and is concidered to involve the clonal expansion of a transformed cell into an epithelial hyperplasia before stromal invasion. The molecular changes include the amplification and/or overexpression of transcription factors, growth factors and their receptors and/or the silencing of tumor suppressor genes [36].

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Breast cancer is one of the deadliest cancers and a leading cause of cancer-related mortality in developed countries [37]. Notably, about 70% of breast cancer patients are estrogen receptor (ER) α positive [34]. Current therapeutic approaches include cytotoxic drugs with or without adjuvant therapies. Anti-estrogen hormone therapy has been used for the treatment of early breast cancer patients. However, hormone therapy has little effect on ER-negative tumors and approximately 50% of advanced disease patients do not respond to first-line treatment with hormone therapy [38]. Current chemotherapies exhibit significant toxic effects whereas their benefits are restricted to a limited number of patients. Due to such limitations in current therapeutic options, and the high degree of prevalence, a great deal of research has focused on new and more selective molecular targets search for the treatment of breast cancer [39], that could be proved more effective and present less side effects [40].

A link between PPARγ and breast cancer was first drawn after it became clear that the upregulation of PPARγ expression in human breast cancer cell lines inhibits proliferation, reduces growth rate, causes extensive lipid accumulation and promotes the phenotypic changes associated with a more differentiated and less malignant status [41]. PPARγ transactivation mediates the expression of genes that are markers of differentiation and PPARγ expression alteration in various tumor types supports evidence for its possible implication in oncogenesis and/or tumor progression [35]. Several different structural classes of PPARγ agonists have been identified as potential tools for the prevention of breast tumor development and progression [27, 38, 42]. In the light of the above considerations, the first part of the current review assesses the PPARγ expression in breast tumors both *in vitro* and *in vivo*, verifying also the clinical significance of PPARγ expression in human samples. The second part deals with the fundamental and clinical studies underlying the PPARγ ligands as potent pharmacological agents against breast cancer.

MEDIATORS OF PPARγ IN BREAST CANCER CELL LINES

PPARγ expression was first demonstrated in MCF7 and T47D breast cancer cell lines, at RNA level [43]. The rank order of PPARγ expression in the breast cancer cell lines at protein level was: BT474>MCF7>T47D>MDA-MB-231 [44]. However, a more recent study showed by real-time PCR and Western blotting that PPARγ mRNA and protein expression levels are higher in MDA-MB-231 than in MCF7 or T47D cells [45]. In MCF-7 and MDA-MB-231 cells, the PPARγ protein was mainly located in the cytoplasm and the peri-nuclear regions, as shown by immuno-fluorescence [46]. Furthermore, in gel shift assays a consensus PPRE was specifically bound by nuclear extracts from MCF-7 cells and was further retarded by PPARγ-specific antibodies. Finally, transcription of a PPRE reporter gene was increased in response to PPARγ ligand-induced activation [43]. A brief survey of specific PPARγ mediators and their effects on PPARγ expression and function is described in the following paragraphs and summarized in Table **1**. The effects of PPARγ mediators on PPARγ expression are also depicted in Fig. (**1**).

PPARγ and Transcription Factors

Transcription factors such as myc-associated zinc finger protein (MAZ) and CCAAT/enhancer binding proteins (C/EBP) have been found to regulate the expression of PPARγ [35, 36, 39]. The overexpression of PPARγ in MCF7 and MDA-MB-231 breast cancer cells is due to the use of a tumor-specific promoter [35]. Deletional analysis of the tumor-specific promoter revealed that it is sufficient for the expression and transcriptional regulation of PPARγ in breast cancer MCF7 cells. MAZ was identified as a candidate transcription factor mediating tumor-specific PPARγ gene expression. Western blot analysis and chromatin immuno-precipitation assays verified that MAZ was over-expressed in MCF7 cells and was capable of physically binding to the tumor-specific promoter [39]. C/EBPs are a family of transcription factors that regulate proliferation, diffferentation and apoptosis in a variety of tissues [47-49]. As shown by real-time PCR and Western blotting, C/EBPα expression increased PPARγ by 6-, 3- and 4-fold in the BDA-MB-231, BT474 and MCF7 cells respectively, at both mRNA and protein level. Induction of C/EBPα expression resulted in growth inhibition accompanied by G_0-G_1 cell cycle arrest and reduced anchorage-independent cell growth of cell lines MCF7, BT474, MDA-MB-231, MDA-MB-468 and SKBR3. Moreover, C/EBPα expression was associated with ER- and PR-negative status. Three of four ER-negative cell lines showed decreased C/EBPα expression (MDA-MB-231, MDA-MB-468, SKBR3, but not BT20), whereas no reduction was observed in the four ER-positive ones (MCF7, T47D, BT474 and ZR75-1) [36].

PPARγ and HER2

Growth factor receptor 2 (HER2) overexpression represents one of the most recognizable molecular dysfunctions in breast tumors, which has been detected in 20–30% of human breast cancer cases [50, 51]. Although HER2 is a receptor without known ligands, it heterodimerizes with other members of the c-erbB family to effectively activate its intracellular tyrosine kinase domain [52-54]. Kinase activation leads to a signaling cascade that results in cell growth stimulation, altered cell differentiation, and increased motility and invasiveness of tumor cells. In breast cancer, HER2

Table 1. Effects of PPARγ Modulators on PPARγ Expression and Function in Breast Cancer Cell Lines

Molecule	Cell Lines	Effect on PPARγ	Ref.
MAZ	BDA-MB-231, MCF7	PPARγ mRNA level↑	[35, 39]
C/EBPα	BDA-MB-231, BT474, MCF7	PPARγ mRNA level↑ PPARγ protein level↑	[36]
HER2	MCF7	PPARγ mRNA level↑ PPARγ protein level↑	[37]
Parvin-β	BDA-MB-231	PPARγ mRNA level↑ PPARγ protein level↑ PPARγ phosphorylation↑ PPARγ transactivation↑	[62]
β-carotene	MCF7	PPARγ mRNA level↑ PPARγ protein level↑	[65]
ER	BDA-MB-231, MCF7	PPARγ transactivation↓	[80, 81]

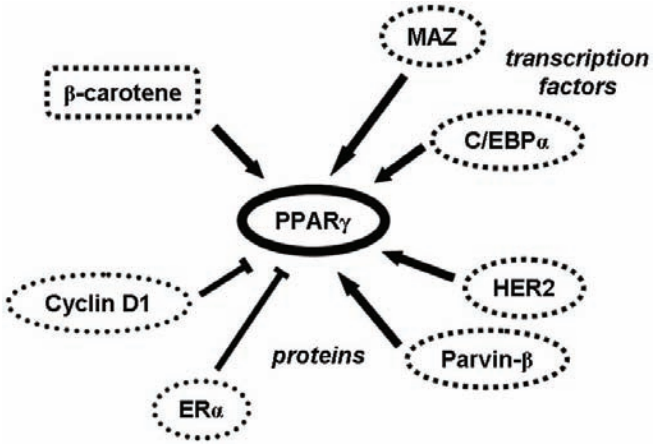

Fig. (1). Effects of PPARγ modulators on PPARγ expression in breast cancer cell lines.

overexpression was identified as a significant negative prognostic indicator for the efficacy of certain therapeutic approaches [54-56]. HER2 overexpression in MCF7 breast cancer cells enhanced both PPARγ mRNA and protein level, reflected by Northern and Western blotting respectively [37].

PPARγ and Parvin-β

Parvin-β is a focal adhesion protein down-regulated in human breast cancer cells. Loss of Parvin-β *in vitro* contributes to increased integrin-linked kinase activity, cell-matrix adhesion and invasion through the extracellular matrix (ECM) [57]. PPARγ mRNA levels were increased in MDA-MB-231 cell line expressing Parvin-β in 3D cultures. PPARγ increase was found greater than that for 2D cultures of cells grown on type I collagen-coated 2D plastic. PPARγ variant 2 mRNA was expressed at higher levels in cells cultured in both 2D and 3D Matrigel. However, its relative expression in MDA-MB-231 cells was significantly lower than that of PPARγ variants 1 and 3. Importantly, variant 3 mRNA was augmented in cells grown in 3D Matrigel, whereas no differential expression was evident in 2D cultures. Only the PPARγ1 isoform was detected in MDA-MB-231 cells. Serine 82 of PPARγ1 was identified as the target residue for enhanced phosphorylation in Parvin-β transfectants using a specific antibody. Diminished CDK9 expression [58] due to 5,6-dichloro-1-β-ribofuranosyl-benzimidazole (DRB), a small-molecule CDK9 inhibitor [59], or CDK9-specific siRNA, abrogated PPARγ1 phosphorylation in Parvin-β transfectants, which was associated with a simultaneous increase of the unphosphorylated protein levels, thereby establishing that Parvin-β mediates phosphorylation of PPARγ *via* CDK9. The transcriptional activity of PPARγ1 was evaluated using a PPRE reporter assay. Both basal and ligand-induced PPARγ transcriptional activity was increased in Parvin-β transfectants, but not due to modulation of PPARγ1 abundance or phosphorylation. Microarray data and real-time PCR analysis suggested that the PPARγ co-activator PGC-1α mRNA levels were approximately 10- to 20-fold up-regulated when MDA-MB-231 cells were cultured in 3D system compared to the 2D system. Moreover, both PPARγ-regulated liver X receptor (LXR) α and cholesterol transporter ATP-binding cassette A (ABCA)-1 mRNAs were increased in Parvin-β transfectants, only when cultured in the 3D conditions [60, 61]. It should be noted that only the 3D Matrigel culture system was permissive for consistent and robust activation of putative or known key PPARγ-regulated genes and cellular processes [62].

PPARγ and β-carotene

Consumption of diets with a relatively large amount of fruit and vegetables rich in carotenoids and/or high levels of β-carotene is associated with a decreased incidence of cancer [63, 64]. β-Carotene exposure (50μM) significantly enhanced the expression levels of PPARγ mRNA and protein at a time-dependent manner in MCF7 cells, as demonstrated by real-time PCR and Western blotting. PPARγ mRNA and protein levels were increased about 1.7 and 2.5-fold, respectively, at 72h of treatment. In addition, β-carotene increased the CDK inhibitor p21 expression and decreased the prostanoid synthesis rate-limiting enzyme cyclooxygenase (COX)-2 expression, as shown by real-time PCR and Western blotting. The regulator effect of β-carotene on p21 and COX-2 was inhibited by the PPARγ antagonist GW9662, indicating that β-carotene modulated p21 and COX-2 through PPARγ. Therefore, PPARγ expression may account partly for β-carotene-mediated anticancer activities [65].

PPARγ and Estrogen Receptors

Estrogens play a crucial role in normal breast development being also implicated in development and progression of breast cancer [66]. The biological effects of estrogen are mediated by ER [67, 68]. Among the two receptors ERα and ERβ, ERα is essential for both normal mammary gland development [69] and also involved in breast cancer development [70-73]. By ligand binding, ERα undergoes a conformational change remodelling chromatin and regulating transcription of target genes [74]. ER contains two activation domains. The activity of activation function-1 (AF1) domain on N-terminus is ligand-independent, while AF2 domain on C-terminus is responsible for transactivation of target genes in response to ligand stimulation [75]. Moreover, ERα binds to the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), regulating diverse processes like cell survival and proliferation [76, 77]. Regarding the interaction between PPAR and ER pathways, the PPAR/RXR heterodimer binds to Estrogen Response Element (ERE) related palindromic sequences. However, it cannot trans-activate ERE due to the non-permissive natural promoter structure [78]. In the MDA-MB-231 and MCF7-K3 breast cancer lines, PPARγ did not have any effect on the ER transactivation of the synthetic ERE at any dose used (20-500ng) [79]. On the other hand, ER negatively interferes with PPRE-mediated transcriptional activity [80, 81]. The expression in MDA-MB-231 cells of either ERα or β lowered both basal and stimulated PPARγ-mediated reporter activity. Interestingly, ERβ inhibited reporter activity in both unstimulated and PPARγ ligand BRL48482-stimulated cells significantly greater than ERα, although no difference in ER expression was seen by Northern blot analysis. Moreover, in BRL48482-simulated MDA-MB-231 cells treated with ICI182780, an ER antagonist, no differences were found in those expressing ERα or ERβ compared with stimulated untreated cells. By contrast, in BRL48482-stimulated cells expressing ERα or ERβ, the treatment significantly inhibited reporter activity compared to stimulated untreated ones not expressing ER. Treatment of stimulated cells with 17β-estradiol had no additional effect on cells lacking or expressing ERβ. However, in BRL48482-stimulated cells expressing ERα, 17β-estradiol treatment significantly inhibited reporter activity. By contrast, 17β-estradiol treatment had no effect on reporter activity in unstimulated cells expressing ERα or ERβ. RNase protection assays showed that the inhibition of PPAR function was not due to a decrease in PPARγ expression. The only significant expression of PPARγ was that of the promoter associated with γ1 isoform. Moreover, the presence of either ERα or ERβ did not inhibit DNA-binding to a PPRE, as shown by gel shift analysis, suggesting that the ER does not prevent PPARγ from recognizing and binding to its response element. Indeed, binding was found 2.2-fold higher in the nuclear extracts of cells expressing ERβ. The deletion of the ER DNA binding domain, rendered the ER unable to inhibit either basal or stimulated PPAR transactivation in MDA-MB-231 cells. Deletion of the AF1 or the AF2 domain also inhibited basal and stimulated reporter activation. Interestingly, the ERα lacking the AF1 domain is a more effective inhibitor than

either the wild type or the protein lacking the AF2 domain. Expression of the wild type ERα imparted estrogen responsiveness to these cells. The DNA binding mutant, by contrast, was unresponsive to transactivation by estrogen treatment. Furthermore, when co-expressed, the DNA binding mutant was able to attenuate the reporter activation by the wild type ERα [81]. In the MCF7 cell line, the PPARγ ligand rosiglitazone (RGZ) and ER antagonists ICI182780 and hydroxytamoxifen were able to stimulate a PPRE reporter gene, an effect reversed by the specific PPARγ antagonist GW9662. Furthermore, the response to RGZ treatment was potentiated by both antiestrogens. It was shown by electrophoretic mobility shift assay that ERα bound to PPRE in MCF7 cells. Additionally, coimmunoprecipitation assays in MCF7 cells indicated that PPARγ was constitutively associated with ERα. Treatment with RGZ or 17β-estradiol slightly decreased PPARγ-ERα association, whereas ICI182780 strongly inhibited it [80]. It should be noted that 24h treatment of MCF7 cells with normal concentrations of 17β-estradiol did not result in the down-regulation of PPAR expression [81]. The physical and functional interactions of ERα and PPARγ involved the p85 regulatory subunit of PI3K. A ChIP assay in MCF7 cells showed that PPARγ as well as ERα bind to the PPRE sequence of the PTEN promoter [82] along with p85 in untreated cells. This ternary complex was formed in an ERα-dependent manner since an ER antagonist was able to abrogate the coprecipitation, while 17β-estradiol did not induce substantial changes. Interestingly, treatment with RGZ and GW9662, enhanced and decreased recruitment of PPARγ to the PTEN promoter sequence, respectively. The overexpression of ERα in MCF7 cells enhanced the PI3K activity, which was further potentiated in the presence of 17β-estradiol. On the contrary, the overexpression of PPARγ in MCF7 cells reduced the PI3K activity, which was further decreased by RGZ treatment. The latter inhibitory effects were no longer noticeable applying a combination of ERα overexpression and 17β-estradiol treatment. Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatise (PTEN) controls several cell functions, including survival and proliferation, by antagonizing the PI3K signaling cascade [83]. A 24-hour exposure of MCF7 cells to 10μM RGZ induced a significant enhancement of PTEN mRNA and protein levels, which was no longer noticeable in the presence of 17β-estradiol. The rapid inhibition of the PI3K/AKT pathway induced by RGZ did not directly involve PTEN, which may only contribute to long-term repression. Interestingly, ERα and PPARγ pathways had an opposite effect on the PI3K/AKT transduction cascade, explaining, at least in part, the divergent response exerted by the cognate ligands 17β-estradiol and RGZ on MCF7 cell proliferation [80]. Breast Cancer Amplified Sequence 2 (BCAS2) was initially identified as a gene that was overexpressed and amplified in some breast cancer cell lines, including MCF7 [84]. BCAS2 interacted with PPARγ in a ligand-independent way, as shown by yeast two-hybrid screening. In addition to PPARγ, BCAS2 also interacted with ERα and potentiated the AF-2 activity, responsible for transactivation of target genes in response to ligand stimulation. Thus, BCAS2 was supported to play an important role in breast cancer development by increasing the ER's function [75].

PPARγ and Retinoid-X-receptor

As demonstrated by Western blotting, PPARγ expression was threefold lower in MCF7 and Hs578T cells than in other lines (MDA-MB-231, MDA-MB-468, T47D and SK-BR-3). RXRα expression was low or undetectable in most cell lines, but it was 3-fold higher in MDA-MB-468 and SKBR3 cells. Immuno-precipitation showed that PPARγ and RXRα interact in all these cell lines. Treatment with 100μM γ-linolenic acid for 24h resulted in 3- to 4-fold induction of a PPAR-responsive promoter activity in all cell lines. Treatment with 100nM of the RXR-selective compound AGN194204 produced 2- to 3-fold increase in promoter activity in these lines. Combination of γ-linolenic acid with AGN194204 resulted in an 8-fold induction of promoter activity in MCF7 and T47D lines, and 15- to 17-fold induction in MDA-MB-468 and SK-BR-3 cells, which express higher levels of RXRα. Therefore, PPAR/RXR heterodimers are functional and RXR-selective ligands can potentiate PPAR-induced transcriptional activation in cells with higher levels of RXR-α.

Treatment with 100nM AGN194204 slightly inhibited proliferation of T47D cells. γ-Linolenic acid produced 30% inhibition of cell growth. Simultaneous treatment with both ligands resulted in an additive effect, producing 40% growth inhibition of T47D cells. In contrast, AGN194204 was a highly effective anti-proliferative compound (70% growth inhibition) when used as a single agent on MDA-MB-468 and SKBR3 cells. γ-Linolenic acid treatment of MDA-MB-468 cells resulted in 20% growth inhibition, but because of the magnitude of the AGN194204 effect additional anti-proliferative activity was not observed when both ligands were used together. BrdU incorporation analysis showed that γ-linolenic acid treatment inhibited S-phase progression by 30-50%. Most alterations in the gene expression that regulate cell cycle progression did not occur until 16h after γ-linolenic acid exposure, as demonstrated by Western blotting. Cyclin E levels decreased by 2-fold at this time point in T47D cells and was undetectable in the MDA-MB-468 line. CDK1 levels also decreased in both cell lines by the 16-hour and 24-hour timepoints. By 24 hours, markedly decreased protein levels of a number of cell cycle regulatory genes were noted, including ERK1, Cyclin A, C-myc and JunB. The majority of these genes are involved in regulating S-phase progression in many cell types. Regulation of G2 phase proteins such as Cyclin B were unaffected by γ-linolenic acid treatment. γ-Linolenic acid-induced changes in gene expression correlated with delayed S-phase progression [85].

IMPORTANCE OF PPARγ IN ANIMAL MODELS

The presence of PPARγ was not required for functional development of the mammary gland during pregnancy and no increase in mammary tumors was observed a mice with a cell-specific deletion of the gene using the Cre-loxP recombination system [86]. To investigate whether the loss of PPARγ could sensitize mice to tumor formation, mice with ablated PPARγ and an equal number of control mice were examined. None of these mice developed tumors over 12 months. After 15 months, mice with ablated PPARγ and 1 control mouse developed breast tumors suggesting that PPARγ is not a strong and dominant tumor suppressor [86]. Transgenic mice that expressed constitutively an active form of PPARγ in mammary gland were indistinguishable from their wild type littermates. This low-level constitutive PPARγ signaling did not interfere with normal mammary gland development, differentiation or function. However, when bred to a transgenic strain prone to mammary gland cancer, and once a tumor-initiating event took place, bigenic animals developed tumors with greatly accelerated kinetics. Surprisingly, in spite of their more malignant nature, bigenic tumors were more secretory and differentiated, and expressed at higher levels differentiation markers such as carbonic anhydrase 2 and mucin 1. Additionally, loss of one allele of PPARγ did not influence breast tumor development. The absence of significant difference in tumor incidence or pathology indicated that PPARγ is not a tumor suppressor gene in mammary neoplasia, as it has been suggested for other cancer types [87]. This finding is consistent with the fact that PPARγ is universally overexpressed in breast tumors [41, 88-90]. The molecular basis of this tumor-promoting effect may be an increase in Wnt signaling, as ligand activation of PPARγ positively regulated Wnt signaling in an *in vivo* model, the developing zebrafish embryo [87]. Wnt signaling arose as an intercellular communication system that relies to a multitude of secreted ligands, ligand-sequestering factors, membrane-bound receptors, and intricate intracellular messenger cascades that control the activity of

diverse nuclear targets [91]. Excessive Wnt signaling in adults led to tumor formation and oncogenic mutations fix the pathway in a permanently activated state. It was also found that Wnt target genes were overexpressed in bigenic tumors. The expression of Cyclin D1 and C-myc, the canonical mammalian Wnt target genes, was significantly up-regulated in multiple mammary tumors. The levels of PGE_2 synthase were also uniformly increased. Among the pathway components examined, β-catenin and the Wnt receptor Frizzled homolog 4 (Fzd4) were overexpressed. In contrast, the levels of Wnt5α (but not Wnt5b), and Fzd9 were down-regulated [87]. In mammary tumors induced by mammary tissue-targeted oncogenes (ErbB2, Ras and Src), the relative abundance of Cyclin D1 was increased and that of PPARγ was decreased compared with those in normal mammary epithelium. Cyclin D1 abundance was also increased in the mammary tumor compared with that in the adjacent mammary epithelium of the same animal, with reciprocal changes in PPARγ expression. In contrast, reduction by 90% of Cyclin D1 protein levels induced PPARγ abundance. The relative abundance of PPARγ1 mRNA was increased 2-fold in the Cyclin D1-deficient mice and their livers displayed the features of hepatic steatosis consistent with increased PPARγ activity [92].

CLINICAL SIGNIFICANCE OF PPARγ IN BREAST NEOPLASIA

A polymorphism in the PPARγ2 isoform resulted in a Pro to Ala amino-acid substitution at codon 12 [93]. The PPARγ2 $Pro^{12}Ala$ led to reduced transcriptional activation of adipose-specific target genes [94]. Carriers of the variant Ala-allele of PPARγ2 $Pro^{12}Ala$ were at lower risk of breast cancer. There was an interaction between non-steroid anti-inflammatory drugs, alcohol consumption and PPARγ2 $Pro^{12}Ala$ genotype in relation to breast cancer risk. Alcohol consumption was associated with a 1.21-fold increased risk of breast cancer per 10g alcohol/day among homozygous wild-type carriers, whereas alcohol was not associated with breast cancer risk among variant allele carriers [95]. Use of non-steroid anti-inflammatory drugs (NSAIDs) has been associated with decreased risk of breast cancer in epidemiological studies [96, 97]. Non users of NSAIDs, who were carriers of the variant allele of PPARγ2 $Pro^{12}Ala$, were at lower risk of breast cancer (IRR=0.44, 95% CI=0.26-0.73) compared with non-users carrying wild-type alleles [95].

A cohort study conducted on post-menopausal women evaluated whether genetic polymorphisms in selected obesity-related genes, including PPARG, were associated with the progression of benign breast disease to cancer and whether the selected polymorphisms modified the association between body mass and breast cancer among women with benign disease. No statistically significant associations were observed in polymorphisms in PPARG and breast cancer risk or between the polymorphisms and body mass index and breast cancer risk [98].

High levels of PPARγ protein expression was noted by immunohistochemistry in human breast adenocarcinoma tissue samples. In contrast, normal breast epithelial cells from individuals with breast cancer expressed low levels of PPARγ protein [44]. Western blotting demonstated that PPARγ expression was dramatically increased in 11 out of 16 tumors compared with the adjacent normal tissue which showed weak or no PPARγ expression. Interestingly, the same tumor samples presented elevated HER2 levels. The codistribution of PPARγ and HER2 was assessed by immunocytochemistry in three of the samples indicating that the membranous and cytoplasmic HER2 overexpression was accompanied by intense nuclear PPARγ immunostaining. Therefore, HER2 overexpression was supported to associate with PPARγ elevated levels in breast tumors [37]. On the other hand, another study referred that, while normal tissues expressed PPARγ, tumour tissues exhibited a reduced level, as observed by Western blotting [46].

PPARγ expression was reduced in human benign breast disease and cancers correlating with increased Cyclin D1 abundance. The majority of human breast cancers were simultaneously Cyclin D1 positive and PPARγ negative, as shown by immunohistochemistry. Thus, in human ERα-positive breast tumors, reduced PPARγ expression was found in conjunction with increased Cyclin D1 levels [92].

S-phase kinase-associated protein 2 (Skp2) is associated with the grade of malignancy and frequently found overexpressed in breast cancer. PPARγ and Skp2 expression were inversely correlated in benign breast desease and cancer, as shown by immunohistochemistry and Western blotting. Moreover, a significant correlation was found between PPARγ expression and ER and PR expression, while high Skp2 expression was associated with loss of tumor differentiation and negative ER or PR expression [99].

Matrix metalloproteinases (MMPs) are involved in the ECM degradation related to cancer invasion, metastasis and angiogenesis [100]. In invasive breast carcinoma, both membrane type 1 (MT1)-MMP and MMP-9 were expressed in the cytoplasm of the malignant cells and the peritumoral stroma. Cytoplasmic MT1-MMP and MMP-9 appeared to be related to well-differentiated tumours, with a low proliferation potential. PPARγ was positively associated with both cytoplasmic MT1-MMP and MMP-9 [101].

The expression of PPARγ was also examined in invasive breast carcinoma. Cytoplasmic PPARγ expression of was detected in 58% of breast carcinoma samples and was inversely correlated with tumor histological grade, and positively with ERβ status. Regarding relapse-free survival, PPARγ expression had a marginally favourable impact being an independent prognosticator for ductal invasive carcinoma (DIC) patients. On the other hand, no association was found between PPARγ expression and overall survival rate. PPARγ expression, possibly in cooperation with ERβ, exhibited a favourable impact on disease-free survival of patients with DIC [102].

Approximately 10% of women with duct carcinoma in-situ (DCIS) receiving breast conservation therapy (BCT) develop in-breast reccurences. However, reccurence can not be accurately predicted using clinical and histopathological criteria [103]. Immunohistochemical evaluation of DCIS tissue showed that nuclear expression of PPARγ was associated with protection from reccurence with 4% vs 27% positivity in women who later developed in-breast reccurence and women who remained free of recurrence. PPARγ positivity was evaluable in 51 women (25 cases and 26 controls); nuclear staining was seen by immunohistochemistry in 8 of 51 women (15.6%). Only one of the women who later developed in-breast reccurence expressed nuclear PPARγ, compared to 7 of 26 (27%) who remained free of recurrence. Interestingly, a highly significant interaction between PPARγ and histopathological grade was observed. The odds ratio for recurrence was 0.11 indicating a protective effect of nuclear PPARγ against recurrence. There was no association between patients' age or HER-2/neu status and PPARγ expression. The protective effect of PPARγ positivity was not changed in multivariate modeling with tamoxifen, radiation therapy, tumor size, estrogen receptor status and margin status [104].

PPARγ LIGANDS AND BREAST CANCER

In the past few years, many studies have investigated the pharmaceutical potential of PPARγ agonists in breast cancer. The next paragraphs focus on the available data regarding the effect of diverse naturally occurring and synthetic PPARγ ligands in breast cancer cell lines, animal models and clinical trials. The PPARγ ligands of interest are depicted in Figs. (**2** and **3**), while the whole data concerning their effects in breast cancer cell lines are summarized in Tables **2** and **3**. Furthermore, an overview of the

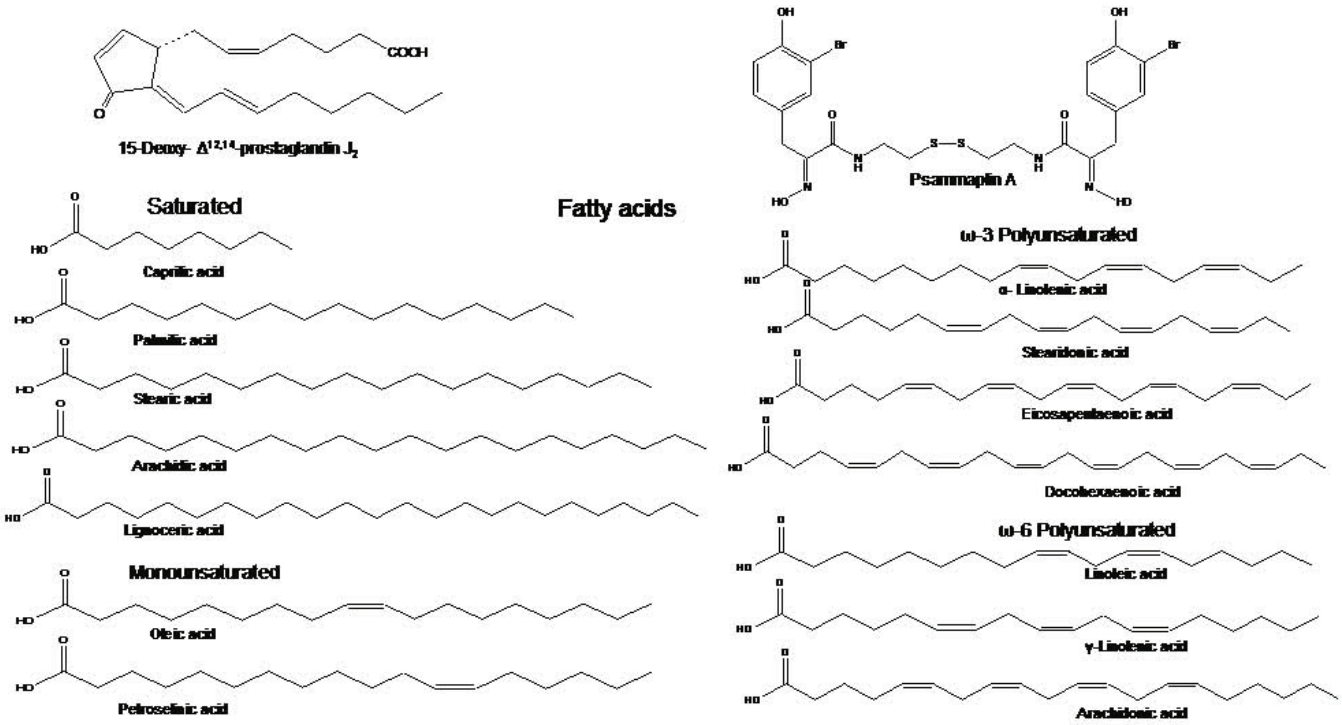

Fig (2). Naturally-occuring PPARγ ligands.

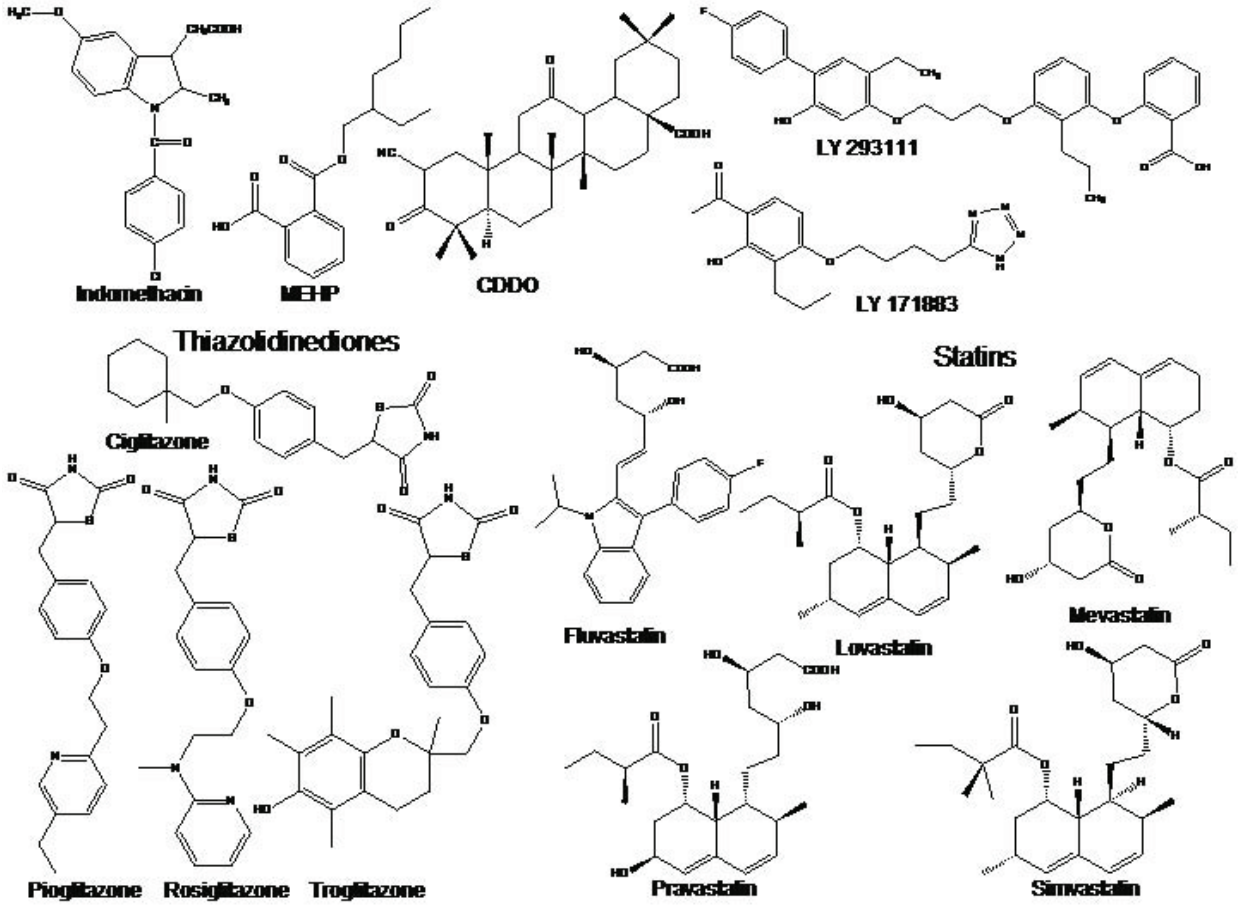

Fig (3). Synthetic PPARγ ligands.

Table 2. Effects of Naturally Occurring PPARγ Ligands in Breast Cancer Cell Lines

PPARγ Ligand			Cell Lines	Dosage	Effects	Ref.
15d-PGJ_2			MCF7 MDA-MB-231 SKBR3 T47D	0,5-25 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Apoptotic death↑ Inhibition of NRG1, NRG2↑ BRCA1, EGR1, NAB2 expression↑ ERK1/2 phosphorylation↑	[34, 44, 105, 106, 107, 108, 109, 110]
Fatty Acids	ω-3 polyunsaturated	α-linolenic acid	MCF7 MDA-MB-231	10-200 μM	PPARγ-induced transcriptional activation↓ PPARγ expression↓ Growth inhibition↑ Eicosapentaenoic acid↑ COX-2, NF-κB expression ↓ Arachidonic acid, prostaglandin E_2↓	[112, 113]
		Stearidonic acid	MCF7 MDA-MB-231	50-250 μM	PPARγ expression↓ Eicosapentaenoic acid↑ COX-2, NF-κB expression↓ Arachidonic acid, prostaglandin E_2↓	[113]
		Eicosapentaenoic acid	MCF7	100 μM	PPARγ-induced transcriptional activation↓	[112]
		Docosahexaenoic acid	MCF7	10-60 μM	PPARγ-induced transcriptional activation↓ SDC-1 expression↑	[112, 115]
	ω-6 polyunsaturated	Linoleic acid	MCF7 MDA-MB-231	250 μM	PPARγ-induced transcriptional activation↑	[112, 113]
		Conjugated linoleic acid	MCF7	60 μM	Change of the intracellular distribution of PPARγ Apoptotic death↑ Re-organization of cytoskeletal proteins Redistribution of β-catenin, E-cadherin	[111]
		γ-linolenic acid	Hs578T MCF7 MDA-MB-231 MDA-MB-468 SKBR3 T47D	60-100 μM	PPARγ-induced transcriptional activation↑ PPARγ phosphorylation↑ Change of the intracellular distribution of PPARγ Growth inhibition↑ Cell cycle arrest (G_1-S)↑ E-cadherin, desmoglein, maspin, nm23, p27 expression↑ Cyclin A, Cyclin E, Cdk1, ERK1, C-myc, JunB expression↓	[85, 114]
		Arachidonic acid	MCF7	250 μM	PPARγ-induced transcriptional activation	[111, 112]
	Saturated - Monounsaturated	Oleic acid	MCF7	350 μM	PPARγ-induced transcriptional activation↑	[112]
		Petroselinic acid		150 μM		
		Caprylic acid		10 μM		
		Palmitic acid		10 μM		
		Stearic acid		250 μM		
		Arachidic acid		100 μM		
		Lignoceric acid		50 μM		
Psammalpin A			MCF7		PPARγ-induced transcriptional activation↑ Apoptotic death↑	[29]
Mycophenolic acid			MCF7 MDA-MB-231	up to 100 μM	PPARγ-induced transcriptional activation↑ PPARγ expression↑ Growth inhibition↑ Cell cycle arrest (G_1-S)↑ Cell differentation ↑ Lipid accumulation↑ Adipsin D, AP2 expression↑	[117]
Thymoquinone			MDA-MB-231	40 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Apoptotic death↑ Bcl-2, Bcl-xL, survivin expression↓	[118]

Table 3. Effects of Synthetic PPARγ Ligands in Breast Cancer Cell Lines

PPARγ Ligand		Cell Lines	Dosage	Effects	Ref.
Thiazoladinediones	TGZ	MCF7 MDA-MB-231	0.1-75 μM	PPARγ-induced transcriptional activation↑ PPARγ expression↑ Growth inhibition↑ Cell cycle arrest (G_1-S) ↑ Apoptotic death↑ Acute cellular acidosis↑ Lipid accumulation↑ EGR1, NAB2, SDC-1, p21, p27 expression↑ Bcl-2, CD36, Cdk2, Cdk4, Cdk6, Cyclin D1, Cyclin D2, Cyclin D3, hTERT, pRb, Sema6B, Skp2 expression↓ ERK1/2 phosphorylation↑ Caspase 3 activation↑ Telomerase activity↓ Intracellular Ca^{2+}↑	[23, 38, 44, 45, 99, 110, 115, 119, 120]
	RGZ	MCF7 MDA-MB-231 MDA-MB-468 NT5	10-50 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Cell cycle arrest (G_1-S)↑ Apoptotic death↑ Cell differentation ↑ AP2, BRCA1, β-casein, caveolin-1 and -2, E-cadherin, FAS, p21, p53, PTEN, SREBP-1c expression↑ AKT, MARK phosphorylation↓	[19, 23, 24, 27, 38, 80, 105, 108, 121, 122, 123]
	CGZ	MCF7 MDA-MB-231	10-100 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Apoptotic death↑ EGR1, NAB2 expression↑ Cyclin D1 expression↓ ERK1/2 phosphorylation↑	[23, 28, 38, 39, 105, 110, 124]
	PGZ	MCF7	up to 100 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Apoptotic death↑ Cyclin D1 expression↓	[23, 125]
	BRL48482	MCF7 MDA-MB-231	5 μM	PPARγ-induced transcriptional activation↑	[81]
Statins	Lovastatin	MCF7 MDA-MB-231 MDA-MB-468 T47D	1-3 μM	PPARγ-induced transcriptional activation↑ Cell cycle arrest (G_1-S)↑ PTEN expression↑ AKT, MARK phosphorylation↓	[23, 24]
	Fluvastatin		up to 25 μM	PPARγ-induced transcriptional activation↑ PTEN expression↑	[24]
	Pravastatin		up to 100 μM		
	Simvastatin		up to 25 μM		
	Mevastatin		up to 25 μM	PPARγ-induced transcriptional activation↑	
CDIMs		BT549 MDA-MB-231 MDA-MB-453	1-20 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Cell cycle arrest (G_1-S) ↑ Caveolin-1, p21, p27, NAG-1, ATF-3 expression↑ Cyclin D1 expression↓	[26, 27]
MEHP		MCF7	0.1-100 μM	PPARγ-induced transcriptional activation↑	[28]
KR62980		MCF7 MDA-MB-231	0.1-10 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Apoptotic death↑ Bax, PTEN expression↑ Bcl-2 expression↓ AKT phosphorylation↓	[121]

Table 3. contd….

PPARγ Ligand	Cell Lines	Dosage	Effects	Ref.
CDDO	MCF7 MDA-MB-468	0,5-5 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Caveolin-1 expression↑ Cyclin D1 expression↓ HER2 phosphorylation↓	[126]
LY 293111 LY171883	BT474 MCF7 SK-BR-3		PPARγ-induced transcriptional activation↑ Cytotoxicity↑	[112, 127]
Indomethacin	MCF7	10 μM	PPARγ-induced transcriptional activation↑ Growth inhibition↑ Apoptotic death↑	[44]

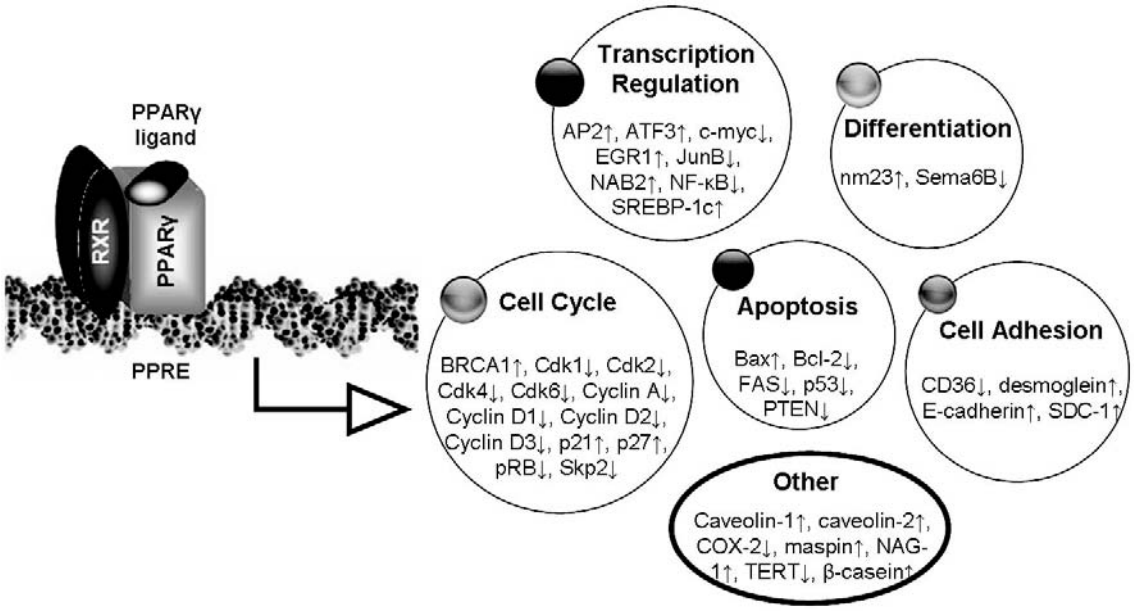

Fig (4). Genes regulated through ligand-mediated PPARγ transcriptional activation in breast cancer cell lines.

genes regulated through ligand-mediated PPARγ transcriptional activation in breast cancer cell lines is depicted in Fig. (**4**).

Effects of Naturally Occuring PPARγ Ligands in Breast Cancer Cell Lines

15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$)

15d-PGJ$_2$, significantly enhanced PPARγ activation in MDA-MB-231 and MCF7 cells, but not in T47D, after an 18h treatment, as shown by a reporter assay [105]. 15d-PGJ$_2$ (10μM) inhibited clonal growth of MCF7 cells in a reversible manner. However, the combination of PPARγ and RAR ligands induced irreversibly the inhibition of clonal proliferation. 15d-PGJ$_2$ (10μM) combined with all-*trans* retinoid acid (1μM) for 4 days induced apoptosis in MCF7 cells [44]. However, it should be noted that the growth inhibitory effect of 15d-PGJ$_2$ on tumor cells can be mediated in part independently of PPARγ activation [34].

BRCA1 has been linked to the genetic susceptibility of many breast cancers. Several lines of evidence indicate that BRCA1 is a tumor suppressor and its expression is downregulated in sporadic breast cancer cases [106, 107]. Increased BRCA1 protein levels were noted in MCF7 cells 16h post-treatment with 15d-PGJ$_2$ (0,5-10μM). Immunofluorescence microscopy analysis showed that BRCA1 protein presented mainly nuclear localization, after treatment with 15d-PGJ$_2$. Treatment of MCF7 cells with 15d-PGJ$_2$ (10μM) resulted in a 9-fold increase in BRCA1 promoter activity, as demonstrated by a reporter assay. Treatment of MCF7 cells with the RXR-specific ligand 9-*cis* retinoic acid (1μM), resulted in a 3-fold increase in promoter activity. Moreover, when the cells were incubated with 9-*cis* retinoic acid and 15d-PGJ$_2$, a synergistic activation of BRCA1 promoter was observed (18-fold). Therefore, 15d-PGJ$_2$ may regulate BRCA1 gene expression through a PPARγ-dependent mechanism. Sequence analysis of the promoter region located between nucleotides -805 and +74, functional analysis by transient transfection of different 5′-flanking region fragments, as well as gel mobility shift assays and mutagenic analysis, suggested that the effects of 15d-PGJ$_2$ were mediated through a PPRE DR1 located between the nucleotides -241 and -229 [108].

PPARγ activation through the 15d-PGJ$_2$ ligand blocked pathways activated by the NRGs. Specifically, preincubation of MCF7, T47D or SKBR3 cells with 15d-PGJ$_2$ (10μM) for 3 and 10h before the addition of the neuregulin factor inhibited of ErbB-2 and ErbB-3 tyrosine phosphorylation induced by NRG1 and NRG2, which were applied for 5 min prior to cell harvesting. NRG-induced Akt phosphorylation and insulin-like growth factor (IGF) phosphorylation were also blocked in MCF7 cells. Moreover, incubation of MCF7, T47D or SKBR3 cells with 15d-PGJ$_2$ (10μM)

for 24h caused a dramatic decrease in basal and neuregulin-induced MCF7, T47D and SKBR3 cell proliferation, accompanied in MCF7 cells by accumulation of cells in the G_0/G_1 compartment of the cell cycle, and a marked increase in apoptotic process. NRG1 and NRG2 induced G_1 progression, which was associated with stimulation of the phosphatidylinositol-3 kinase (PI3-K) pathway, whereas survival was dependent on ERK1/ERK2 activation. Both pathways were inhibited by preincubation of MCF7 cells with 15d-PGJ_2 10h before the addition of NRG1 or NRG2. Furthermore, 15d-PGJ_2 abolished the NRG1 and NRG2-induced increase in anchorage-independent growth of MCF7 cells. Such results suggested that 15d-PGJ_2 treatment can restore normal cellular growth characteristics and abrogate the transforming effects of the neuregulins on MCF7 cells, although the observed effects could be due to the potent anti-mitogenic activity of 15d-PGJ_2. Finally, treatment with 15d-PGJ_2 (10μM) for 48h can stimulate a morphological change in the appearance of MCF7 cells, which became filled with neutral lipids that stained with Nile red [109].

Treatment of MCF7 cells with 15d-PGJ_2 (25μM) increased EGR1 mRNA and protein expression levels and the mRNA levels of the EGR1 target gene NAB2, as shown by RT-PCR and Western blotting. Use of a PPARγ antagonist and a PPARγ-specific siRNA suggested that the induction of EGR1 expression is PPARγ-independent. However, 15d-PGJ_2 (25μM) induced ERK1/2 phosphorylation, indicating that the ERK1/2 pathway could be involved in EGR1 expression stimulation. Pretreatment of MCF7 cells for 30min with a Ca^{+2} chelator before their exposure to 15d-PGJ_2 (25μM) abolished the ERK1/2 activation and the increase in EGR1 mRNA level [110].

Fatty Acids

A range of fatty acids, including the polyunsaturated fatty acids, was found to bind to PPARγ and regulate gene transcription [111]. Whereas omega-3 fatty acids appeared to inhibit transactivation of PPARγ to levels below control, omega-6, monounsaturated and saturated fatty acids stimulated the activity of the transcriptional reporter, indicating that individual fatty acids differentially regulate the transcriptional activity of PPARγ by selectively acting as agonists or antagonists. As shown by reporter assay in MCF7 cell line, omega-3 polyunsaturated fatty acids inhibit the transcriptional activity of PPARγ. The strongest inhibition was observed by eicosapentaenoic acid (100μM), which suppressed reporter activity to 66%. α-Linolenic acid (10μM) resulted in 80% reporter activity. Docosahexaenoic acid (10μM) also inhibited PPARγ activity to 89%. By contrast, docosapentaenoic acid did not suppress the transcriptional activity of PPARγ [112]. Treatment with γ-linolenic acid (100μM) resulted in up to 4-fold induction of PPARγ-responsive promoter activity in MCF7, Hs578T, MDA-MB-231, MDA-MB-468, T47D and SK-BR-3 cell lines after 24h [85]. Treatment of MCF7 cells with linoleic acid (250μM) and arachidonic acid (250μM) resulted in a 1.57- and 1.52-fold induction, respectively. Similar results were documented in MDA-MB-231 cells where linoleic acid (250μM) significantly inducted reporter activity 4.2-fold over control. Monounsaturated and saturated fatty acids with increasing chain lengths also exerted an effect on PPRE-mediated reporter activity in MCF7 cells. Oleic acid (350μM) increased reporter activity 1.24-fold whereas 150μM petroselinic acid (150μM) stimulated reporter activity by 1.85-fold. Both caprylic and palmitic acid (10μM each) weakly stimulated reporter activity 1.15- and 1.11-fold, respectively. Lignoceric acid (50μM) treatment stimulated reporter activity 1.25. A 1.2-fold induction was found with arachidic acid (100μM) treatment and stearic acid (250μM) increased reporter activity 1.4-fold. Therefore, individual fatty acids appear to selectively function as agonists or antagonists of PPARγ. Although variability exists between individual fatty acids within a class, clear differences exist between the classes of fatty acids themselves [112].

Stearidonic acid and α-linolenic acid treatments for 24h suppressed the transcription of PPARγ in the MDA-MB-231 cells, as shown by real-time PCR. Suppression of COX-2 mRNA and protein levels as well as NF-κB mRNA levels was associated with the reduction in the PPARγ mRNA levels. Cells treated with linoleic acid (50μM) demonstrated the highest levels of PPARγ mRNA compared with the stearidonic acid and α-linolenic acid treatments, while the addition of linoleic acid with stearidonic acid to the cell cultures prevented the lowering effect of stearidonic acid on PPARγ mRNA [113].

Immunoprecipitation and Western blotting indicated that γ-linolenic acid was able to induce PPARγ serine and tyrosine residues phosphorylation. Treatment of both MCF7 and MDA-MB-231 cells with γ-linolenic acid also resulted in translocation of PPARγ from the cytoplasm to the nucleus, detected as early as 30min post-exposure [114]. The same effect, evident by Western blotting and immunofluorescence, was observed after treatment with conjugated linoleic acid (60μM) for 72h [111].

Treatment of MCF7, T47D, MDA-MB-468 and SKBR3 cell lines with hydroxyeicosatetraenoic acid (100nM) resulted in 35-50% apoptotic cells after 24h, as demonstrated by TUNEL assay. Patterns of caspase activation showed that hydroxyeicosatetraenoic acid-mediated apoptosis was the result of intrinsic pathways [85]. Treatment with conjugated linoleic acid (60μM) for 72h led to a significant reduction of cell viability in MCF7 cells, accompanied by a cytotoxic side-effect. Notably, the PPARγ antagonist GW9662 co-administration abolished conjugated linoleic acid effects in all cases. Furthermore, the overexpression of PPARγ positively correlated with cell proliferation inhibition and modulation of ERK signaling induced by conjugated linoleic acid [111]. Moreover, treatment with γ-linolenic acid (100μM) produced cell growth inhibition by 30% in T47D and SKBR3 and 20% in MDA-MB-468 cells. It also inhibited S-phase progression in T47D and MDA-MB-468 by 30-50%, as demonstrated by bromodeoxyuridine incorporation and fluorescence microscopy. Most alterations noted in the expression of genes regulating cell cycle progression occur 16h after γ-linolenic acid exposure, as shown by Western blotting. Cyclin E levels decreased by 2-fold at this time point in T47D cells and were undetectable in the MDA-MB-468 line. Cdk 1 levels were also decreased in both cell lines by the 16 and 24h timepoints. Markedly decreased protein levels of a number of cell cycle regulatory genes, including ERK1, Cyclin A, c-Myc and JunB, were noted by 24h in culture. G_2 phase regulation proteins such as Cyclin B was unaffected by γ-linolenic acid treatment. γ-Linolenic acid-induced changes in gene expression correlated with delayed S-phase progression [85]. Moreover, α-linolenic acid (25μM and 50μM) treatments for 24h lowered the MDA-MB-231 cell number in a statistically significant manner [113].

Polyunsaturated fatty acids exerted cytotoxic activities against cancer cells and were capable of being involved in cytokine-mediated cell growth and regulating cell matrix adhesion, expression of cell adhesion molecules such as E-cadherin, desmoglein and b-catenin, and also expression of tumour suppressors and motility regulators, such as nm23, maspin, and p27. Finally, these fatty acids were found to affect the activity of MAP kinase together with a reduction in the cellular c-Jun levels. Several mechanisms have been proposed to explain the effects, such as the production of lipid superoxide and other free radicals from cancer cells. However, the complexity of the responses to the fatty acids, was shown to include many different mechanisms of controlling cell proliferation and movement [114]. The treatment of MCF7 cells with conjugated linoleic acid (60μM) for 72h resulted in the up-regulation and the redistribution of β-catenin and E-cadherin, as shown by immunoprecipitation, Western blotting and immuno-fluorescence. The increase in cytosolic β-catenin fraction induced by conjugated linoleic acid treatment was accompanied by a

reduction in the nuclear fraction. The significant increase in membrane β-catenin levels most likely reflected an increase in the β-catenin-associated membrane fraction of E-cadherin. It should be noted that cadherins and catenins connect cellular adhersion complex with cytoskeleton: E-cadherin binds to both actin cytoskeleton and β-catenin protein that, in addition to E-cadherin, binds to APC protein. Conjugated linoleic acid did not affected APC distribution, but caused a redistribution of actin filaments, which were localized above all near the plasma membrane with a lower density at cytoplasmic level [111]. γ-Linolenic acid treatment of MCF7 and MDA-MB-231 cells was able to up-regulate the expression of E-cadherin, desmoglein, maspin, nm23 and p27. It also regulated the function of β-catenin, focal adhesion kinase (FAK) and paxillin. Cells treated with PPARγ antisense oligo (2nM) had lost their response to γ-linolenic acid, which appeared to be PPARγ-dependent [114].

Stearidonic acid and α-linolenic acid (50μM and 200μM) treatment for 24h and their combinations reduced prostaglandin E_2 production, but not arachidonic acid, as demonstrated by competitive enzyme immunoassay. Treatment of MDA-MB-231 cells with stearidonic acid (50μM), but not α-linolenic acid, demonstrated a significant increase in the concentrations of total polyunsaturated fatty acids in the cellular lipids, as shown by gas chromatography. Moreover, stearidonic acid (200μM) increased the level of eicosapentaenoic acid by 5-fold compared with a 2.6-fold increase with α-linolenic acid treatment. The concentration of arachidonic acid is similarly lowered by the stearidonic acid, α-linolenic acid and combination treatment [113].

Syndecan-1, a heparin sulfate proteoglycan, expressed on the surface mammary epithelial cells, is known to regulate many biological processes, including cytoskeletal organization, growth factor signaling, and cell-cell adhesion. It was shown to act as a tumor suppressor molecule by induction of apoptosis and inhibition of cell growth. In MCF7 cells, treatment with n-3-enriched low-density lipoproteins but not n-6-enriched low-density lipoproteins resulted in significantly greater synthesis of syndecan-1 mRNA and protein, as observed by real-time reverse transcription PCR and ^{35}S sulfate incorporation respectively, in a dose-dependent manner, with a maximal effective time at 8h post-treatment. No effect of either low-density lipoproteins was observed in MCF-10A cells. The optimal concentration of n-3 low-density lipoproteins was 100mM, while toxic effects were evident at 200mM. Docosahexaenoic acid (30μM or 60μM) significantly increased the level of SDC-1 mRNA at a level similar to that of n-3 low-density lipoproteins. On the other hand, eicosapentaenoic acid or linoleic acid, principal components of n-6 low-density lipoproteins, were not effective in stimulating proteoglycan synthesis. Therefore, regulation of SDC-1 by n-3 low-density lipoproteins was assessed to be primarily an effect of docosahexaenoic acid. The effect was mimicked by the PPARγ agonist troglitazone (TGZ), being eliminated by the presence of PPARγ antagonist GW259662, which supported that regulation of the SDC-1 gene by n-3 low-density lipoproteins is mediated by the PPARγ transcriptional pathway [115]. The polyunsaturated fatty acid arachidonic acid, as well as, the PPARγ ligand ciglitazone was also documented that alter breast cancer cell motility through modulation of the plasminogen activator system [116].

More Naturally Occuring PPARγ Ligands

Psammaplin A

Bioassay-guided fractionation and isolation of an active extract from *Pseudoceratina rhax* yielded the known histone deacetylase (HDAC) inhibitor psammaplin A. Psammalpin A activated PPARγ in a MCF7 cell-based reporter assay and induced apoptosis *in vitro*. Molecular modeling studies suggested that it may interact with binding sites within the PPARγ ligand-binding pocket [29].

Mycophenolic Acid

Mycophenolic acid has recently been documented to induce adipocyte-like differentiation and reversal of malignancy of breast cancer cells, in part, through PPARγ [117].

Thymoquinone

Thymoquinone was able to increase PPARγ activity and down-regulate the expression of Bcl-2, Bcl-xL and survivin genes in breast cancer cells. The increase in PPARγ activity was suppressed by the use of PPAR-γ antagonist and PPAR-γ dominant negative plasmid, suggesting a receptor-dependent mechanism of action [118].

Effects of Naturally Occuring PPARγ Ligands in Animal Models

Fatty Acids

High fat diets have been associated with a greater incidence of cancer. Recently, concern in the role that dietary fat plays in carcinogenesis has shifted from the amount consumed to the type of fat, that is n-3 versus n-6 polyunsaturated fatty acids [113]. Human epidemiological and animal model studies support a role for n-3 polyunsaturated fatty acids in prevention or inhibition of breast cancer [115]. A significant inverse relationship between the consumption of fish oil rich in long chain n-3 polyunsaturated fatty acids and breast cancer rate was suggested [113]. Overexpression of human epidermal growth factor receptor 2 (HER-2/neu) is considered to characterize a molecular subtype or breast cancer associated with poor clinical outcome. Interestingly, it was shown that dietary fish oil inhibits mammary tumor incidence in MMTV-HER-2/neu transgenic mice. Fish oil-based diets suppressed breast tumor incidence by 30%, relative to the corn oil-based ones, as well as tumor multiplicity and mammary gland dysplasia. These findings demonstrate a potent preventive effect of (n-3) PUFA on HER-2/neu-mediated mammary carcinogenesis, without interaction with a synthetic PPARγ activator. PPARγ expression was demonstrated in MMTV-HER-2/neu mouse mammary tumor specimens and PPARγ ligands exerted anti-proliferative effects on HER-2/neu transgenic mouse mammary tumor cells. The NT5 cell line was derived from an FVB/N-TgN(MMTVneu)202Mul transgenic mouse mammary tumor and it was shown to express PPARγ protein. However, HER-2/neu receptor expression and phosphorylation in NT5 cells were not significantly altered by PPARγ ligand treatment, as demonstrated by Western blotting [42].

Effects of Synthetic PPARγ Ligands in Breast Cancer Cell Lines

Troglitazone (TGZ)

TGZ exhibits antihyperglycemic and antiproliferative actions. In growth factor-induced cell growth, the antiproliferative activity of TGZ is related to signalling *via* PPARγ pathway and downregulation of cyclins and cyclin-related kinases as well as hypophosphorylation of the negative regulatory retinoblastoma protein. However, in serum-independent tumor cell growth, TGZ was shown to reduce proliferation by both PPARγ-dependent and PPARγ-independent pathways. Studies on PPARγ-negative cancer cell lines have shown that TGZ reduced proliferation in a dose-dependent manner by partial intracellular Ca^{2+} depletion and Ca^{2+}-mediated inhibition of translation initiation, Cyclin D and E expression, or expression of the hyperphosphorylated form of the retinoblastoma tumor suppressor gene product as well as Cyclin E formation. Therefore, depending on the concentration and physiologic conditions, the antitumorigenic action of TGZ appears to involve both PPARγ-dependent and -independent pathways [119].

Treatment of MDA-MB-231 cells with TGZ induced up-regulation of PPARγ, as determined by Western blotting, and

inhibited cell growth, as determined by MTT assay, in a dose-dependent manner [38, 99]. The MCF7 cells were the most sensitive to the inhibitory effects of TGZ (MCF7>MDA-MB-231>BT474>T47D), with an effective dose of 0.1μM resulting in the inhibition of 50% clonal growth. The combination of various concentrations of TGZ together with all-*trans* retinoid acid, a RAR-specific ligand, enhanced this inhibition in MCF7 cells. Growth inhibition by either TGZ (10μM) or all-*trans* retinoid acid (1μM) was partially reversible. In contrast, exposure to both TGZ (10μM) and all-*trans* retinoid acid (1μM) irreversibly inhibited 80% clonogenic growth [44].

TGZ treatment also induced apoptosis in MDA-MB-231 and MCF7 cells. The number of apoptotic MDA-MB-231 cells was elevated by 2.5-fold at 10μM, to 3.1-fold at 50μM and to 3.5-fold at 75μM of TGZ treatment [38]. Moreover, TGZ treatment of PPARγ-overexpressing MDA-MB-231 cells increased caspase 3 activation by cleavage, as shown by Western blotting [99]. The percentage of apoptotic MCF7 cells was only slightly increased after 4 days of TGZ (1μM) treatment. In contrast, the combination of TGZ and all-*trans* retinoid acid significantly increased the number of MCF7 cells undergoing apoptosis and decreased Bcl-2 protein to nearly undetectable levels. However, levels of PPARγ protein in MCF7 cells slightly decreased by culturing with TGZ (83%), all-*trans* retinoid acid (48%), or a combination of all-*trans* retinoid acid and TGZ (66%). Therefore, the observed apoptosis mediated by TGZ and all-*trans* retinoid acid was probably related to the striking down-regulation of Bcl-2, as forced over-expression of Bcl-2 in MCF7 cells cultured with TGZ and all-*trans* retinoid acid blocked cell death [44].

Moreover, TGZ (50μM) induced G_1 arrest to MDA-MB-231 cells. Furthermore, TGZ treatment, applied in a dose-dependent manner (10μM, 25μM, 50μM and 75μM) caused a marked decrease in pRb, Cyclin D1, Cyclin D2, Cyclin D3, Cdk2, Cdk4 and Cdk6 expression as well as a significant increase in p21 and p27 expression [38]. TGZ also down-regulated Cyclin D1 in MCF7 cell line, as shown by Western blotting. On the other hand, stimulation with TGZ did not alter PTEN expression in a wide range of concentrations (0-100μM), at any time-point tested (0-72h) [23].

Skp2 is associated with the grade of malignancy and frequently found overexpressed in breast cancer. PPARγ and Skp2 expression were inversely correlated in MCF7, MDA-MB-231 and MDA-MB-435 cell lines, as shown by Western blotting. Treatment of MDA-MB-231 cells with TGZ for 48h induced a dose-dependent down-regulation of Skp2, in a PPARγ-dependent manner [99].

TGZ was identified as an effective stimulator of proteoglycan synthesis in MCF7 cells, as showed by proteoglycan isolation. Syndecan-1, a heparin sulfate proteoglycan, expressed on the surface mammary epithelial cells, is known to regulate many biological processes, including cytoskeletal organization, growth factor signaling, and cell-cell adhesion. It was shown to act as a tumor suppressor molecule by induction of apoptosis and inhibition of cell growth. Real-time PCR indicated that SDC-1 expression by TGZ (10μM) was up-regulated at the level of transcription [115].

Treatment of MCF7 cells with TGZ strongly decreased the semaphorin 6B (Sema6B) mRNA. Semaphorins are signalling molecules that control a broad range of functions, including cancer. The drop in Sema6B mRNA and protein levels was more important when the treatment combined the action of TGZ and 9-*cis*-retinoic acid [120].

Untreated MCF7 cells were almost negative for lipid accumulation, as measured by staining with Oil-red O, but expressed the lipid metabolism-associated CD36 protein, as demonstrated by immunohistochemistry and flow cytometry. Exposure of MCF7 cells to TGZ (10μM) for 4 days, increased lipid accumulation and CD36 protein expression. Exposure to all-*trans* retinoid acid alone decreased CD36 expression without a change in lipid accumulation compared with untreated cells. In contrast, the combination of both all-*trans* retinoid acid (1μM) and TGZ (10μM) dramatically decreased lipid accumulation and CD36 expression in MCF7 cells compared with TGZ-treated MCF7 cells. Although TGZ induced lipid accumulation in MCF7 cells, these cells did not change their pattern of differentiation either to adipocytes as measured by expression of adipocyte-associated transcripts for CyEBPa, AP2, lipoprotein lipase, or adispin, or to more differentiated breast cancer cells as measured by β-casein and E-cadherin [44].

TGZ (25μM) induced acute cellular acidosis in both MCF7 and MDA-MB-231 cells without increasing acid production. Acid extraction was markedly blunted or eliminated in MDA-MB-231 and MCF7 cells, respectively. Chronic exposure to TGZ resulted in Na^+/H^+ exchanger activity reduction and a dose-dependent decrease in DNA synthesis (<75% inhibition at 100μM) associated with a decreased number of viable cells. Sustained acidosis was consonant with decreased proliferation and growth that was not reversed by a PPARγ antagonist suggesting that TGZ exerted its effect independent of PPARγ as a Na^+/H^+ exchanger-mediated action [119].

Treatment of MDA-MB-231 cells with troglitagone (20, 40 and 80 μM) for 24 and 48h reduced telomerase activity in a dose- and time-dependent manner, as measured by the TRAP assay. Moreover, TGZ (20μM) suppressed the telomerase reverse transcriptase (hTERT) transcription in MDA-MB-231 cells after 24h, as shown by real-time PCR. However, use of PPARγ antagonists and PPARγ knocked-out MDA-MB-231 cells by shRNA interference showed that the reduction in telomerase activity is PPARγ-independent [45].

TGZ (5 and 25μM) increased EGR1 mRNA and protein expression levels in a dose-dependent manner in MCF7 cells after 3h, as shown by RT-PCR, Western blotting and immunocytochemistry. Moreover, TGZ (25μM) treatment for 9h increased the mRNA levels of the EGR1 target gene NAB2. Use of a PPARγ antagonist and a PPARγ-specific siRNA suggested that the induction of EGR1 expression is PPARγ-independent. However, TGZ (25μM) induced ERK1/2 phosphorylation and increased intracellular calcium, indicating that these pathways could be involved in EGR1 expression stimulation. Pretreatment of MCF7 cells for 30min with a Ca^{+2} chelator before their exposure to TGZ (25μM) abolished the ERK1/2 activation and the increase in EGR1 mRNA level [110].

HER2 caused resistance of breast cancer cells to PPARγ ligand response. HER2 up-regulation resulted in a partial inhibition of transcriptional activity of the endogenous PPARγ, suppression of differentiation-inducing function and resistance to TGZ-mediated inhibition of anchorage-independent growth of breast cancer cells. The elevated expression of PPARγ in HER2-overexpressing cells did not result in hyperresponsiveness to TGZ. In contrast, PPARγ up-regulation was accompanied with resistance to PPARγ activation. Conversely, down-regulation of HER2 by anti-HER2 monoclonal antibody Herceptin led to a decreased level of PPARγ protein and sensitization of breast cancer cells to the inhibitory effects of TGZ. Therefore, combination of Herceptin and PPARγ ligand therapy may lead to significant anti-growth activity in breast cancer cells [37].

Rosiglitazone (RGZ - BRL4653)

RGZ (10μM) significantly enhanced PPARγ activation in the MDA-MB-231, MDA-MB-453 and MCF7 cell lines, but not in T47D, after an 18h treatment, as shown by a reporter assay [27, 80, 105]. Specifically, RGZ induced 120-fold increase in PPARγ-mediated transcriptional activation in MCF7 cells [24].

Treatment with RGZ (10μM) inhibited cell proliferation in a time- and dose-dependent manner, as determined by MTT assay, and induced apoptosis, as shown by Annexin V staining, in MDA-MB-231 [38] and MCF7 cell lines [121]. RGZ also inhibited the

proliferation of NT5 cell line, which was derived from an FVB/N-TgN(MMTVneu)202Mul transgenic mouse mammary tumor, after a 24h treatment [42]. PPARγ is involved in the RGZ-induced anti-proliferative and apoptotic actions [121]. The potent irreversible PPARγ selective antagonist 2-chloro-5-nitrobenzanilide (GW9662) did not prevent RGZ-mediated growth suppression. Co-treatment of MDA-MB-231 cells with both RGZ (50μM) and GW9662 (10μM) resulted in an additive effect upon cell survival. However, it seemed that PPARγ activation is not involved in growth suppression induced by both RGZ and GW9662 [122].

In MCF7 cell line, PPARγ induced G_0-G_1 cycle arrest exerted by a 24h exposure to RGZ in a concentration-dependent manner (1μM, 10μM, 50μM) with a concomitant decrease in the proportion of cells entering in S phase [123]. Moreover, unsyncronised cells stimulated with RGZ (30μM) for 48h showed a 10% increase in G1 cell cycle arrest [23]. Furthermore, rosiglitasone (1-50μM) was able to up-regulate mRNA and protein levels of the tumor suppressor gene p53 and its effector p21 in a time- and dose-dependent manner, as shown by real-time PCR and Western blotting. Moreover, RGZ increased the recruitment of PPARγ to the p53 gene promoter and transactivated it in a concentration-dependent manner after a 24h treatment. The NF-κB sequence was required for the transcriptional response to RGZ: PPARγ bound directly to the NF-κB site located in the promoter region of p53, as shown by EMSA and ChIP experiments, and RGZ increased the recruitment of PPARγ on the p53 promoter sequence. Therefore, PPARγ has the ability to stimulate the transcription of p53 in a NF-κB-independent manner. Both PPARγ and p53 were involved in the cleavage of caspase-9, which is an important component of the intrinsic apoptotic process, and DNA fragmentation induced by RGZ. Therefore, it appeared that RGZ promoted the growth arrest and apoptosis, at least in part, through a cross-talk between p53 and PPARγ [123]. Finally, RGZ induced cell differentiation, by up-regulating differentiation marker expression, such as E-cadherin, β-casein, caveolin-1 and -2, and transcription of the well-known breast cancer lipogenesis markers, AP2, SREBP-1c and FAS, as shown by Western blotting [121].

BRCA1 has been linked to the genetic susceptibility of a majority of familial breast and ovarian cancers. Several lines of evidence indicated that BRCA1 is a tumor suppressor and its expression is downregulated in sporadic breast and ovarian cancer cases. In MCF7 cells, treatment with RGZ (2-30μM) for 16h increased the levels of BRCA1 protein. Specifically, treatment of MCF7 cells with RGZ (30μM) resulted in an 8-fold increase in BRCA1 promoter activity, as demonstrated by a reporter assay. Treatment of MCF-7 cells with 9-*cis* retinoic acid (1μM), an RXR-specific ligand, resulted in a significant increase by 3-fold in promoter activity. Moreover, when the cells were incubated with 9-*cis* retinoic acid and RGZ, a synergistic activation of the BRCA1 promoter by 15-fold was observed. Functional analysis by transient transfection of different 5´-flanking region fragments, as well as gel mobility shift assays and mutagenic analysis, suggested that the effects of RGZ may be mediated through a functional DR1 located between the nucleotides -241 and -229, which is a canonical PPARγ type response element [108].

RGZ (30μM) induced the tumor suppressor gene PTEN transcription, as shown by real-time PCR, and functional PTEN protein in a dose- and time-dependent manner. RGZ stimulation of the MCF7 cell line resulted in a maximum of 1.5-fold increase in PTEN protein levels, as shown by Western blotting, with an increase in PTEN expression observed at 24h post-stimulation and maximal effects at 48h post-stimulation. At 72h, PTEN levels were near basal. Rosiglitasone-induced PTEN expression was accompanied by an increase in G_1 arrest and a decrease in phosphorylated-AKT, indicating an increase in PTEN lipid phosphatase activity, and phosphorylated-MAPK, indicating an increase in PTEN protein phosphatase activity. RGZ specifically induced PTEN production in a PPARγ-dependent manner. Cells lacking PTEN or PPARγ were unable to induce PTEN-mediated cellular events in the presence of RGZ, indicating the critical role of PTEN in the antiproliferative effects of PPARγ activation [121] [23].

Ciglitazone (CGZ)

In MDA-MB-231 and MCF7 cells, CGZ (10μM) significantly enhanced PPARγ activation, as shown by a reporter assay [105]. Specifically, In MCF7 cells, PPARγ was transactivated approximately 3 to 5-fold [28]. However, the transcription activity of PPARγ stimulated by CGZ (10μM) in MCF7 cells over-expressing HER2 was significantly lower [39].

CGZ inhibited cell growth and induced apoptosis of MDA-MB-231 cells, as determined by MTT assay, annexin-V straining and flow cytometry [38]. Restriction of specific aminoacids known to inhibit proliferation and induce apoptosis sensitized the cell line to CGZ, and the combined effects were greater than the individual effects of either treatment. Proliferation/viability was inhibited by about 30-40% by methionine deprivation, and proliferation/viability was decreased by about one half with concurrent deprivation of methionine and CGZ (25μM). Proliferation/viability was essentially non-existent in cells cultured in methionine-free medium also treated with CGZ (50μM) [124]. Finally, stimulation with CGZ of MCF7 cells down-regulated Cyclin D1 in a dose-dependent manner (0-100μM). However, it did not induce a change in PTEN expression in a wide range of concentrations (0-100μM), at any time point tested (0-72h), as demonstrated by Western blotting [23].

Treatment of MCF7 cells with CGZ (25μM) increased EGR1 mRNA and protein expression levels and the mRNA levels of the EGR1 target gene NAB2, as shown by RT-PCR and Western blotting. Use of a PPARγ antagonist and a PPARγ-specific siRNA suggested that the induction of EGR1 expression is PPARγ-independent. However, CGZ (25μM) induced ERK1/2 phosphorylation, indicating that the ERK1/2 pathway could be involved in EGR1 expression stimulation. Pretreatment of MCF7 cells for 30min with a Ca^{+2} chelator before their exposure to CGZ (25μM) abolished the ERK1/2 activation and the increase in EGR1 mRNA level [110].

Other TZDs

Pioglitazone (PGZ)

In breast cancer cells, PGZ inhibited proliferation and induced apoptosis, both *in vitro* and *in vivo*. It also inhibited tumor angiogenesis and invasion [125]. Stimulation of MCF7 cell line with PGZ did not alter PTEN expression in a wide range of concentrations (0-100μM), at any time point tested (0-72h), but inhibited Cyclin D1 in a dose dependent manner, as demonstrated by Western blotting [23].

BRL48482

Treatment with BRL48482 (5μM) for 24h significantly stimulated PPARγ-mediated reporter activity in MCF7 and MDA-MB-231 cells, although the basal and stimulated levels of reporter activity were significantly higher in the MDA-MB-231 than in MCF7 cells [81].

Statins

Statins, fermentation-derived (lovastatin, simvastatin, pravastatin and mevastatin) and synthetic (fluvastatin), are considered to have some anti-carcinogenic properties. A reporter assay demonstrated that 48h of lovastatin, simvastatin, pravastatin, mevastatin or fluvastatin stimulation induced PPARγ-mediated transcription in MCF7 cell line, by 100-, 80-, 70-, 120- and 70-fold, respectively [24].

In MCF7 cells, lovastatin induced PTEN mRNA and protein levels in a dose- and time-dependent manner, as demonstrated by real-time PCR and Western blotting. Induction of PTEN protein

levels began at 1μM and was maximal at 3μM, with a 1.8-fold increase. An increase in PTEN expression was observed at 24h post-stimulation, and maximal effects at 48h post-stimulation. At 72h, PTEN levels were near basal. Lovastatin-induced (3μM) PTEN expression was accompanied by an increase in G_1 cell cycle arrest and a decrease in phosphorylated-AKT, indicating an increase in PTEN lipid phosphatase activity, and phosphorylated-MAPK and indicating an increase in PTEN protein phosphatase activity [23].

Simvastatin, parvastatin and fluvastatin also induced PTEN mRNA and protein expression in a dose-dependent manner in MCF7 cells, as demonstrated by real-time PCR and Western blotting. The greatest increase in PTEN expression occurred at 3, 30 and 18μM for simvastatin, pravastatin and fluvastatin, respectively. A 1.5-, 1.4- and 1.6-fold induction of PTEN protein levels was observed for simvastatin, pravastatin and fluvastatin, respectively. MCF7 cells stimulated with simvastatin (3μM) for 48h had a 1.7-fold increase in PTEN transcript. A 1.8-fold induction of PTEN transcript was observed after pravastatin (30μM) stimulation. Furthermore, a 1.8-fold induction of PTEN transcript was also observed after stimulation with fluvastatin (18μM). In contrast, mevastatin (0-25μM) did not cause any changes to PTEN mRNA or protein levels [24].

Up-regulation of sterol response element-binding protein (SREBP) in MCF7 cells induced PPARγ expression and increased PTEN expression. A combination of statin treatment and SREBP up-regulation led to a significant inhibition of PPARγ-mediated transcription. Therefore, it was speculated that statins did not signal through SREBP to induce PTEN expression, but these two signaling pathways antagonize each other. Serial deletion and electromobility shift assays of the full-length PTEN promoter led to the identification of a region between -854 and -791, where the as-yet-unidentified transcription factor through which the statins induce PTEN expression, binds. Statin stimulation and SREBP up-regulation also resulted in 1.8-fold increased PTEN expression in MDA-MB-435, MDA-MB-231 and T47D cell lines [24].

Methylene-substituted Diindolylmetyhanes (CDIMs)

1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl)methanes containing *para*-trifluomethyl, *t*-butyl, phenyl [27] and biphenyl (CDIM9) [26] groups are CDIMs that function as PPARγ agonists that exhibit both receptor-dependent and independent antitumor activities.

CDIMs activated PPARγ in MDA-MB-231 and MDA-MB-453 breast cancer cells [27]. CDIMs at 1, 5 and 10μM exhibited selective cytotoxicity and anti-proliferative effects in MDA-MB-231 and BT549 cells, after 48h treatment [26, 27]. Moreover, C-DIM treatment increased the percentage of MDA-MB-231 cells in G_0/G_1 and decreased the percentage of cells in the S phase, as shown by fluorescence-activated cell sorting (FACS). In contrast, they have minimal effects on the distribution of MDA-MB-453 cells in G_0/G_1, S and G_2/M phase of the cell cycle [27].

Treatment of MDA-MB-231, but not MDA-MB-453, cells with CDIMs (10μM) for 12 and 24h caused a decrease in the Cyclin D1 expression levels, as shown by Western blot analysis. Cotreatment of MDA-MB-231 cells with CDIMs and the proteasome inhibitor MG132, but not GW9662, inhibited the effects of CDIMs on Cyclin D1 expression, indicating that this response was proteasome-dependent, not PPARγ-dependent [27].

CDIM (5-20μM) treatment for 24h increased p27 expression levels in MDA-MB-231, but not BT549 cells at a concentration-dependent manner in a PPARγ-dependent way, as shown by Western blotting [26, 27]. CDIM9 at 10 and 20μM increased p27 expression 2.1- and 8-fold in MDA-MB-231 cells, respectively [26]. Levels of p21 were slightly increased in MDA-MB-231 cells [27] and in BT549 cells treated with CDIM9 (5 to 20 μM) [26].

C-DIMs induced caveolin-1 expression concentration-independently in MDA-MB-231, but not in MDA-MB-453 or BT549 cells, as shown by Western blot analysis [26, 27]. In MDA-MB-231 cells, caveolin-1 expression was up-regulated 1.7-, 2.4- and 3.4-fold after treatment with 5, 10 and 20μM CDIM9, respectively, in a PPARγ-dependent way [26].

NAG-1, a transforming, pro-apoptotic and growth inhibitory growth factor β-like peptide, and activating transcription factor-3 (ATF-3) were also induced by C-DIMs (10μM), as shown by Western blotting [26, 27]. In contrast, C-DIM compounds did not affect expression of the stress protein GRP78 in MDA-MB-231 or MDA-MB-453 cells. However, inhibition of cell growth, induction of NAG-1 and activation of kinases by CDIMs were not PPARγ-mediated. Apoptotic cell death was not observed in MDA-MB-231 or MDA-MB-453 cells by TUNEL assay [27].

Phthalates

The phthalates di(2-ethylhexyl)phthalate (DEHP) and di-*n*-butyl phthalate (DBP) are environmental contaminants with significant human exposures. Both compounts are known reproductive toxins. DEHP and DBP are metabolised to their respective monoesters, mono-(2-ethylhexyl)phthalate (MEHP) and mono-*n*-butyl phthalate (MBP). MEHP was able to significantly transactivate PPARγ in MCF7 cells, at concentrations as low as 0.1μM, with an approximate 2-fold increase. MEHP (100μM) treatment resulted in an approximate 9.5-fold increase in PPARγ transactivation. Confirmation of the potent agonist ability of MEHP for PPARγ was shown in the presence of suboptimal levels of ciglitazone, as well as maximal levels of ciglitazone. MBP was unable to activate PPARγ. Experiments where PPARγ was activated by suboptimal and maximal concentrations of ciglitazone in the presence of MBP (100μM) revealed that MBP was an antagonist for PPARγ [28].

Other Synthetic PPARγ Ligands

KR62980

KR62980, 1-(trans-methylimino-N-oxy)-3-phenyl-6-(3-phenyl-propoxy)-1H-idene-2-carboxy-lic acid ethyl ester is a novel synthetic PPARγ agonist. Treatment of MDA-MB-231 and MCF7 cells with KR-62980 (0.1-10μM) inhibited cell proliferation in a time- and dose-dependent manner, as determined by MTT assay, and induced apoptosis, as shown by Annexin V staining, and induced DNA fragmentation. KR62980 caused a significant decrease in Bcl-2 protein expression, as determined by Western blotting, while Bax protein expression was markedly and concentration-dependently elevated. Moreover, KR62980 time and dose-dependently increased the PTEN protein level, showing a maximum effect at 24h. In correlation with decreased PTEN expression, KR62980 also suppressed the Akt phosphorylation. PPARγ is involved in and PTEN activation is required for the KR62980-induced anti-proliferative and apoptotic actions. Finally, KR-62980 did not alter the differentiation pattern of MCF7 cells and had little effects on the lipid accumulation and the expression of lipogenesis markers. It induced nor differentiation marker expression, such as E-cadherin, β-casein, caveolin-1 and -2, neither transcription of the well-known breast cancer lipogenesis markers, AP2, SREBP-1c and FAS [121].

CDDO

The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) induced the transactivation of the PPARγ in MCF7 cells. Cell growth and colony formation were preferentially suppressed in MCF7 and MDA-MB-435 HER2-overexpressing cell lines at low concentrations of CDDO (0.5-2μM), whereas growth-inhibitory effects at high concentrations (≥2μM) did not correlate with the expression level of HER2. This finding implied that at low concentrations of CDDO HER2 may be the primary target for the reduction of colony formation. At higher concentrations of CDDO, other mechanisms may be triggered, such as cell cycle regulation and/or apoptosis induction. CDDO dose-dependently inhibited

phosphorylation of HER2 in HER2-overexpressing cells and diminished HER2 kinase activity and protein level *in vitro*, as shown by Western blotting. The growth inhibition at lower concentrations of CDDO correlated with the induction of caveolin-1 protein expression in both MCF7 and MDA-MB-435 cells at 24h, as shown by Western blotting. Caveolin-1, a potent suppressor of mammary tumor growth and metastasis induced in cancer cells by PPARγ activation, negatively regulated HER2. CDDO also reduced Cyclin D1 mRNA and protein expression at 24h in a dose-dependent manner, with the complete disappearance of cyclin D1 protein at CDDO (5μM), as demonstrated by Western blotting and real-time PCR. No change to the Cyclin E expression was noted [126].

LY293111

LY293111 is an oral agent known to be a leukotriene B4 antagonist, a 5-lipoxygenase inhibitor and a PPARγ agonist with cytotoxic properties in cell lines. After a 72h incubation, LY293111 displayed synergism with 5´-DFUR (the active metabolite of capecitabine) in two breast cancer lines. LY293111 was additive with 5´-DFUR in BT474 and synergistic with 5´-DFUR in SK-BR-3, two cell lines representing aggressive breast cancer. LY293111 and SN-38 demonstrated additivity in SKBR3. The combination of LY293111, cisplatin and gemcitabine gave additive effects in three cell lines. As proteosome inhibition is a new targeted therapy undergoing extensive clinical trials, the proteosome inhibitor MG 132 was combined with LY 293111, giving synergistic effects in MCF7/wt and antagonistic effects in MCF7/adr and SK-BR-3 cell lines [127].

LY171883

The synthetic PPARγ ligand LY171883, an oral agent known to be a leukotriene B4 antagonist, stimulated reporter activity in MCF7 cells [112].

Indomethacin

Indomethocin (10μM) inhibited clonal growth of MCF7 cells and this inhibition was reversible. However, combination with RAR ligands induced irreversible inhibition of clonal proliferation. Indomethocin (10μM) combined with all-*trans* retinoid acid (1μM) for 4 days induced apoptosis [44].

In Vivo Activity of Synthetic PPARγ Ligands

Troglitazone (TGZ)

TGZ alone or in combination of all-*trans* retinoid acid inhibited the tumor growth of MCF7 cells in triple-immunodeficient mice as measured by tumor size and weight. Histological analysis of MCF7 tumors from untreated mice revealed poorly differentiated infiltrating adenocarcinomas almost without apoptotic changes, while mice treated with TGZ showed apoptosis at a low extent as measured by morphology and TUNEL assay. However, in mice treated with the combination of both TGZ and all-*trans* retinoid acid, almost all MCF7 tumor cells were either apoptotic or necrotic while extensive fibrosis of the tumors was observed [44].

Both normal and breast adenocarcinoma tissues cultured for 4 days with either TGZ (10μM) or all-*trans* retinoid acid (1μM) showed no changes either in morphology or apoptosis. However, in combination caused massive apoptosis (80% of cells as measured by TUNEL) in each tumor sample but not in the accompanied normal breast epithelial cells (10% apoptotic cells) [44].

While clinical trials among different patient populations might uncover subtle effects on tumor differentiation, PPARγ activation by TGZ has little apparent clinical value among patients with treatment refractory metastatic breast cancer. TGZ was generally well tolerated and none of the patients developed serious or life-threatening hepatotoxicity on study. No objective tumor responses (either complete or partial) were observed. Preclinical data suggested that PPARγ activation might induce changes in expression of tumor antigens, such as CA27.29 and CEA. All patients with elevated tumor markers at baseline showed rising markers by 8 weeks of therapy. Nevertheless, TGZ was withdrawn from the market on March 21, 2000 by the manufacturer following FDA warnings over rare but life-threatening risk of hepatotoxicity [128].

Rosiglitazone (RGZ - BRL4653)

RGZ did not affect mammary carcinogenesis in MMTV-HER-2/neu transgenic mice. Mice consumed ~1.2mg RGZ /kg×d. The drug treatment was not significant and RGZ affected neither mammary tumor incidence nor tumor multiplicity by palpation [42].

Treatment with RGZ (8mg/d) for 2-6 weeks of 38 women with early stage breast cancer, administered between the time of diagnostic biopsy and definitive surgery, did not elicit significant effects on breast tumor cell proliferation. In tumors notable for nuclear expression of PPARγ, down-regulation of nuclear PPARγ expression occurred following RGZ administration. RGZ was well tolerated without serious adverse events. Thus, short-term RGZ therapy in early-stage breast cancer patients was supported to lead to local and systemic effects on PPARγ signaling that may be relevant to breast cancer [129].

Methylene-substituted Diindolylmetyhanes (CDIMs)

Antitumor growth activities of CDIM9 were assessed in MDA-MB-231 basal-like breast tumor xenografts in athymic nude mice. CDIM9 (40mg/kg daily, intraperitoneally (ip), for 35 days) inhibited the growth of subcutaneous MDA-MB-231 tumor xenografts by 87%, and produced a corresponding decrease in proliferation index. Nearly half of the treated mice (46%) had complete durable remissions, confirmed with histology. The growth of an established tumor was inhibited by CDIM9 treatment (60mg/kg daily, ip, for 10 days). CDIM9 induced a dramatic increase (7.2-fold) in tumor caveolin-1 expression and a moderate one (2.9-fold) in p27, which may contribute to its antitumor activity in basal-like breast cancer. In conclusion, CDIM9 showed potent antiproliferative effects on basal-like breast cancer cell in tissue culture and dramatic growth inhibition in animal models at safe doses [26].

Other Synthetic PPARγ Ligands

GW7845

GW7845 is a tyrosine analogue which has been optimized for potency on PPARγ and is significantly more potent than either RGZ or TGZ. GW7845 was tested as an inhibitor of experimental mammary carcinogenesis, using the classic rat model with nitrosomethylurea as carcinogen. Rats were first treated with a single dose of nitrosomethylurea (50 mg/kg BW). Starting 1 week later, they were fed GW7845, at either 60 or 30 mg/kg of diet, for 2 months. GW7845 was well tolerated and significantly reduced tumor incidence, tumor number, and tumor weight at both doses. To evaluate possible synergy with tamoxifen, a very low dose of this agent was used. Although some statistically significant additive effects were seen with the combination of GW7845 and tamoxifen, there was little evidence for a strong synergy between the two [40].

CDDO

In vivo studies showed complete abrogation of the growth of the highly tumorigenic MCF7 HER2-overexpressing cells in a murine breast cancer xenograft model. CDDO reduced HER2-positive breast cancer growth in immunodeficient mice and induced tumor cell apoptosis, by 7-fold. Furthermore, CDDO significantly decreased HER2 phosphorylation and nuclear Cyclin D1 expression in tumors. Therefore, CDDO has a therapeutic potential in advanced breast cancer [126].

CONCLUSIONS

PPARγ seems to be highly overexpressed in breast tumors, both invasive and non-invasive, primary and metastatic, due to the recruitment of a distal, tumor-specific promoter element, although contradictory evidence does exist [46]. It is still inconclusive whether it promotes or suppresses tumor development, however, it is agreed that it has ability to induce cell differentation. Evidence which supports the first hypothesis is derived from *in vivo* studies on transgenic mice expressing low levels of a ligand-independent PPARγ mutant. In this specific animal model, enhanced PPARγ signaling on a background prone to breast cancer accelerated tumor development and progression [87]. Evidence supporting the notion that PPARγ acts as a tumor suppressor comes from breast cancer cell lines, case studies and clinical histopathological material, but it is mostly indirect, through the examination of PPARγ expression and activity in relation with proteins of defined function, such as HER2 and Cyclin D1. However, animal studies showed that it is not a strong tumor suppressor [86]. Furthermore, the receptor's prognostic value varies among different studies. It should be noted that a PPARγ variant associated with reduced breast cancer risk was identified [95]. More importantly, PPARγ expression was associated with lack of reccurence in duct carcinoma patients [102, 104]. In this aspect, it should be kept on mind that some studies have implicated PPARs in the promotion and development of cancer, whereas others have suggested a protective role for these receptors against cancer. These contradictory findings have been ascribed to PPAR receptor-independent effects, cancer stage-specific effect and/or differences in essential ligand-related pharmacokinetic behaviour [130].

Human breast cancer cells were found functionally responsive to synthetic and naturally occurring PPARγ ligands. PPARγ ligands were able to induce anti-proliferative responses, cell-cycle arrest and apoptosis, through transcriptional activation of a number of genes implicated in the aforementioned processes. These responses can be potentiated by an RXR-selective [85] or an RAR-selective [44] ligand. PPARγ activation was shown to exert different effects on cancer cells depending on the type of agonists used and PPARγ ligands exhibited distinct activities within a cell type and between tumor cells derived from the same tissue. It should be noted that both ER-positive and ER-negative cell lines are functionally responsive to several PPARγ ligands. *In vivo* studies with animal models provided evidence that PPARγ ligands were able to suppress mammary tumor development or abrogate tumor growth. Importantly, the effects of PPARγ agonists have been shown to involve both receptor-dependent and -independent mechanisms. Importantly, recent evidence has expanded the repertoire of PPARγ ligands as potential therapeutic agents in the treatment of breast cancer, suggesting that they are capable of inhibiting leptin signaling mediated by MAPK/STAT3/Akt phosphorylation and counteracting leptin stimulatory effect on estrogen signaling [131].

The combination of the receptor overexpression in breast tumors and the physiological effects of its ligands on cancer cells render PPARγ as a target of chemotherapeutic agents. Unfortunately, none of the case studies conducted, using the PPARγ ligands TGZ and rosiglitasone, was particularly promising. The first showed that PPARγ activation appears to have little clinical benefit in women with heavily pretreated, refractory breast cancer. TGZ was withdrawn from the commercial market following reports of rare instances of liver toxicity [128]. The second study demonstrated that short-term treatment with RGZ did not significantly alter tumor cell proliferation in early-stage breast cancer patients [129]. Future studies should be orientated to the use of PPARγ agonists in pharmacological treatment of various breast cancer types. Possibly combining a PPARγ ligand with another agent may improve the efficacy of such treatment approaches. In this context, a recent promising study further suggests that multidrug regimens, such as a combination of thiazolidinedione and hydralazine can promote antiproliferative and apoptotic effects in triple-negative breast cancer cells and decrease the proliferation index in tumor xenografts [132]. Moreover, the production of new synthetic PPARγ ligands, with increased selectivity and potency, may prove extremely useful in breast cancer treatment.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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