Biotinylation enhances the anticancer effects of 15d-PGJ2 against breast cancer cells

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Abstract. 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) is a natural agonist of peroxisome proliferator-activated receptor γ (PPARγ) that displays anticancer activity. Various studies have indicated that the effects of 15d-PGJ2 are due to both PPARγ-dependent and -independent mechanisms. In the present study, we examined the effects of a biotinylated form of 15d-PGJ2 (b-15d-PGJ2) on hormone-dependent MCF-7 and triple-negative MDA-MB-231 breast cancer cell lines. b-15d-PGJ2 inhibited cell proliferation more efficiently than 15d-PGJ2 or the synthetic PPARγ agonist, efatutazone. b-15d-PGJ2 was also more potent than its non-biotinylated counterpart in inducing apoptosis. We then analyzed the mechanisms underlying this improved efficiency. It was found not to be the result of a free biotin receptor-mediated increased incorporation, since free biotin in the culture medium did not decrease the anti-proliferative activity of b-15d-PGJ2 in competition assays. Of note, b-15d-PGJ2 displayed an improved PPARγ agonist activity, as measured by transactivation experiments. Molecular docking analyses revealed a similar insertion of b-15d-PGJ2 and 15d-PGJ2 into the ligand binding domain of PPARγ via a covalent bond with Cys285. Finally, PPARγ silencing markedly decreased the cleavage of the apoptotic markers, poly(ADP-ribose) polymerase 1 (PARP-1) and caspase-7, that usually occurs following b-15d-PGJ2 treatment. Taken together, our data indicate that biotinylation enhances the anti-proliferative and pro-apoptotic activity of 15d-PGJ2, and that this effect is partly mediated via a PPARγ-dependent pathway. These results may aid in the development of novel therapeutic strategies for breast cancer treatment.

Introduction

Breast cancer is the most prevalent type of cancer affecting women worldwide and represents the leading cause of cancer-related mortality after lung cancer (1). Three biomarkers are used in clinical practice for tumor characterization and treatment: estrogen receptor α, progesterone receptor and epidermal growth factor receptor 2. Nevertheless, the benefits of therapies targeting these mechanisms are often limited due to resistance. Indeed, approximately one-third of patients with early-stage breast cancer will develop resistance against tamoxifen over a 5-year treatment period, and the majority of patients will become resistant against trastuzumab over a 1-year period (2,3). Moreover, no targeted therapies exist for the aggressive triple-negative tumors, which express none of these three markers. On the whole, epidemiological data reveal the urgent need for novel therapeutic options.

Prostaglandins (PGs) are a family of biologically active endogenous metabolites of arachidonic acid. 15-Deoxy-D12,14-prostaglandin J2 (15d-PGJ2) is a dehydrated derivative of PGD2, that displays potent anticancer properties (4-11). 15d-PGJ2 may also be mediated through PPARγ-independent pathways (17-24). In vivo, these effects have been confirmed...
in several mouse tumor models (25-27). In breast cancer cell lines, the anti-proliferative effects of 15d-PGJ2 remain modest compared to several synthetic PPARγ ligands (20). Recent clinical trials relaunched PPARγ agonist perspectives for the treatment of cancer. Indeed, pioglitazone in combination with imatinib can erode the cancer stem cell pool and efatutazone alone or in combination with olifirin has been shown to provide some degree of control for metastatic cancer (28-30).

In order to adapt 15d-PGJ2 for clinical use, more potent derivatives are required. Several studies have demonstrated that biotin-conjugated compounds are able to increase the uptake of anticancer drugs in tumor cells (31-34). Previously, we demonstrated that two biotinylated derivatives of Δ2-troglitazone, PPARγ-inactive analogues of troglitazone (TGZ), were more potent than the original compound in inhibiting cell proliferation (35,36).

In the present study, we examined the anti-proliferative activity of a biotin-conjugated 15d-PGJ2 (b-15d-PGJ2) on estrogen-dependent MCF-7 and triple-negative MDA-MB-231 breast cancer cells. We demonstrate that b-15d-PGJ2 is more efficient than 15d-PGJ2 in inhibiting cell proliferation and inducing apoptosis. Molecular docking analysis indicated that b-15d-PGJ2 was able to bind the ligand-binding domain of PPARγ, in a similar manner as 15d-PGJ2. This compound also displayed an improved PPARγ agonist activity, as measured by transactivation experiments. RNA interference experiments revealed that a PPARγ-dependent pathway was involved in b-15d-PGJ2-induced apoptosis. This study provides a better understanding of the anti-proliferative effects of PPARγ ligands and supports the concept of more efficient derivatives, which may be used for the development of novel therapeutic strategies for breast cancer.

Materials and methods

Reagents and cell lines. 15d-PGJ2 and 15-deoxy-Δ²,14-prostaglandin J2-biotinamide (b-15d-PGJ2) were purchased respectively, from Merk-Millipore (Fontenay-sous-Bois, France) and Cayman Chemical Co. (Ann Arbor, MI, USA). Efatutazone (RS5444; CS-7017) was obtained from ChemScene, France). Other chemicals were purchased from Sigma-Aldrich (Lyon, France). 1,2,3,4-Propanetetraol (DMSO), fetal calf serum (FCS), L-glutamine and all other chemicals were purchased from Eurogentec (Angers, France).

The human breast cancer cell lines, MCF-7 and MDA-MB-231, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco’s modified Eagle’s medium (DMEM), Leibovitz’s L-15 medium, trypsin-EDTA and PBS were purchased from Life Technologies (Saint Aubin, France). Biotin, ethanol (EtOH), dimethylsulfoxide (DMSO), fetal calf serum (FCS), L-glutamine and all other chemicals were purchased from Sigma-Aldrich (Lyon, France).

Cell culture and treatment. The MCF-7 and MDA-MB-231 cells were cultured at 37°C under 5% CO₂ in DMEM or without CO₂ in L-15 medium, respectively. Both media were supplemented with 10% fetal calf serum FCS and 2 mM L-glutamine. The cells were treated with 0.1% EtOH (vehicle) or various concentrations of 15d-PGJ2, b-15d-PGJ2 and efatutazone in 1% FCS-containing medium. 15d-PGJ2 and efatutazone were dissolved at 50 mM in sterile DMSO. b-15d-PGJ2 was dissolved at 1.6 mM in EtOH. All these compounds were stored frozen at -20°C.

Cell proliferation assays. The MCF-7 and MDA-MB-231 cells (0.8x10⁵ cells/ml) were seeded in 12-well plates and treated with various concentrations of 15d-PGJ2, b-15d-PGJ2, efatutazone (2 µM up to 50 µM) and EtOH (control) for 24 h. For competition experiments, various concentrations of free biotin (5 or 25 µM) were added together with 15d-PGJ2 or b-15d-PGJ2. Each treatment was performed in triplicate. Cell proliferation was measured using CellTiter-Glo™ Luminescent Cell Viability assay (Promega, Charbonnières, France).

For the different compounds, the concentration leading to a decrease of 50% in the number of viable cells (IC₅₀) was determined. Efatutazone was used as a gold standard, since it is a PPARγ activator currently evaluated for clinical trials (37).

Transient transfection assays. The MCF-7 cells (1.6x10⁵ cells/ml) were seeded in 24-well plates and transfected with pPPREγ-tk-luc reporter (1 µg/well) and pCMV-β-galactosidase (β-Gal) (0.6 µg/well), as an internal control plasmid, in the presence of a human PPARγ expression vector (2 µg/well). pPPREγ-tk-luc reporter comprises three copies of PPRE from the promoter of the rat ACO gene. hPPARγ2 was cloned into the pcDNA3 vector. pCMV-β is a plasmid encoding β-galactosidase under the control of the cytomegalovirus promoter. The pPPREγ-tk-luc, the human PPARγ expression vector and the pCMV-β-galactosidase construct were a gift from Professor P. Becuwe, Dr L. Domenjoud and Professor O. Nusse, respectively. Cell transfection was performed using Exgen 500 (Euromedex) according to the manufacturer’s instructions. Following transfection, the cells were allowed to grow for 24 h in DMEM supplemented with 10% FCS stripped in dextran-coated charcoal and were treated with 10 µM of 15d-PGJ2, b-15d-PGJ2, efatutazone and ethanol as a control for 24 h. Luciferase and β-Gal activities were measured as previously described (35).

RNA interference. The MCF-7 and MDA-MB-231 cells (2x10⁵ cells/ml) were seeded in 6-well culture plates and were transfected with 100 nM of PPARγ siRNA duplex mix or control scRNAs [negative control (OR-0030-neg05)] using Oligofectamine™ reagent (Life Technologies) according to the manufacturer’s instructions. The siRNA sequences against human PPARγ (PPARγ-siRNAs) were as follows: 5’-GUA-CCA-AAG-UGC-CAA-AATT-3’ and 5’-UUU-GAU-UGC-ACU-UUG-GUA-CTT-3’ for duplex no. 1, 5’-CAA-UCA-GAU-UCA-AGC-UUA-UTT-3’ and 5’-AUA-AGC-UUC-AAU-CUG-AU-U-GTT-3’ for duplex no. 2. Twenty hours later, the cells were exposed to 15d-PGJ2 (10 µM), b-15d-PGJ2 (10 µM) or 0.1% EtOH (vehicle control) for 24 h and harvested for RT-PCR or western blot analyses.

Semi-quantitative RT-PCR. The MCF-7 and MDA-MB-231 cells (2x10⁵ cells/ml) were seeded overnight in 6-well plates and were exposed to 15d-PGJ2 (10 µM), b-15d-PGJ2 (10 µM) or EtOH (0.1%) for 24 h. RT-PCR was performed as previously described (38). Briefly, cDNA was further amplified by PCR with specific primers: 5’-GACCAGT CCCACTCCTTT-3’
5'-CGACATTCAATTGCCATGAG-3' for PPARγ, 5'-TACA TGGGTGTTGGTGTGAA-3' and 5'-AAGAGAAGGCATCCT CACCC-3' for β-actin. Amplification was carried out under the following conditions: i) initial denaturation 94˚C for 2 min; ii) 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 45 sec; iii) 10 min extension step at 72˚C. Subsequently, 20 µl of the PCR products were mixed with loading buffer (5 µl) and submitted to electrophoresis in a 1.5% agarose gel at 90 V for 35 min at room temperature. The gel was stained with ethidium bromide, viewed and photographed on a UV-transilluminator (Gel Doc 2000, Bio-Rad Laboratories, Marnes-la-Coquette, France). The intensity of the PPARγ signal was normalised to β-actin using Gel Doc 2000 and a software package (Quantity One v.4·3·1) (both from Bio-Rad Laboratories).

**Western blot analysis.** The MCF-7 and MDA-MB-231 cells (2x10^5 cells/ml) were seeded in 6-well plates and were treated as described below for 24 h and subjected to western blot analysis as previously described (38). The antibodies against cleaved PARP-1 (F21-852, BD Biosciences, Le Pont-de-Claix, France) and caspase-7 (9494, Cell Signaling Technology, Danvers, USA) were diluted at 1:1,000. The monoclonal antibody against tubulin (EP1332Y, Epitomics, Burlingame, CA, USA) and the polyclonal antibody against β-actin (SC-1615, Santa Cruz Biotechnology, Dallas, TX, USA) were used diluted at 1:2,000. Non-specific binding sites were blocked in TNT buffer (50 mM Tris·HCl, 150 mM NaCl, 0.1% Tween-20) with 5% non-fat powder milk and the membranes were incubated with the primary antibodies diluted in blocking solution overnight at 4˚C. The membranes were probed with appropriate horseradish peroxidase-conjugated secondary antibodies (SC-2005 for mouse antibody and SC-2004 for rabbit antibody, Santa Cruz Biotechnology) for 1 h at room temperature. On some western blots, the intensity of the bands corresponding to cleaved PARP-1 and caspase-7 was normalized to β-actin or tubulin by using Gel Doc 2000 and a software package (Quantity One v.4·3·1) (both from Bio-Rad Laboratories).

**Nuclear staining.** The MCF-7 and MDA-MB-231 cells (2x10^5 cells/ml) were seeded in 6-well plates and were treated with 15d-PGJ2 or b-15d-PGJ2 (10 µM) for 24 h. Following centrifugation (10 min, 1,200 rpm), the pellet was resuspended with 15d -PGJ2 or b -15d-PGJ2 (10 µM) for 24 h. Following treatment (IC50, P=0.016).

**Statistical analysis.** The results of each experiment are expressed as the mean ± standard error of the mean (SEM) of 3 to 5 different experiments. Bars represent the means ± SEM. Statistical differences were tested using analysis of variance (ANOVA) followed by Bonferroni, Student-Newman-Keuls or Student’s t-test post hoc comparisons (SPSS v11.0 Computer Software). Differences in which the P-value was <0.05 were considered statistically significant.

**Molecular docking.** Molecular docking of 15d-PGJ2 and b-15d-PGJ2 in the ligand binding domain (LBD) of PPARγ was performed using the Autodock software, version 4.2 (39). The target protein structure corresponds to the Protein Data Bank (PDB) entry 3V9V (40). This structure was preferred over PDB entry 2ZK1 (41), which corresponds to 15d-PGJ2 bound to PPARγ as the former structure has a better resolution (1.60 Å for 3V9V vs. 2.60 Å for 2ZK1, respectively) and as its LBD pocket can accommodate larger ligands. Ligands were manually built with the GaussView software (42). After a semi-empirical PM6 (43) geometrical optimization with Gaussian 09 (44), atomic charges were computed using the AM1-BCC (45) scheme by the Antechamber program from the AmberTools suite of programs (46). Atomic charges for the protein were assigned according to the Amber ff12SB force field (47). A grid of 78x78x78 points centered on the Ca of Cys285 (Helix3 of the LBD of PPARγ) was built with a spacing of 0.375 Å using AutoGrid. The number of total docking runs was set to 30, each with a population of 150 individuals, a maximum number of generations set at 27,000 and a maximum number of energy evaluations set at 108. All other parameters were given default values.

**Results**

**b-15d-PGJ2 markedly decreases cell viability compared to the PPARγ agonists, 15d-PGJ2 and efatutazone.** The anti-proliferative effects of 15d-PGJ2, its biotinylated derivative, b-15d-PGJ2, and the PPARγ agonist, efatutazone (Fig. 1A) were measured on hormone-dependent MCF-7 and triple-negative MDA-MB-231 breast cancer cell lines. The cells were exposed to increasing concentrations of each compound or corresponding ethanol concentrations (control cells). Cell viability was determined after 24 h of treatment (Fig. 1B). b-15d-PGJ2 induced a potent dose-dependent inhibition of MCF-7 (IC50, 9.8±1.2 µM) and MDA-MB-231 (IC50, 6.0±0.1 µM) cell proliferation, compared to 15d-PGJ2 or efatutazone used as a positive control (IC50, both >50 µM). The anti-proliferative effect of b-15d-PGJ2 differed significantly from that of 15d-PGJ2 and efatutazone, from concentration as low as 15 and 10 µM in the MCF-7 and MDA-MB-231 cells, respectively. Of note, the MDA-MB-231 cells were significantly more sensitive than the MCF-7 cells to b-15d-PGJ2 treatment (IC50, P=0.016).

**b-15d-PGJ2 is a more potent inducer of apoptosis.** First, we investigated the activation of the executioner caspase, caspase-7 (since caspase-3 is not expressed in MCF-7 cells), as well as PARP-1 cleavage, since it is a well-known caspase substrate (Fig. 2A). At the concentration of 10 µM b-15d-PGJ2, western blot analysis revealed the cleavage of caspase-7 and the cleavage of PARP-1 after 24 h of treatment compared with the 15d-PGJ2-treated or the control cells. To ascertain our results, we examined the effects of b-15d-PGJ2 on nuclear morphology in Hoechst-stained cells. Both cell lines were treated for 24 h with b-15d-PGJ2 (10 µM). As depicted in Fig. 2B, the treated cells exhibited characteristics of apoptosis, such as cell shrinkage, nuclear condensation and fragmentation compared to the untreated controls. Taken together, these results provide insight into the induction of apoptosis in both cell lines following treatment with 10 µM b-15d-PGJ2.
Biotin group is not responsible for the enhanced anti-proliferative effects of b-15d-PGJ2. We then performed competition experiments with free biotin to determine whether the enhanced anti-proliferative effect of b-15d-PGJ2 could be the result of an increased internalization mediated by a biotin membrane receptor (Fig. 3). We examined viability of...
the MCF-7 and MDA-MB-231 cells following 24 h of treatment with b-15d-PGJ2 (0, 5 and 10 µM) in the presence of increasing concentrations of free biotin (0, 5 or 25 µM). In both cell lines, free biotin up to 25 µM in the culture medium did not significantly modify the effects of b-15d-PGJ2 on cell viability. This result thus excluded biotin receptors as a cause for the enhanced anti-proliferative effects of b-15d-PGJ2.

*b-15d-PGJ2 is a potent PPARγ agonist.* In order to examine whether the biotin moiety could affect PPARγ activation, we compared the effects of b-15d-PGJ2 to the PPARγ agonists, efatutazone and 15d-PGJ2. The MCF-7 cells were transiently transfected with a pPPRE₃-tk-luc vector in the presence of a human PPARγ expression vector. Following 24 h of treatment with 10 µM efatutazone, 15d-PGJ2 or b-15d-PGJ2, luciferase activity was stimulated 4.2-fold by efatutazone and 5.5-fold by b-15d-PGJ2, whereas 15d-PGJ2 induced only a 3.2-fold stimulation (Fig. 4). This result clearly indicated that b-15d-PGJ2 is a potent PPARγ agonist.

*b-15d-PGJ2 docks into the LBD of PPARγ, in a similar manner as 15d-PGJ2.* In order to determine the mechanisms through which b-15d-PGJ2 activates PPARγ, we performed docking of both ligands into the ligand binding pocket of the receptor. The docking of 15d-PGJ2 in the LBD of PPARγ yielded a pose in the 3V9V structure that was very similar to the X-ray pose (PDB entry code 2ZK1). It is important to note that in the 2ZK1 entry, a covalent bond between 15d-PGJ2 and Cys285 was present, which cannot be reproduced by Autodock. However, in the best pose, the distance that we obtained between the sulfur atom of Cys285 and C-13 of 15d-PGJ2 (the exocyclic Michael acceptor) remained small and compatible with a subsequent chemical bonding (Fig. 5).

Our docking protocol being able to reproduce in the 3V9V structure a correct pose for 15d-PGJ2, we docked b-15d-PGJ2 into the same target protein using the same protocol. In solution, each of the two amide bonds of b-15d-PGJ2 can adopt configurations Z or E. Therefore, we considered in our docking study, 4 different types of structures for b-15d-PGJ2 (i.e., ZZ, ZE, EZ, or EE). Our docking results were very similar for all 4 isomers. In all cases, the biotin moiety lies out of the PPARγ ligand-binding pocket. The only significant interaction of this molecular fragment was the interaction of the first amide bond (i.e., the amide bond linked to the 15d-PGJ2 moiety) and Ser342. Regardless of its configuration, the amide bond formed a hydrogen bond with either the backbone of Ser342 (N-H of Ser342 donates to the C=O of the amide bond) and/or the side chain of Ser342 (the amide bond N-H donates to the hydroxyl oxygen of Ser342). The ‘15d-PGJ2’ moiety adopted
a similar conformation than the best 15d-PGJ2 docking pose with a closer distance between Cys285 and C-13 of 15d-PGJ2 (3.1 vs. 3.8 Å between the two atoms for the b-15d-PGJ2 pose vs. the 15d-PGJ2 pose, respectively). Taken together, our results indicated that b-15d-PGJ2 activated PPARγ via a covalent bond with Cys285 in a very similar manner to what has been reported for 15d-PGJ2 (41).

b-15d-PGJ2-induced apoptosis is attenuated with PPARγ silencing. To determine whether b-15d-PGJ2-induced apoptosis was mediated by PPARγ, we used siRNA directed against this receptor in the MCF‑7 cells. First, we verified the silencing of PPARγ by performing a functional assay, in which the MCF‑7 cells were co-transfected with the reporter construct, pPPRE₃-tk-luc, a human PPARγ-expressing vector and PPARγ siRNA or a scramble sequence. The cells were then treated with 15d-PGJ2, b-15d-PGJ2 (10 µM, 24 h) or ethanol as a control. In the presence of PPARγ siRNA, we observed a potent and significant decrease in luciferase activity following treatment with 15d-PGJ2 (3.2-fold) and following treatment with b-15d-PGJ2 (2.9-fold) compared to scramble sequence (Fig. 6A). These data were then confirmed by the analysis of the PPARγ mRNA levels. (Fig. 6B). We then also examined the effect of PPARγ silencing on the b-15d-PGJ2-induced apoptosis of both cell lines. After PPARγ silencing, the pro-apoptotic signals usually induced by b-15d-PGJ2 were significantly reduced in the MCF‑7 cells: the level of cleaved PARP-1 and cleaved caspase-7 exhibited a 2- and 3-fold decrease, respectively (Fig. 7A). PPARγ silencing led to a similar trend in the MDA-MB-231 cells, with a decrease of cleaved PARP-1 and caspase-7 of 1.8- and 1.6-fold, respectively; however, no significant difference was observed (Fig. 7B).

Discussion

De novo or acquired resistance to current therapies constitutes a main obstacle in breast cancer therapy. In this context, the development of alternative treatments remains essential. The PPARγ agonist, 15d-PGJ2, has been broadly studied for its anticancer properties (4-11,48,49). Recently, we described that biotinylation enhanced the anti-proliferative activity of TGZ, a synthetic agonist of PPARγ, in breast cancer cells (36).
The aim of the present study was to determine whether the biotinylation of the natural PPARγ agonist, 15d-PGJ2, could further enhance its activity on breast cancer cells.

In this context, we examined the effects of b-15d-PGJ2 on two breast cancer cell lines: MCF-7 (estrogen-dependent) and MDA-MB-231 (triple-negative) cells. According to previous studies, 15d-PGJ2 inhibited the proliferation of various cell lines with an IC50 value >50 µM (20,50). By contrast, the linkage of a biotin group to the terminal carboxylic acid of 15d-PGJ2 greatly enhanced its anti-proliferative effects on both cell lines. b-15d-PGJ2 significantly reduced MCF-7 cell viability with an IC50 value of 9.8±1.2 µM. b-15d-PGJ2 appeared more potent on the MDA-MB-231 cells, with an IC50 value of 6.0±0.1 µM. No viable cells remained in the presence of b-15d-PGJ2 (10 µM), whereas 80% of the cells were viable in presence of 15d-PGJ2 (10 µM). In previous studies, we observed that the MDA-MB-231 cells were more sensitive than the MCF-7 cells to the biotinylated derivatives of TGZ (35,36). Of note, the b-15d-PGJ2 inhibition efficiency was significantly higher than that of efatutazone, a recent PPARγ agonist undergoing clinical investigation, and whose activity has been demonstrated in vitro and in xenograft models (51,52). Moreover, it has been described that PPARγ ligands and 15d-PGJ2 induce apoptosis (53,54). In the present study, we observed strong apoptotic signals in response to a 24 h treatment with b-15d-PGJ2 (10 µM), while no signal was detectable in response to treatment with 15d-PGJ2 (10 µM) in both cell lines.

Subsequently, we investigated the cause of this improved efficiency. One might suggest that biotin can improve the cellular intake of b-15d-PGJ2 through receptor-mediated internalization (31-34,55). Biotin competition experiments were performed to test this hypothesis. However, the presence of free biotin did not decrease the anti-proliferative activity. We also investigated whether biotin could improve the general cellular uptake. To shed light into this matter, we performed cooperation experiments between biotin and 15d-PGJ2. In the MCF-7 and MDA-MB-231 cells, no significant differences were observed in the anti-proliferative effects of 15d-PGJ2 in presence of 5 or 10 µM of biotin (data not shown). Thus, the improved activity was not the result of an increased uptake of the molecule mediated by the biotin group. Most likely, the structural modification of 15d-PGJ2 elicited a better activity of the biotinylated compound as we observed previously using biotinylated derivatives of TGZ (35,36). One might suggest that biotinylation could alter the lipophilicity of 15d-PGJ2 and increase its entry into the cell. We then calculated the logP of both molecules. The introduction of the biotin group induces a slight decrease of the ClogP of 0.21, which is not so significant. However, the non-biotinylated molecule bears a carboxylic acid group, which is ionized at the pH of the culture medium. This may lead to a lower diffusion of 15d-PGJ2 through the cellular membrane, and could explain a lower activity than the biotinylated compound.

Subsequently, we determined whether biotin could modify the 15d-PGJ2 PPARγ agonist activity. Co-transfection
experiments demonstrated a clear improvement of PPARγ stimulation. Indeed, b-15d-PGJ2 induced a 5.5-fold stimulation compared to the solvent control. This activation was not statistically different from those induced by etafatuzone. Moreover, in this condition, both ligands were significantly more efficient than 15d-PGJ2.

The question of whether the biotinylation of 15d-PGJ2 could improve its binding to PPARγ still remained unanswered. Thus, to assess this question, we performed a docking analysis of b-15d-PGJ2 into PPARγ. It appeared that b-15d-PGJ2 potentially docked into the LBD of PPARγ in a very similar manner as 15d-PGJ2. However, there were two differences: i) the distance between Cys285 of the receptor and C-13 of the prostaglandin derivative is shorter (3.1 Å) in the case of the biotinylated derivative than for the non-biotinylated molecule (3.8 Å). Since this distance may affect the formation of a beneficial covalent bond between the two species, this could lead to a different transactivation ability of both molecules; ii) in the case of b-15d-PGJ2, a new H-bond interaction was seen with the receptor, compared to 15d-PGJ2. This interaction is related to the amide bond between 15d-PGJ2 and the 1,5-diaminopentane linker used for the biotinylation. Indeed, it may establish a hydrogen bond with Ser342 and this could also lead to a different transactivation ability of both molecules. As a perspective, these two issues could be addressed as follows. First, as previously reported by Schopfer et al (56), a covalent binding of b-15d-PGJ2 to PPARγ LBD could be checked by means of HPLC-MS/MS after incubation. If this linkage was confirmed, a co-crystallization experiment could permit X-ray diffraction analysis, enabling the study of tight interactions between both partners. Second, a new and non-commercial analog of b-15d-PGJ2 could be chemically synthetized, with a structure which would not permit the hydrogen bond with Ser342. For example, this could be obtained by replacing both amide bonds of the linker by ester bonds. If this interaction was significant, such a new compound should be less active.

It may also be interesting to investigate whether the presence of b-15d-PGJ2 may improve interaction with co-activators or allow recruitment of additional activators. Moreover, we questioned the exact role played by PPARγ in the pro-apoptotic activity of b-15d-PGJ2. We observed a PPARγ-dependent effect, since the pro-apoptotic response was significantly reduced following PPARγ silencing in MCF-7 cells. Despite the similar tendency, this decrease was not significant in the MDA-MB-231 cells, suggesting the possibility of a different mechanism of action. PPARγ-independent mechanisms are probably also involved, as illustrated in other cell lines for 15d-PGJ2. For instance, as previously demonstrated, 15d-PGJ2 induced apoptosis through both PPARγ-dependent and PPARγ-independent pathways in Jurkat T lymphocytes, in spite of a specific role of PPARγ in cell death (57). The level of these two pathways may differ between the MCF-7 and MDA-MB-231 cells. Nevertheless, both pathways can result from the covalent binding of 15d-PGJ2 by Michael's addition to nucleophilic components targeting proteins of intracellular signaling pathways (17,20,58) or the PPARγ LBD (59,60). In conclusion, the biotinylation of 15d-PGJ2 markedly enhanced the anti-proliferative and pro-apoptotic activities in breast cancer cells, with a higher efficiency toward the triple-negative MDA-MB-231 cells compared to the estrogen-sensitive MCF-7 cells.

The present study contributes to the understanding of the mechanisms of action of b-15d-PGJ2 and suggests that its biotinylation may be a promising tool for the further development of novel therapeutic agents for triple-negative breast cancer. As a perspective, this study could be extended by in vivo experiments using mouse tumor xenografts derived from breast cancer cells. A number of studies have demonstrated the in vivo therapeutic effects of 15d-PGJ2 in inflammatory diseases, as well as in cancer (26,27,61); however, no in vivo data obtained with b-15d-PGJ2 are available to date, at least to the best of our knowledge. Nevertheless, the in vivo administration of other biotinylated compounds has already been described. For instance, biotinylated nanoparticles containing doxorubicin solution in terms of cardio-compatibility and overall effect on morphology of vital organs (62). Besides, when administrated once weekly to patients with deep venous thrombosis, idraparinux, the biotinylated form of the anticoagulant idraparinux, appeared to be at least as effective and safe as idraparinux during the 6-month treatment period (63). These data may encourage the commencement of in vivo studies with b-15d-PGJ2. The future of this compound will depend on the demonstration of its safety and efficacy.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

CCo provided substantial contributions to the design of the study and acquisition, analysis, and interpretation of data for the study, and also revised the draft critically for important
intellectual content. MM and AK provided substantial contributions to data acquisition and interpretation of the data, and also revised the draft critically for important intellectual content. CCe, MD, SF, MB and GM provided substantial contributions to the conception and design of the study and the acquisition of data, and also drafted the manuscript. All authors gave final approval of the version be published, and agree to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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