New troglitazone derivatives devoid of PPARγ agonist activity display an increased antiproliferative effect in both hormone-dependent and hormone-independent breast cancer cell lines

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Pre Clinical Study

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Abstract

Numerous recent studies indicate that most anticancer effects of PPARγ agonists like thiazolidinediones are the result of PPARγ-independent pathways. These conclusions were obtained by several approaches including the use of thiazolidinedione derivatives like Δ2-Troglitazone (Δ2-TGZ) that does not activate PPARγ. Since biotinylation has been proposed as a mechanism able to increase the specificity of drug delivery to cancer cells which could express a high level of vitamin receptor, a biotinylated derivative of Δ2-TGZ (bΔ2-TGZ) has been synthetized. In the present article, we have studied the *in vitro* effects of this molecule on both hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cells. In both cell lines, bΔ2-TGZ was more efficient than Δ2-TGZ to decrease cell viability. bΔ2-TGZ was also more potent than Δ2-TGZ to induce the proteasomal degradation of cyclin D1 in both cell lines and those of ERα in MCF-7 cells. However, in competition experiments, the presence of free biotin in the culture medium did not decrease the antiproliferative action of bΔ2-TGZ. Besides, other compounds that had no biotin but that were substituted at the same position of the phenolic group of the chromane moiety of Δ2-TGZ decreased cell viability similarly to bΔ2-TGZ. Hence we concluded that the increased antiproliferative action of bΔ2-TGZ was not due to biotin itself but to the functionalisation of the terminal hydroxyl group. This should be taken into account for the design of new thiazolidinedione derivatives able to affect not only hormone-dependent but also hormone-independent breast cancer cells in a PPARγ-independent pathway.

Keywords: Breast cancer, PPARγ, Troglitazone, cyclin D1, biotin

Introduction

Breast cancer is the most frequent cancer in women and represents the second leading cause of cancer death in this population after lung cancer. Breast tumours can be separated in at least four individual subgroups defined by Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal growth factor Receptor 2 (HER2) status [1]. These markers led to the development of endocrine agents against hormone receptor-positive tumours and targeted therapeutics against HER2-expressing tumors. However, almost 25% of ER-positive breast cancer patients do not respond to the anti-estrogen tamoxifen and half of the patients receiving tamoxifen eventually die due to creation of tamoxifen-resistant phenotype [2]. Similarly, *de novo* and acquired resistance to the anti-HER2 monoclonal antibody trastuzumab (Tzb; Herceptin®) also exist [3]. Indeed, the majority of patients who achieve an initial response to Herceptin-based regimens generally acquire resistance within 1 year. Besides, no targeted therapy is available for patients with triple-negative breast cancer, lacking expression of hormone receptors and HER. These limitations in the therapy of breast cancer provide a strong stimulus for developing new therapeutic agents. In this context, ligands of peroxisome proliferator–activated receptor gamma (PPARγ), mainly thiazolidinediones, have been proposed as anticancer agents and tested in clinical trials for breast cancer [4, 5].

PPARs are ligand-activated transcription factors that belong to the nuclear receptor superfamily [6]. Three types of receptors have been described: α, β (also called δ) and γ [7-9]. Their activation involves ligand binding, heterodimerization with the retinoid X receptor (RXR) and interaction with a specific response element (PPRE) leading to the transcription modulation of the target gene facilitated by coactivators recruitment [10-13]. PPARγ is activated by endogenous ligands, the polyunsaturated fatty acids, mainly arachidonic derivatives such as 15d-PGJ(2) [14-16]. Thiazolidinediones including troglitazone (TGZ), rosiglitazone (RGZ), pioglitazone and ciglitazone (CGZ) are synthetic PPARγ agonists belonging to a class of drugs used in the treatment of type II diabetes [17].

PPARγ ligands inhibit proliferation and induce differentiation of breast cancer cell lines *in vitro* and *in vivo* [18, 19-21]. Growth inhibition is associated with apoptosis and alteration in cell-cycle progression through the over-expression of the cdk inhibitors p21 and p27 and the repression of cyclin D1 expression [22, 23]. Recently, in the hormone-independent breast cancer cells MDA-MB-231, a nonapoptotic and nonautophagic cytoplasmic vacuolation death was observed in response to 15d-PGJ(2) whereas autophagy

was described in response to thiazolidinediones [24, 25]. Besides, PPARγ agonists inhibit breast tumour angiogenesis and cell migration [26, 27].

Nevertheless, the involvement of PPARγ in these effects is not clear since numerous PPARγ-independent events have been demonstrated [28, 29]. The PPARγ-independence of these events was shown using PPARγ antagonists, transfection of dominant negative PPARγ isoforms, PPARγ-targeted RNA interference as well as PPARγ-inactive TZD analogs like Δ2-TGZ [30-32]. Such compounds were obtained by introducing a double bond adjoining the terminal thiazolidine-2,4-dione ring [31]. These Δ2 derivatives were inactive in PPARγ activation according to a PPARγ transcription factor ELISA as well as transfection assays using a reporter construct that contains PPAR response element [31, 33]. The loss of PPARγ activity was explained by the structural rigidity induced by the double bond introduction surrounding the thiazolidinedione ring. We recently used Δ2-TGZ, together with RNAi and PPARγ antagonists, to demonstrate the PPARγ-independence of TZD-induced ERα proteasomal degradation in two hormone-sensitive breast cancer cell lines [32]. Both Δ2-TGZ and Δ2-CGZ were used to demonstrate PPARγ-independent cyclin D1 proteolysis in the hormone-dependent cell line MCF-7 [34]. However, our knowledge about the action of these molecules in hormone-independent breast cancer cells is very poor. Moreover, these derivatives devoid of PPARγ agonist activity were only modestly more potent than TGZ and CGZ in inhibiting cell proliferation and inducing cyclin D1 proteolysis [34]. Hence, the use of these compounds for breast cancer therapy needs to improve their antiproliferative effects.

Recently, several studies showed that biotin-conjugated macromolecular carriers were able to increase the uptake of anticancer drugs in tumor cells [35, 36]. Biotin, a member of the vitamin family (vitamin H), is a growth promoter of cells. Its content in cancerous tumors is significantly higher than in normal tissue and cancer cells often over-express biotin-specific receptors on the cell surface. Thus, the specific interactions between biotin and its receptors may be exploited for targeted drug delivery. In the present study, we have assessed the *in vitro* effects of a biotinylated derivative of Δ2-TGZ not only in hormone-dependent (MCF-7) but also in hormone-independent (MDA-MB-231) breast cancer cells. The biotinylated derivative was more potent in affecting cell proliferation than Δ2-TGZ. This was correlated to a higher potency to induce cyclin D1 proteolysis. However, the addition of other substitutents to the terminal hydroxyl function of Δ2-TGZ also resulted in an increase in the antiproliferative activity. This suggested that rather than biotinylation, the functionalisation of

the terminal hydroxyl function of Δ2-TGZ was a key element for the antiproliferative effect of the molecule. This important result should be taken into account for the design of new thiazolidinedione derivatives able to affect various types of breast cancer cells in a PPARγ-independent pathway.

Material and methods

Cell culture and reagents

MCF-7 and MDA-MB231 human breast cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Both cell lines were grown at 37°C under 5% CO_2, in phenol red Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Saint-Quentin Fallavier, France) for MCF-7 and in L-15 medium (Gibco, Invitrogen, UK) for MDA-MB-231. These media were supplemented with 10% fetal calf serum (FCS, Eurobio, Les Ulis, France) and 2 mM L-glutamine.

TGZ was purchased from Sigma-Aldrich. The synthesis of compound **1** (carbonic acid *tert*-butyl ester 2-{4-[2,4-dioxothiazolidin-(5Z)-ylidenemethyl]phenoxymethyl}-2,5,7,8-tetramethylchroman-6-yl ester) was achieved according to slight modifications of known procedures [37, 38]. Then, Δ2-TGZ resulted from trifluoroacetic acid treatment of compound **1**. Biotinylated-Δ2-TGZ (b-Δ2-TGZ) was obtained from Δ2-TGZ by simple esterification of the phenol group of the chromane moiety by carboxylic acid group of biotin. Compound **2** (*O*-(*N*-*tert*-butoxycarbonyl-8-aminocapryloyl)Δ2-TGZ) and compound **3** (*O*-(8-aminocapryloyl)Δ2-TGZ trifluoroacetate) were obtained during the synthesis of another form of bΔ2-TGZ in which biotin was associated to Δ2-TGZ by the use of a linker. Synthetic and analytical details will be published elsewhere. The identity and purity of these synthetic derivatives were verified by nuclear magnetic resonance, mass spectrometry and elemental analysis. TGZ, Δ2-TGZ, b-Δ2-TGZ, and compounds **1**-**3** were dissolved in DMSO.

Cell proliferation assay

Cells were seeded in 6-well plates at the density of 8.10^4 cells/well in 2 mL of medium supplemented with 10% FCS and 2 mM L-glutamine. After 24 h of cell attachment, the medium was replaced by fresh medium supplemented with 1% FCS, 2 mM L-glutamine and investigated compounds. Cell proliferation was studied after 24 h of treatment. Control wells received 0.1% DMSO. At the end of the treatment, cells were washed with PBS, trypsinized

and counted with the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Charbonnieres, France). Each treatment was performed in triplicate. For the different compounds, the concentration leading to a decrease of 50% in the number of viable cells (IC_{50}) was measured.

Transient transfection assay

MCF-7 cells were seeded in 6-well plates at the density of 2.10^5 cells/well in 2 mL of medium supplemented with 5% FCS and 2 mM L-glutamine, and allowed to adhere for 24 h. For PPAR transcriptional activity measurement, cells were transfected with pPPRE3tk-luc reporter (1 µg/well) and SV40-driven 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). Transfections were performed using Exgen 500 (Euromedex, France) according to the manufacturer's protocol. After transfection, cells were allowed to grow for 24 h in phenol-red DMEM supplemented with 10% FCS stripped in dextran-coated charcoal and 2 mM L-glutamine. Cells were then treated with different compounds for 24 h in fresh medium. In each sample, luciferase activity was measured by using the Bright-Glo Luciferase Assay System (Promega, Charbonnières, France). β-Gal activity was measured using the β-Galactosidase Enzyme Assay System (Promega). Luciferase activity was detected using a luminometer and normalized with respect to β-Gal activity measured using a spectrophotometer.

Western immunoblotting

At the end of the treatment, MCF-7 and MDA-MB-231 cells were washed twice with PBS in T-75 flasks, scrapped and collected by centrifugation at 180 g for 10 min. The pellets were washed with PBS and suspended in 200 µL of lysis buffer containing: 25 mM MOPS pH 7.2, 60 mM β-glycerophosphate, 15 mM nitrophenylphosphate, 15 mM EGTA, 15 mM $MgCl_2$, 2 mM DTT, 0.1 mM sodium orthovanadate and protease inhibitors (10 µg/mL aprotinin, 5 µg/mL pepstatin, 10 µg/mL leupeptin, 10 µg/mL soybean trypsin inhibitor and 100 µM benzamidine). An aliquot of cell lysate was used for protein concentration determination (RC-DC kit, Bio-Rad Laboratories). After addition of 2X Laemmli buffer (1v/1v), samples were heated at 100°C for 10 min. Thirty µg of total proteins were subjected to SDS-PAGE (10%). After electrophoresis, proteins were transferred onto nitrocellulose membrane. The membrane was blocked in TNT buffer (5 mM Tris-HCl, 15 mM NaCl, 0.1%

Tween 20) with 5% non-fat powder milk and incubated with ERα antibody (clone F-10, SC-8002, Santa Cruz Biotechnology) diluted at 1:500, cyclin D1 antibody (Cell Signaling) diluted at 1:1000, cyclin B1 antibody (clone H-433, SC-752, Santa Cruz Biotechnology) diluted at 1:500, or β-Actin antibody (clone C-11, SC-1615-R, Santa Cruz Biotechnology) diluted at 1:3000 in TNT buffer overnight at 4°C. Then, the membranes were washed five times with TNT and incubated for 1 h with a peroxidase-linked secondary antibody (Santa Cruz Biotechnology). After five washes with TNT, the membranes were developed with ECL detection reagent using chemiluminescence (Amersham, Orsay, France) and observed using a GelDoc 2000 imaging system (Bio-Rad Laboratories).

Statistical analysis

The results of IC_{50} and those of luciferase/β-Gal activities were expressed as mean ± standard error of the mean of three different experiments. Differences among treatment groups were tested using analysis of variance (ANOVA). Differences in which *P* was less than 0.05 were statistically significant. When significant differences were detected, specific post-hoc comparisons between treatment groups were examined with the Bonferroni test (SPSS v11.0 Computer Software).

Results

MDA-MB-231 cells are more sensitive than MCF-7 to TGZ and Δ2-TGZ

The antiproliferative effects of Δ2-TGZ (the PPARγ inactive analog of TGZ) had mainly been tested on the hormone-dependent MCF-7 cell line. Hence, we started our study by analyzing its effects on the hormone-insensitive cell line MDA-MB-231. We compared its effects to those of TGZ and both compounds were also applied on MCF-7 cells as a reference (Fig. 1).

After 24 h of treatment with TGZ, an inhibition of MCF-7 cell proliferation was observed: at 25 µM, the number of viable cells was 81% of those in control cultures treated with DMSO whereas at 50 µM TGZ, only 11% of viable cells were present. The IC_{50} was 35.4 ± 1.3 µM. This compound was more potent in inhibiting MDA-MB-231 cell proliferation. Indeed, after 24 h of exposure, at 15 µM, viable cells were 56% of control and at 25 µM there was approximately no viable cells. The IC_{50} was 15.8 ± 0.3 µM which was significantly different from the IC_{50} measured in MCF-7 cells ($P < 0.05$). Thus, the hormone-insensitive cell line was more sensitive to TGZ.

After 24 h of treatment with Δ2-TGZ, an inhibition of proliferation was also observed in both cell lines. In MCF-7 cells, this inhibition was significantly higher than with TGZ since the IC_{50} was 29.7 ± 2 µM ($P < 0.05$). At 50 µM there was approximately no viable cells. In MDA-MB-231, the IC_{50} was 16.6 ± 0.9 µM and it was not significantly different from those observed with TGZ.

The antiproliferative effect of Δ2-TGZ is potentiated by biotinylation

Then, we studied the effects of a biotinylated derivative of Δ2-TGZ (bΔ2-TGZ) whose structure is shown in Figure 2A. First we verified that bΔ2-TGZ did not activate PPARγ. This was performed using MCF-7 cells transfected with a pPPRE3tk-luc reporter. In contrast to TGZ used as a positive control, bΔ2-TGZ did not induce a significant increase in luciferase activity (Fig. 2B). Thus biotinylation did not modify the properties of Δ2-TGZ. Then, both MCF-7 and MDA-MB-231 were treated with bΔ2-TGZ. In both cell lines, after 24 h of exposure to this derivative, we observed a higher inhibition of proliferation than with Δ2-TGZ (Fig. 2C). Indeed, in MCF-7 cells, the IC_{50} was 11.3 ± 1.8 µM (significantly different from 29.7 µM in the case of Δ2-TGZ, $P < 0.05$). In MDA-MB-231, the IC_{50} was 3.4 ± 0.1 µM (significantly different from 16.6 µM in the case of Δ2-TGZ, $P < 0.05$). Once again, the

hormone-insensitive cell line displayed a higher sensitivity than the hormone-dependent cell line.

Then we analyzed cyclin D1 in breast cancer cells that had been exposed to the biotinylated derivative of Δ2-TGZ. Indeed, the inhibition of MCF-7 cell proliferation induced by Δ2-TGZ is associated with a proteolysis of cyclin D1 which occurs in a PPARγ-independent manner. We studied cyclin D1 by western blotting in whole cell lysates from both MCF-7 and MDA-MB-231 cells exposed for 24 h to bΔ2-TGZ. We compared the effect of this molecule with those of Δ2-TGZ.

In the hormone-dependent MCF-7 cells exposed to 1 or 5 µM Δ2-TGZ, the intensity of the signal obtained with the anti-cyclin D1 antibody was similar to those of control cells (Fig. 3A). However, the signal intensity decreased significantly in lysates of cells exposed to 25 µM of the compound. In the case of bΔ2-TGZ-treated MCF-7 cells, a dose-dependent decrease in cyclin D1 detection was observed: at 5 µM bΔ2-TGZ, a significant decrease of the staining was observed whereas in the presence of 25 µM bΔ2-TGZ, the signal almost completely disappeared (Fig. 3A). The decrease in cyclin D1 detection in case of Δ2-TGZ or bΔ2-TGZ treatment (25 µM) was not observed when the proteasome inhibitor epoxomicin (10 µM) was present in the culture medium demonstrating a proteasome-dependent degradation (Fig. 3A). β-actin detection was identical in all samples demonstrating the loading of equal amount of proteins.

In the hormone-independent cell line MDA-MB-231 which was more sensitive to bΔ2-TGZ in regard to its antiproliferative effect, we used only 3 µM bΔ2-TGZ which is approximately the IC_{50} value measured in our proliferation analyses. Cyclin D1 was not detected in lysates of MDA-MB-231 cells that had been exposed for 24 h to such a concentration of bΔ2-TGZ (Fig. 3B). At a similar concentration, neither TGZ nor Δ2-TGZ could induce a modification of cyclin D1 signal intensity in comparison to control cells (Fig. 3B). In the same cell lysates, cyclin B1 detection was not affected as previously reported in MCF-7 cells exposed to Δ2-TGZ (Fig. 3B). As observed in MCF-7, the disappearance of cyclin D1 did not occur in case of bΔ2-TGZ exposure in the presence of epoxomycin (Fig. 3C). Thus, bΔ2-TGZ that we have shown to have a more potent antiproliferative action than Δ2-TGZ is also a higher cyclin D1-ablative agent. Besides, the higher sensitivity of MDA-MB-231 cells to bΔ2-TGZ in regard to cell proliferation is associated with a higher sensitivity to cyclin D1 proteolysis.

bΔ2-TGZ has also a more potent ERα-ablative effect in MCF-7 cells

Since in hormone-dependent cell lines, Δ2-TGZ is known to induce ERα proteolysis in a PPARγ-independent manner, we also studied ERα in MCF-7 cells. This receptor was studied by western blotting in whole cell lysates from cells exposed to bΔ2-TGZ, Δ2-TGZ or TGZ used at 1, 5 and 25 µM for 24 h. The intensity of the signal obtained with the anti-ERα antibody decreased significantly in lysates of MCF-7 cells exposed to 25 µM of either TGZ or Δ2-TGZ. In the presence of 25 µM bΔ2-TGZ, the signal almost completely disappeared whereas at a dose as low as 5 µM, a decrease of the staining was observed (Fig. 4A). These changes in ERα detection were related to its proteasomal degradation as demonstrated using epoxomicin (Fig. 4A). The decrease in ERα immunodetection was not only obtained at lower doses of bΔ2-TGZ but it was also obtained faster. Indeed, when used at 25 µM, this compound induced a complete disappearance of the signal as soon as 8 h after the beginning of the treatment whereas no decrease could be observed in case of treatment with 25 µM Δ2-TGZ (Fig. 4B). Thus bΔ2-TGZ also displays a higher ERα-ablative effect than Δ2-TGZ.

Biotin is not responsible for the increased antiproliferative effects of bΔ2-TGZ

In order to determine if the increase in the antiproliferative effects of bΔ2-TGZ was the result of the functionalisation by biotin which could be internalized by a membrane receptor, we performed competition experiments with free biotin (Fig. 5). In both MCF-7 and MDA-MB-231 cells, we could not observe a lower antiproliferative effect of bΔ2-TGZ after 24 h in the presence of free biotin used up to 25 µM. This result did not agree with an interaction between biotin and its receptors to explain the increased antiproliferative effects of bΔ2-TGZ.

In order to get more information on the increase in the antiproliferative effects of bΔ2-TGZ, we studied the effects of other compounds that were substituted at the same position (Fig. 6). Compound **1** was carbonic acid *tert*-butyl ester 2-{4-[2,4-dioxothiazolidin-(5Z)-ylidenemethyl]phenoxymethyl}-2,5,7,8-tetramethylchroman-6-yl ester. This compound was obtained during the synthesis of Δ2-TGZ which resulted from trifluoroacetic acid treatment of compound **1**. Compounds **2** and **3** were obtained during the synthesis of other Δ2-TGZ derivatives. Compound **2** was *O*-(*N*-*tert*-butoxycarbonyl-8-aminocapryloyl)Δ2-TGZ and compound **3** was *O*-(8-aminocapryloyl)Δ2-TGZ trifluoroacetate.

When these compounds were studied in cell proliferation asssays, we observed that despite the absence of biotin, they had antiproliferative effects on both MCF-7 and MDA-MB-231 cells. When applied on the hormone-dependent cell line MCF-7 during 24 h, the IC_{50} of compounds **1**, **2** and **3** were 7.7 ± 0.9 µM, 13 ± 2.6 µM and 3.2 ± 0.1 µM respectively (Table 1). These values were lower than the IC_{50} values measured for Δ2-TGZ (29.7 µM ; $P < 0.05$) and not significantly different from those measured for bΔ2-TGZ (11.3 µM). In the hormone-independent cell line MDA-MB-231, the IC_{50} of compounds **1**, **2** and **3** were 3.3 ± 0.1 µM, 3.2 ± 0.5 µM and 5 ± 1.3 µM respectively that is lower than the IC_{50} value measured for Δ2-TGZ (16.6 µM ; $P < 0.05$) and not significantly different from those measured for bΔ2-TGZ (3.4 µM) (Table 1). Thus the potentiation of the antiproliferative effects of Δ2-TGZ did not appear to be associated with the presence of biotin but rather to the functionalisation of the terminal OH group of the molecule.

Discussion

Advances in the characterisation of breast tumour molecular phenotypes, led to an improvement of therapy. Nevertheless, *de novo* and acquired resistance mechanisms to endocrine or targeted therapies on the one hand, and the absence of targeted therapy for patients with triple-negative breast cancer on the other hand, are two important reasons for developing new therapeutic agents. In this purpose, thiazolidinediones that are synthetic PPARγ ligands have been subjected to numerous studies due to their anti-tumour activity in preclinical models of breast cancer. However, mounting evidence indicates that the anti-tumour effects of thiazolidinediones occur independently of PPARγ activation. The dissociation of the anti-tumour effects of thiazolidinediones from their PPARγ agonist activity provided a rationale for using thiazolidinediones as scaffolds for lead optimisation to develop a novel class of anti-tumour agents. Such compounds were obtained by introducing a double bond adjoining the terminal thiazolidine-2,4-dione ring [31]. For instance, Δ2-TGZ was a derivative of TGZ devoid of PPARγ activating activity [34]. However, Δ2-TGZ was only modestly more potent than TGZ in inhibiting MCF-7 cell proliferation (IC_{50} = 55 µM versus 65 µM as determined graphically) [34]. A similar modest increase in cell proliferation inhibiting potency was observed in prostate cancer cell lines (IC_{50} = 20 µM versus 30 µM in PC-3 cells for Δ2-TGZ and TGZ respectively) [31]. Hence it is necessary to improve the effects of this kind of derivative. The specific interactions between biotin and its receptors

may be exploited for targeted drug delivery [35,36]. Thus, the aim of our work was to study the *in vitro* effects of a biotinylated derivative of Δ2-TGZ.

We studied the effect of bΔ2-TGZ on two breast cancer cell lines: MCF-7 which are hormone sensitive cells which were studied previously for their response to Δ2-TGZ and MDA-MB-231 which are hormone-insensitive and whose response to Δ2-TGZ has not been described. In MCF-7 our results confirm the antiproliferative action of TGZ (IC_{50} = 35.4 µM) and the modest increase in antiproliferative action of Δ2-TGZ (IC_{50} = 29.7 µM) previously reported [34]. We observed that MDA-MB-231 cells were more sensitive to TGZ (IC_{50} = 15.8 µM). This is consistent with a previous study that also demonstrated a TGZ-induced acidification of intracellular pH in both MCF-7 and MDA-MB-231 [39]. In this hormone-independent cell line, we showed that the PPARγ-inactive compound Δ2-TGZ also inhibited cell proliferation but we did not observe a significant difference with TGZ (IC_{50} = 16.6 µM). It might be interesting to determine if Δ2-TGZ also acts on cellular pH and to study other hormone-independent breast cancer cell lines to determine if Δ2-TGZ could be used for the therapy of both hormone-sensitive and –insensitive tumours.

Then, we studied the effects of a biotinylated TGZ derivative. bΔ2-TGZ could not stimulate the PPRE-driven expression of luciferase in MCF-7 transfected cells, demonstrating that biotinylation did not modify the property of Δ2-TGZ in regard to PPARγ activation. The level of luciferase expression measured in MCF-7 cells exposed to TGZ that we used as a positive control was similar to previously reported values [32]. bΔ2-TGZ was more potent than TGZ and Δ2-TGZ in inhibiting cell proliferation in both MCF-7 and MDA-MB-231 cell lines. Its antiproliferative potency was 2.6-fold and 4.9-fold higher than that of Δ2-TGZ in MCF-7 and MDA-MB-231 respectively. This result is consistent with the fact that vitamin-mediated targeting is a potential mechanism to increase drug uptake by cancer cells [35]. Interestingly, bΔ2-TGZ also exhibited higher potency in inducing cyclin D1 proteasomal degradation compared to TGZ or Δ2-TGZ in both cell lines. This result indicates that in the hormone-independent MDA-MB-231 cells, cyclin D1 proteolysis can also occur in a PPARγ-independent manner as in MCF-7 cells exposed to either Δ2-TGZ or some of its derivatives [34, 38]. These PPARγ-inactive compounds also induce cyclin D1 proteolysis in prostate cancer cells and this has been recently shown to be mediated by a $SCF^{\beta TrCP}$ mechanism [33, 40]. It could be interesting to determine if a similar mechanism is involved in cyclin D1 proteolysis in both hormone-dependent and hormone-independent breast cancer cells.

In the hormone-dependent cell line MCF-7, there was also an improvement of ERα proteasomal degradation by bΔ2-TGZ. The proteolysis of this receptor occurred earlier and more completely than in case of Δ2-TGZ treatment. It could be interesting to compare MCF-7 with other hormone-dependent breast cancer cell lines to determine if this is a general feature of this compound. The mechanism of this proteolysis that is observed not only with TGZ derivatives but also with 15d-PGJ(2) is still unknown [32]. It was recently reported that 15d-PGJ(2) was able to bind covalently to ERα *in vitro* and *in vivo* [41]. One might suggest that such a modification could trigger ERα proteolysis. We studied the possibility of a covalent binding of bΔ2-TGZ to ERα in MCF-7 cells but we failed to observe it (data not shown).

Since biotinylation of Δ2-TGZ improved its antiproliferative and both cyclin D1- and ERα-ablating activities, we checked that it was related to the presence of biotin which could lead to a better internalization of Δ2-TGZ in case of a receptor-mediated event. We were surprised to observe that the inhibition of proliferation could not be reduced in the presence of biotin in the culture medium. Indeed, free biotin should have realized a competition with bΔ2-TGZ for the receptor as expected from experiments in which unlabeled biotin was able to inhibit [^{3}H]biotin uptake in epithelial cells [42]. This negative result suggested that the presence of biotin *per se* was not the reason for the increased activity of Δ2-TGZ. This hypothesis was confirmed by the use of other Δ2-TGZ derivatives that possessed substituents at the same position and that were as potent as bΔ2-TGZ in inhibiting cell proliferation. These results were in accordance with previous studies using Δ2-TGZ as a lead compound for structural modifications [34, 38]. Structural optimisation of Δ2-TGZ led for instance to Δ2-TG-6 which possessed an additional allyl moiety on the terminal hydroxyl function of Δ2-TGZ. This derivative exhibited IC_{50} of 8 µM in inhibiting MCF-7 cell viability after 24 h of exposure [34]. In regard to cyclin D1 proteolysis, on western blots, in extracts of cells exposed to 7.5 µM Δ2-TG-6 the level of this protein was only 2% of that of control cells [34, 40]. Other Δ2-TGZ derivatives having other substituents on the terminal hydroxyl group like 3,3-dimethylallyl or benzyl also exhibited a higher cyclin D1 ablative activity than Δ2-TGZ: at 7.5 µM, cyclin D1 levels were reduced to 5 and 12% respectively [38]. The highest potency in cyclin D1 proteolysis induction and proliferation inhibition was obtained with STG28. In addition to an allyl-functionalisation of the terminal hydroxyl group, this Δ2-TG-6 derivative bore an additional methoxy moiety at the central phenyl ring [38]. In MCF-7 cells exposed during 24 h to 7.5 µM STG28, cyclin D1 levels were reduced to 1% of that in control cells

and the IC_{50} in MCF-7 cell proliferation was 5 µM [38]. It might be interesting to determine if a similar substitution could also potentiate the activity of our derivatives, especially compound 3 that was the most efficient molecule on both MCF-7 and MDA-MB-231 cells.

To conclude, our results demonstrate that PPARγ-inactive troglitazone derivatives have anti-proliferative activity not only in hormone-dependent but also in hormone-independent breast cancer cells. In both hormone-dependent and hormone-independent cell lines, biotinylation of Δ2-TGZ increases its antiproliferative effect and its cyclin D1 proteasomal degradation. This potentiation is due to the modification of Δ2-TGZ on its hydroxyl terminal group rather than to biotin *per se*. This optimisation of the antiproliferative action of Δ2-TGZ is very promising for the design of new synthetic compounds able to target various forms of breast cancer.

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Figure legends

Fig. 1 - Analysis of cell proliferation of MCF-7 and MDA-MB-231 cells exposed to TGZ and its PPARγ inactive analog Δ2-TGZ. A) Structure of TGZ and Δ2-TGZ which possesses a double bond adjoining the terminal thiazolidine-2,4-dione ring. B) Cells were treated with 10, 15, 25 or 50 µM TGZ or Δ2-TGZ. Control cells were treated with DMSO. Cell numbers were evaluated after 24 h using the CellTiter-Glo^{TM} Luminescent Cell Viability Assay. Results are given as mean ± s.e.m. of 3 different counts.

Fig. 2 - Analysis of cell proliferation of MCF-7 and MDA-MB-231 cells exposed to Δ2-TGZ and its biotinylated derivative. A) Structure of bΔ2-TGZ that was obtained by coupling of the terminal hydroxyl function of Δ2-TGZ with biotin. B) MCF-7 cells were transiently co-transfected with pPPRE3tkLuc and pCMV-βGal in the presence of a human PPARγ expression vector. The cells were treated for 24 h with TGZ or bΔ2-TGZ (25 µM). Luciferase reporter activity was measured, normalized with the β-Gal activity and expressed as percentage of the control assumed as 100%. The values represent the means ± s.e.m. of 3 different experiments. In each experiment, the activities of transfected plasmids were assayed in duplicate transfections. *, Significantly different from untreated control ($P < 0.05$). C) Cells were treated with increasing concentrations of Δ2-TGZ or bΔ2-TGZ. Control cells were treated with DMSO. Cell numbers were evaluated after 24 h using the CellTiter-Glo^{TM} Luminescent Cell Viability Assay. Results are given as mean ± s.e.m. of 3 different counts.

Fig. 3 - Western blot analysis of Cyclin D1 in MCF-7 and MDA-MB-231 cells exposed to bΔ2-TGZ. A) MCF-7 cells were treated with TGZ, Δ2-TGZ or bΔ2-TGZ (at 1, 5 and 25 µM) for 24 h. Other cells were exposed to 25 µM Δ2-TGZ or bΔ2-TGZ in presence of epoxomicin (10 µM) which was also used alone. Control cells received DMSO. 30 µg of total proteins were subjected to SDS-PAGE and Western blotting analysis using an anti-cyclin D1 polyclonal antibody. β-Actin was used as an internal control. B) Similar analysis performed after 24 h of culture of MDA-MB-231 cells in presence of 3 µM of TGZ, Δ2-TGZ or bΔ2-TGZ. Control cells were exposed to DMSO alone. Cyclin B1 was also detected in addition to cyclin D1 and β-Actin. C) MCF-7 cells were exposed for 24 h to 3 µM bΔ2-TGZ used alone or in addition to epoxomicin (10 µM). Other cells were exposed for the same time only to epoxomicin or to DMSO (Ctrl).

Fig. 4 - Western blot analysis of ERα in MCF-7 cells exposed to different PPARγ ligands. A) MCF-7 cells were treated with TGZ, Δ2-TGZ or bΔ2-TGZ (at 1, 5 and 25 µM) for 24 h. Other cells were exposed to 25 µM Δ2-TGZ or bΔ2-TGZ in presence of epoxomicin (10 µM) which was also used alone. Control cells received DMSO. 30 µg of total proteins were subjected to SDS-PAGE and Western blotting analysis using an anti-ERα monoclonal antibody. β-Actin was used as an internal control. B) Similar analysis performed after 8 h of culture in presence of 25 µM of the different compounds.

Fig. 5 - Analysis of cell proliferation of MCF-7 and MDA-MB-231 cells exposed to bΔ2-TGZ in presence of biotin. MCF-7 and MDA-MB-231 cells were treated with Δ2-TGZ in presence of increasing concentration of biotin (0, 5 and 25 µM). Cell numbers were evaluated after 24 h using the CellTiter-Glo^{TM} Luminescent Cell Viability Assay. Results are given as mean ± s.e.m. of 3 different counts.

Fig. 6 – Chemical structure of various *O*-substituted Δ2-TGZ derivatives. **1**: *O*-*tert*-butoxycarbonylΔ2-TGZ. **2**: *O*-(*N*-*tert*-butoxycarbonyl-8-aminocapryloyl)Δ2-TGZ. **3**: *O*-(8-aminocapryloyl)Δ2-TGZ trifluoroacetate

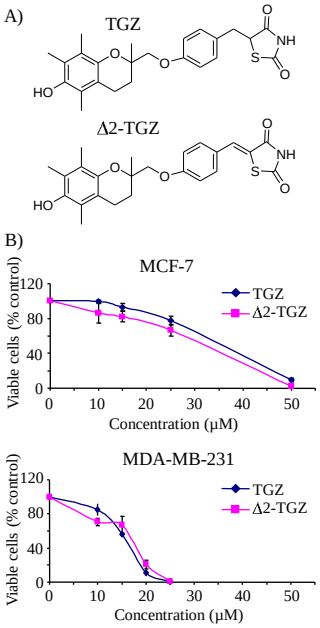

A)
TGZ
HO
O
O
NH
S
O
Δ2-TGZ
B)
MCF-7
Viable cells (% control)
Concentration (μM)
TGZ
Δ2-TGZ
MDA-MB-231

Figure 1

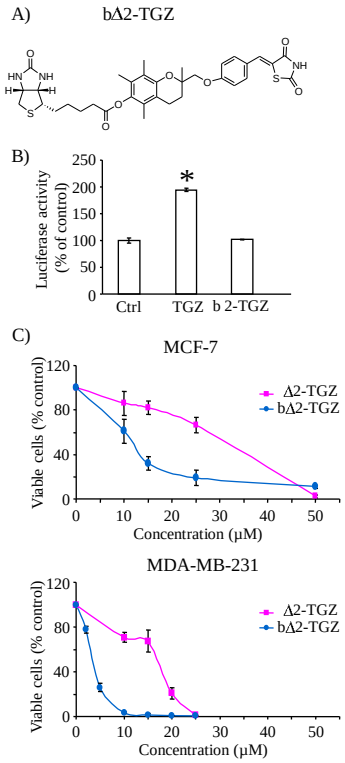

A)
bΔ2-TGZ
B)
Luciferase activity (% of control)
250
200
150
100
50
0
*
Ctrl
TGZ
b 2-TGZ
C)
MCF-7
Viable cells (% control)
120
80
40
0
Δ2-TGZ
bΔ2-TGZ
0
10
20
30
40
50
Concentration (μM)
MDA-MB-231
Viable cells (% control)
120
80
40
0
Δ2-TGZ
bΔ2-TGZ
0
10
20
30
40
50
Concentration (μM)

Figure 2

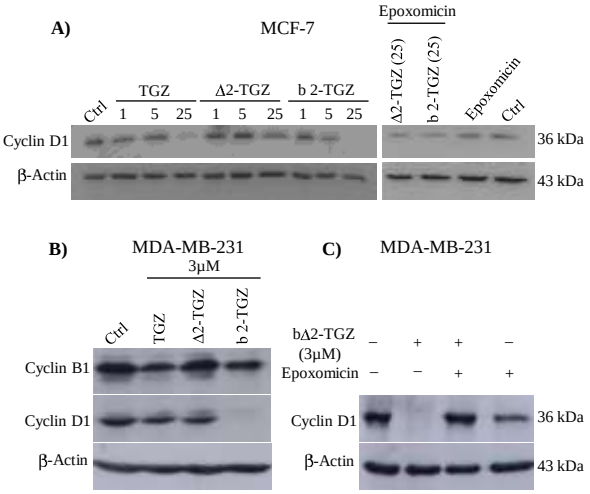

A)
MCF-7
Epoxomicin
Ctrl
TGZ
Δ2-TGZ
b 2-TGZ
1 5 25 1 5 25 1 5 25
Δ2-TGZ (25)
b 2-TGZ (25)
Epoxomicin
Ctrl
Cyclin D1
β-Actin
36 kDa
43 kDa
B)
MDA-MB-231
3μM
Ctrl
TGZ
Δ2-TGZ
b 2-TGZ
Cyclin B1
Cyclin D1
β-Actin
C)
MDA-MB-231
bΔ2-TGZ (3μM)
Epoxomicin
– + + –
– – + +
Cyclin D1
β-Actin
36 kDa
43 kDa

Figure 3

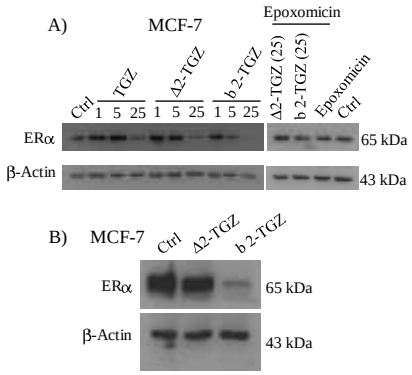

A)
MCF-7
Epoxomicin
Ctrl
TGZ
Δ2-TGZ
b 2-TGZ
1 5 25 1 5 25 1 5 25
Δ2-TGZ (25)
b 2-TGZ (25)
Epoxomicin
Ctrl
ERα
65 kDa
β-Actin
43 kDa
B)
MCF-7
Ctrl
Δ2-TGZ
b 2-TGZ
ERα
65 kDa
β-Actin
43 kDa

Figure 4

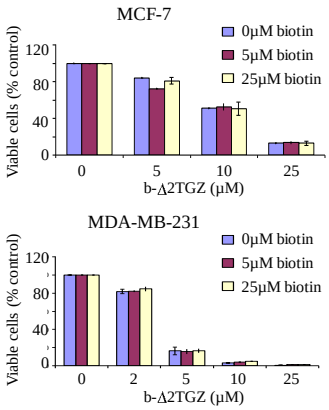
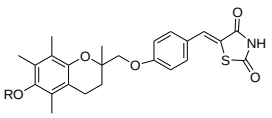

MCF-7
0μM biotin
5μM biotin
25μM biotin
Viable cells (% control)
120
80
40
0
0
5
10
25
b-Δ2TGZ (μM)
MDA-MB-231
0μM biotin
5μM biotin
25μM biotin
Viable cells (% control)
120
80
40
0
0
2
5
10
25
b-Δ2TGZ (μM)

Figure 5



1: R = -COO*t*-Bu
2: R = -$(CH_2)_7$NHCOO*t*-Bu
3: R = -$(CH_2)_7NH_3^+$, CF_3COO^-

Figure 6

Compound	IC_{50} (µM)	
	MCF-7	**MDA-MB-231**
TGZ	35.4	15.8
Δ2-TGZ	29.7	16.6
bΔ2-TGZ	11.3	3.4
1	7.7	3.3
2	13	3.2
3	3.2	5

Table 1 : Antiproliferative effects of various Δ2-TGZ derivatives.