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Cytotoxic effect of a family of Peroxisome Proliferator-Activated Receptor antagonists in colorectal and pancreatic cancer cell lines

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Abstract

Recent studies report an interesting role of Peroxisome Proliferator-Activated Receptors (PPAR) antagonists in different tumor models, being these compounds able to perturb metabolism and viability in cancer cells. In this work the identification of a novel PPAR antagonist, showing inhibitory activity on PPARα and a weaker antagonism on PPARγ, is described. The activity of this compound and of a series of chemical analogues was investigated in selected tumor cell lines, expressing both PPARα and PPARγ. Data obtained show a dose-dependent cytotoxic effect of the novel PPAR antagonist in colorectal and pancreatic cancer models.

Keywords

antitumor agents, CPT1A expression, cytotoxicity, PPAR antagonist, sulfonimides

Since their discovery in 1990, Peroxisome Proliferator-Activated Receptors (PPARs) represent important pharmacological target to modulate metabolic pathways in the organism. They are transcription factors directly binding to DNA, able to control many physiological pathways in the cells. The distribution of the three PPAR isotypes (PPARα, PPARγ, and PPARβ/δ) is related to different effects in liver, kidney, heart, skeletal muscle and adipose tissue.[1-3]

After heterodimerization with 9-*cis* retinoic acid receptor (RXR), PPARs undergo conformational changes stimulating the expression of target genes, involved in glucose, lipid and energetic homeostasis.[4-6] PPARα is mainly involved in lipid metabolism, leading to an increase of fatty acid oxidation.[7-8] Potent and selective PPARα agonists have been developed in the last decades, as useful drugs to treat hypercholesterolemia and related metabolic disorders.[9-10] PPARγ agonists play an important role in insulin sensitivity and adipocyte differentiation: synthetic agonists, the thiazolidinediones, are currently used to improve insulin sensitivity in type-2 diabetes.[11-12] To date no PPARδ agonists are marketed, also if it is well known their beneficial effect on fatty acid catabolism and energetic homeostasis.[13-14] In addition to selective PPAR agonists, dual agonists and PPAR-pan agonists have been developed.[15-17]

A recent field of research is focused on compounds able to repress PPAR activation, including full antagonists and partial agonists.[18-20] In different pathologies, in fact, a repression of PPARs could be advantageous to obtain therapeutic effects. To this aim, selective PPAR antagonists have been identified, showing beneficial effects in metabolic and tumor diseases.[21-23]

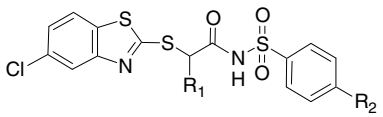
There is a rising interest in the possibility to interfere with metabolic pathways in cancer cells, that exhibit different energetic needs to survive and proliferate, with an aberrant glycolysis and fatty acid oxidation (FAO).[24-26] In this scenario, PPARα is emerging as a new target to interfere with metabolic needs of cancer cells, that utilize FAO for ATP generation.

The exact role of PPARα in cancer is still controversial, and to date it is uncertain whether this receptor functions as a tumor suppressor or an oncoprotein.[27] Antitumor effects of PPARα antagonists have been recently described in leukemia and kidney carcinoma. A specific PPARα antagonist, GW6471, induces cell cycle arrest and apoptosis in renal cell carcinoma, showing a synergistic cytotoxic behavior when used in combination with a glycolysis inhibitor.[28] Spaner *et al.* tested MK886 in a chronic lymphocytic leukemia (CLL) model; in those experiments the PPARα antagonist killed circulating CLL cells and caused proliferating cells to undergo immunogenic death, suggesting a possible therapeutic role of PPARα antagonists in leukemia.[29]

In addition, also PPARγ repression produces antitumor effects: T0070907 and CDDO-Me, PPARγ antagonists, displayed antitumor effects in different cancer cell lines.[30-31]

In the search for novel PPARα ligands, we identified sulfonimide derivatives **1a-d** (**Figure 1**) showing a PPARα antagonistic profile in the micromolar range.[32-34] These molecules also showed a PPARγ antagonism, whereas no PPARδ activity was observed.[34] In agreement with these observations, the sulfonimide derivatives were able to repress carnitine palmitoyl transferase 1 (CPT1A), a key gene controlled by PPARα and PPARγ, involved in fatty acid transport in mitochondria.

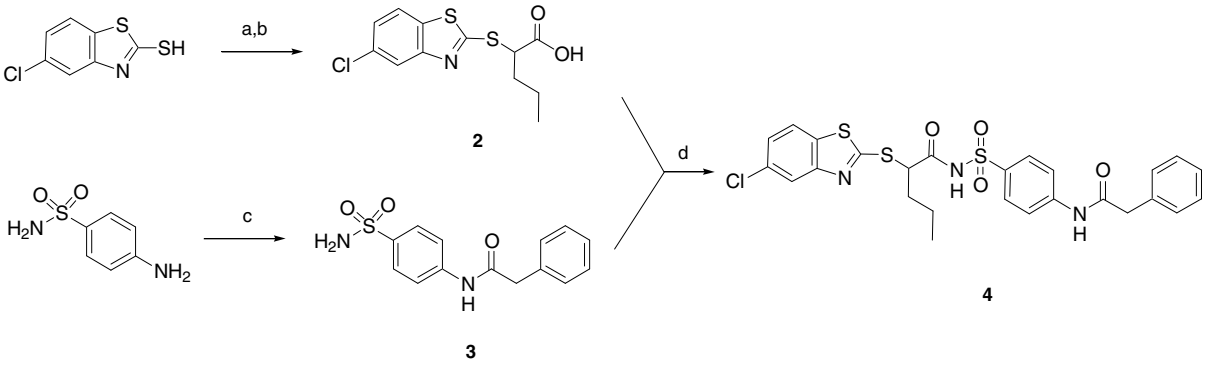
In the present work the synthesis of a novel sulfonimide derivative with PPARα and PPARγ antagonistic effects is described, and the cytotoxic evaluation of this family of sulfonimide compounds in different tumor cell lines, including colorectal and pancreatic cancer cell lines, expressing both PPARα and PPARγ, was reported.



1a R_1= Ph; R_2 = H
1b R_1= *n*Pr; R_2 = OMe
1c R_1= *n*Pr; R_2 = NO_2
1d R_1= Ph; R_2 = NO_2

Figure 1. General structure of benzenesulfonimide derivatives **1a-d**. [34]

The novel sulfonimide derivative, compound **4**, was obtained by inserting an amide group in *p*-position to benzenesulfonimide scaffold; the synthetic route is illustrated in **Scheme 1**.



Scheme 1. Synthesis of compound **4**. Reagents and conditions: a) ethyl 2-bromopentanoate, Na, EtOH, reflux, 4 h, 89%; b) NaOH 2N, THF, r.t., 24 h, 92%; c) phenyl acetyl chloride, pyridine, DCM, 0°C-r.t., 15 h, 99%; d) EDC, DMAP, dry DCM, 0°C-r.t., N_2, 20 h, 77%.

The 2-mercapto-5-chlorobenzothiazole was reacted with ethyl 2-bromopentanoate, in S_N2 conditions, and the intermediate ester was hydrolyzed by NaOH to afford acid **2**.[35] Sulfanilamide was acylated in standard conditions by phenyl acetyl chloride, in the presence of pyridine, to obtain the derivative **3**, that was directly coupled to acid **2**, using EDC as condensing agent, to synthesize the target compound **4**.

In **Figure 2** is depicted a summary of SAR studies performed on our benzothiazole PPAR ligands;[32,33,35] while carboxylic acids are PPARα agonists, benzenesulfonimide bioisosteres showed antagonistic properties. The introduction of substituents in *para* position of aromatic ring was tolerated, and the presence of amide groups resulted in compounds with better potency.

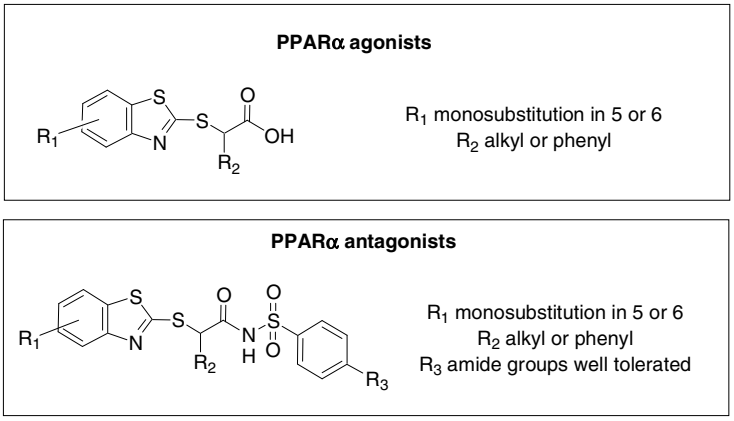


Figure 2. Summary of SARs of benzothiazole PPARα agonists and antagonists.

Transactivation assays have been chosen to assess PPAR activity of synthesized compound. These assays were performed on three PPAR isoforms by Gal4 fusion reporter assays (using Gal4-PPAR-LBD) in human embryonic kidney 293 cells (HEK293).

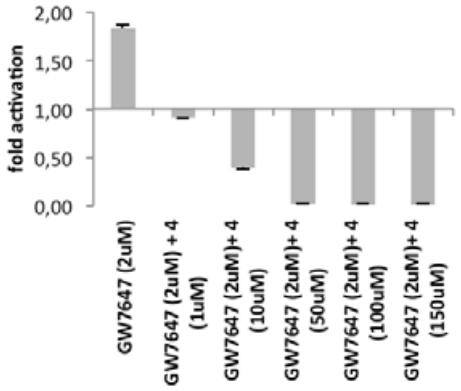
When tested at 150 μM, compound **4** showed an antagonistic behavior on PPARα (calculated as fold activation), being able to decrease the basal receptor activity, while it was inactive on PPARδ and showed a slight antagonism on PPARγ (data not shown). Because the weak PPARγ antagonism at

high concentration (150 μM), we decided to not further analyze the action of compound **4** on this isoform.

Dose-response experiments with compound **4** on PPARα were performed, at concentrations up to 150 μM, in the presence of GW7647 (2 μM), a potent PPARα agonist. In these conditions, compound **4** showed a dose-dependent inhibition profile. Notably, the IC_{50} of this compound, corresponding to its capability to decrease by 50% the maximum receptor activity obtained in the presence of the agonist GW7647 alone, was in the submicromolar range (IC_{50} 0.98 μM) (**Figure 3, panel A**).

In line with these results, compound **4**, alone or in combination with GW7647, was able to repress the PPARα/γ target gene CPT1A (**Figure 3, panel B**). Based on these findings, compound **4**, similarly to other molecules in this family,[34] shows a PPARα antagonism and a weaker PPARγ antagonism.

A)



	FA
GW7647 (2uM)	1.83
GW7647 (2uM) + 4 (1uM)	0.91
GW7647 (2uM) + 4 (10uM)	0.39
GW7647 (2uM) + 4 (50uM)	0.02
GW7647 (2uM) + 4 (100uM)	0.02
GW7647 (2uM) + 4 (150uM)	0.01

B)

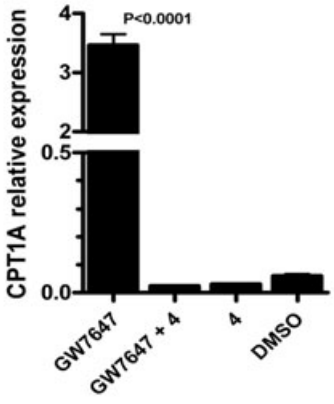


Figure 3. Dose-response inhibition of compound **4** on PPARα in the presence of GW7647 (**panel A**) and evaluation of CPT1A mRNA expression by RT-qPCR, using 150 μM of compound **4**. (**panel B**).

To assess the cytotoxicity of compounds **1a-d** and **4**, MTT assays were performed in different tumor cell lines. Colorectal (HT-29 and SW480) and pancreatic (Capan-2 and AspC-1) cancer cell lines were selected, because all of them express PPARα and PPARγ according to the Expression Atlas database (https://www.ebi.ac.uk/gxa/home). Preliminary experiments were conducted incubating the four cell lines for 72 hours with PPARα/γ antagonists **1a-d** and **4**. Cells were also incubated with PPARα agonist WY-14643 or PPARγ agonist rosiglitazone. Compounds were used at a single, high concentration (75 μM) to identify the molecule most active on cell viability (**Figure 4**). Interestingly, tested compounds significantly reduced viability in pancreatic cancer cell lines, and the novel sulfonimide derivative **4** was the most potent in this series**.** Notably, when used at the same concentration (75 μM), compound **4** showed a threefold lower toxicity on normal human gingival fibroblasts as compared to the commercial PPARα antagonist GW6471 (fibroblast viability: 74% with compound **4** vs. 25% with GW6471). In addition, this novel compound had the most pronounced effect on the viability of colorectal cancer cell lines. Conversely, treatments with WY-14643 or rosiglitazone had no relevant effect on colorectal cancer cell viability.

Based on these results, the most active compound **4** was selected for further characterization of its dose-dependent cytotoxic effects. This molecule was incubated with the four cancer cell lines at concentrations ranging from 25 to 150 μM to obtain dose-response curves (**Figure 5**). Incubation for 72 hours significantly reduced cell viability in a dose-dependent fashion in pancreatic and colorectal cancer cell lines. Colorectal cell lines were more sensitive to the novel molecule with an IC_{50} of 73.3 μM in SW480, 72.3 μM in HT-29, whereas pancreatic cancer cell lines appeared to be less sensitive, with an IC_{50} of 153.6 μM in Capan-2 and 122 μM in AspC-1, as calculated using the CompuSyn software.[36]

These IC_{50}s are similar to those observed with other antitumor molecules.[37-38] It should be also noted that the distinctive IC_{50} values observed among the analyzed cancer cell lines could be related to the inherent differences in their genetic profile, suggesting cell-line specific effects.

Further studies will be necessary to exclude the possibility that the reduction in tumor cell viability observed in this study could be due to effects of the molecule on different targets, as previously shown for other compounds used in human therapy.[39]

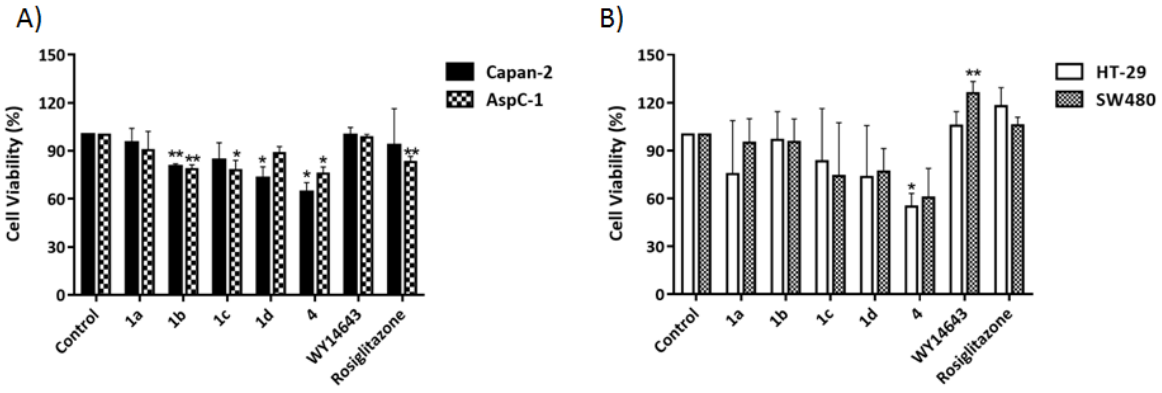


Figure 4. Effect of compounds tested in this study on viability in pancreatic (**panel A**) and colorectal (**panel B**) tumor cell lines. Cell viability was assessed by MTT assay using compounds at 75 µM. Data shown are the means +SD of three experiments with quintuplicate determinations. *Significant differences between control and each compound (*p<0.05; **p<0.01).

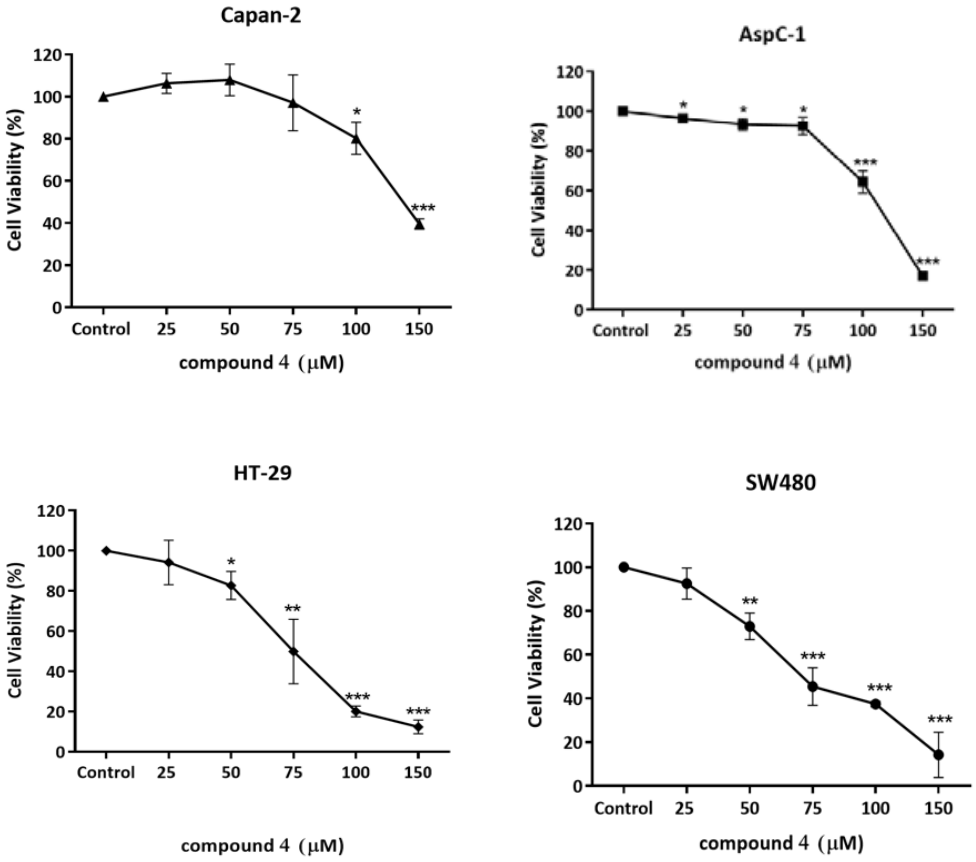


Figure 5. Dose-response curves of the effect of compound **4** on viability of pancreatic (Capan-2 and AspC-1) and colorectal (HT-29 and SW480) tumor cell lines assessed by MTT assay. Compound **4** was incubated with the four cancer cell lines at concentrations ranging from 25 to 150 μM for 72 hours. Data shown are the means +SD of three experiments with quintuplicate determinations. *Significant differences between control and each molecule concentration (*p<0.05; **p<0.01; ***p<0.001).

In conclusion, the synthesis of a novel sulfonimide derivative with PPARα antagonistic activity and a weaker PPARγ antagonism was described. The cytotoxic potential of this novel compound and of a group of related PPAR antagonists was evaluated in pancreatic and colorectal cancer cell lines. The novel sulfonimide **4** emerged as the most promising compound in this series with antitumor activity, showing an interesting cytotoxic effect in selected tumor cell lines, more pronounced in colorectal cancer cell lines.

In line with other studies [25-29] our experiments support the hypothesis that a repression of PPARα and PPARγ could be an advantageous strategy in cancer therapy.

Experimental Section

General

Commercial reagents were used as received from Aldrich or Fluka. Flash chromatography was performed on silica gel 60 (Merck) and thin layer chromatography (TLC) on F_{254} silica gel 60 TLC plates. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Infrared spectra were recorded on a FT-IR 1600 Perkin-Elmer spectrometer. NMR spectra were run at 300 MHz on a Varian instrument using TMS as an internal standard; chemical shifts (δ) are reported in ppm. Microanalyses were carried out with an Eurovector Euro EA 3000 model analyzer. Analyses indicated by the symbols of the elements were within ± 0.4 % of the theoretical values.

*General procedure for the preparation of compounds **3** and **4***

The synthesis of compound **2** was previously described.[35]

Compound **3** was synthesized by reaction of sulfanilamide (1 eq) with phenylacetyl chloride (1.3 eq), in the presence of pyridine (2 eq) in dichlorometane (5 mL) at 0°C, allowing the reaction to reach r.t.

and stirring for 15h. The solvent was then evaporated under reduced pressure, water was added and the solid precipitate was collected under reduced pressure and recrystallized.

***N*-[4-(aminosulfonyl)phenyl]-2-phenylacetamide (3)**

Colourless needles (from water), 99% yield; m.p. 204-206 °C; IR (KBr) 3350, 3230, 1691, 1594, 1535, 1324, 1153 cm^{-1}; ^{1}H NMR (CD_3OD) δ 3.69 (s, 2H, CH_2), 7.26-7.35 (m, 5H, CH_{Ar}), 7.73 (d, 2H, *J* = 9.0 Hz, CH_{Ar}), 7.82 (d, 2H, *J* = 9.0 Hz, CH_{Ar}); ^{13}C NMR (CD_3OD) δ 43.5, 119.3, 126.8, 126.9, 128.4, 128.9, 135.2, 138.6, 142.3, 171.4. Anal. Calcd. for $C_{14}H_{14}N_2O_3S$: C, 57.92; H, 4.86; N, 9.65. Found: C, 57.70; H, 4.87; N, 9.63.

To a cooled mixture (0-5°C) of **2** (1 eq) in dry dichloromethane (15 mL), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, 1 eq) and 4-dimethylaminopyridine (DMAP, 1 eq) were added, under stirring in a nitrogen atmosphere. After 15 minutes, **3** (1.1 eq) was added and the mixture was allowed to warm to r.t. After stirring overnight, the reaction was diluted with dichloromethane, washed with 2N HCl, dried over Na_2SO_4. After evaporation of solvent under reduced pressure, crude product was purified on silica gel (eluent dichloromethane/methanol 95:5).

2-[(5-chloro-1,3-benzothiazol-2-yl)thio]-*N*-({4-[(phenylacetyl)amino]phenyl}sulfonyl) pentanamide (4)

Colourless solid, 77% yield; m.p. 202-204 °C (dec); IR (KBr) 3310, 3267, 1706, 1671, 1540, 1403, 1363, 1173 cm^{-1}; ^{1}H NMR (DMSO) δ 0.82 (t, 3H, *J* = 7.2 Hz, C$H_3$$CH_2$), 1.19-1.35 (m, 2H, C$H_2$$CH_3$), 1.72-1.85 (m, 2H, C$H_2$CH), 3.65 (s, 2H, C$H_2$), 4.49 (t, 1H, *J* = 7.2 Hz, C*H*S), 7.22-7.36 (m, 6H, CH $_{Ar}$), 7.65 (d, 1H, *J* = 2.1 Hz, CH_{Ar}), 7.71 (d, 2H, *J* = 9.0 Hz, CH_{Ar}), 7.82 (d, 2H, *J* = 9.0 Hz, CH_{Ar}), 7.96 (d, 1H, *J* = 8.4 Hz, CH_{Ar}), 10.55 (bs, 1H, N*H*), 12.56 (bs, 1H, NH_{Ar}); ^{13}C NMR (DMSO) δ 14.0, 20.2, 33.9, 43.9, 51.6, 119.0, 121.2, 123.9, 125.4, 127.3, 129.0, 129.6, 129.8, 131.8, 133.0, 134.2, 136.1, 144.5, 153.7, 166.7, 169.4, 170.5. Anal. Calcd. for $C_{26}H_{24}ClN_3O_4S_3$: C, 54.39; H, 4.21; N, 7.32. Found: C, 54.57; H, 4.22; N, 7.30.

In vitro PPAR transactivation assay

Human embryonic kidney 293 cell line (HEK293) was maintained in growth medium composed of DMEM (Sigma) supplemented with 10% FBS (Gibco), 1% penicillin/streptomycin (Sigma), 1% MEM non-essential amino acid (Sigma) and 1% sodium pyruvate MEM (Sigma) at 37 °C, 5% CO_2. The PPARα or PPARγ ligand-binding activity of the test compound was determined using transient transfection assay. The HEK293A cells were placed in white 96-well plates and cultured until 70-80% confluency for 16 h.

Before transfection, the culture medium was replaced by fresh serum-free medium. The cells were transiently transfected with 50 ng of reporter plasmid, 20 ng of renilla, 40 ng of pGEM, and 30 ng

of each receptor expression plasmid per well by the calcium phosphate coprecipitation method.

Test compound, dissolved in DMSO, was added after 6 h; the final concentration of DMSO did not exceed 0.1% (v/v) in the sample. After 16-18 h treatment, the cellular luciferase activity was determined using commercial fire-fly luciferase assay according to the supplier's instructions (Promega). Luciferase and renilla activities were measured by a dual luciferase assay kit using a luminometer (Labsystems Ascent Luminoskan reader). The results were normalized to the renilla activity to correct the transfection efficiencies.

Cell culture, RNA extraction and gene expression analysis

Human HepG2 cells were maintained in growth medium composed of DMEM (Sigma) supplemented with 10% FBS (Gibco), at 37°C, 5% CO_2. Cells were seeded in 6-well plates at a density of $5x10^5$ cells/well in 2 mL of medium per well and incubated for 24 h. After this, medium was replaced to serum free medium containing test compound (150 µM) vehicled by DMSO, GW7647 (2 µM) or GW7647 in combination with test compound, respectively. Cells were then incubated for 48 h. After the treatment period, cells were washed with PBS and total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. RNA was quantified using the Nanodrop 2000 (Thermo Scientific) and RNA integrity was checked by visualization on agarose gel electrophoresis. Total RNA (300 ng) was retro-transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies) following the manufacturer's instruction. RT-qPCR assays were performed in 96-well optical reaction plates using the ABI 7900HT instrument (Applied Biosystem). Samples were analyzed in duplicate plates, with each sample analyzed in triplicate on each of the duplicate using the following reaction mixture per well: 5 µL of Power SYBR Green buffer

(Life Technologies), 1.2 µL of primers at a final concentration of 150 nM, 0.8 µL of RNAase free water, 3 µL of cDNA. For all experiments the following conditions were used: denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, then at 60°C for 60 s. Baseline values of amplification plots were set automatically, and threshold values were kept constant to obtain normalized cycle times and linear regression data. Quantitative normalization was performed using GAPDH as internal control. Relative quantification was performed using the DDCT method using as a calibrator the cDNA obtained from cells cultured adding only the compound's vehicle (DMSO) in the medium. Validated primers for RTqPCR were 50TGCCATGGATCTGCTGTATATCC30 (FW) e 50GCGTTGCCGGCTCTTG30 (RW) for the human CPT1A mRNA and 50CAACTTTGGTATCGTGGAAGGAC30 (FW) e 50ACAGTCTTCTGGGTGGCAGTG30 (RW) for the human GAPDH.

After treatments significant expression differences of CPT1A were evaluated using the two tailed t-test performed with Graph-Pad Prism software (version 4; San Diego, CA, USA).

MTT assay

Stock solutions were prepared dissolving compounds **1a-d** and **4** (80 mM) in DMSO that were then diluted to the final concentration in tissue culture medium. In this way the working solutions were completely clear and devoid of any undissolved material by microscopic inspection. The final concentration of DMSO in all points of our MTT experiments was 0.19% and showed no cell toxicity. Human Capan-2, AspC-1 and SW480 cells were cultured in RPMI (Euroclone), whereas human HT-29 cells were cultured in DMEM (Sigma), all supplemented with 10% FBS (Gibco), at 37°C, 5% CO_2. Cell viability was tested by MTT assay (Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's instructions. Briefly, cells were seeded in 96-well plates ($4x10^3$ cells/well) and incubated with the indicated treatments (5 replica wells per each condition) for 72 hours. At the end, cells were incubated with the MTT solution for at least 3 hours, until purple precipitate was visible. Then, the MTT solution was removed and crystalline precipitate in each well was dissolved in DMSO. Absorbance of each well at 570 nm was quantified using a microplate reader (SoftMaxPro, Molecular Devices).

Statistical analysis

Comparisons of mean values were performed using the independent samples t-test using the Dunnett's test for multiple comparisons where appropriate. A *p*-value of 0.05 was considered

statistically significant. IC_{50} values were calculated using the CompuSyn software based on the Chou-Talalay algorithm.[36]

Aknowledgements

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Conflict of interest

Authors declare no conflict of interest.

Figure legends

Figure 1. General structure of benzenesulfonimide derivatives **1a-d**. [34]

Scheme 1. Synthesis of compound **4**. Reagents and conditions: a) ethyl 2-bromopentanoate, Na, EtOH, reflux, 4 h, 89%; b) NaOH 2N, THF, r.t., 24 h, 92%; c) phenyl acetyl chloride, pyridine, DCM, 0°C-r.t., 15 h, 99%; d) EDC, DMAP, dry DCM, 0°C-r.t., N_2, 20 h, 77%.

Figure 2. Summary of SARs of benzothiazole PPARα agonists and antagonists.

Figure 3. Dose-response inhibition of compound **4** on PPARα in the presence of GW7647 (**panel A**) and evaluation of CPT1A mRNA expression by RT-qPCR, using 150 μM of compound **4**. (**panel B**).

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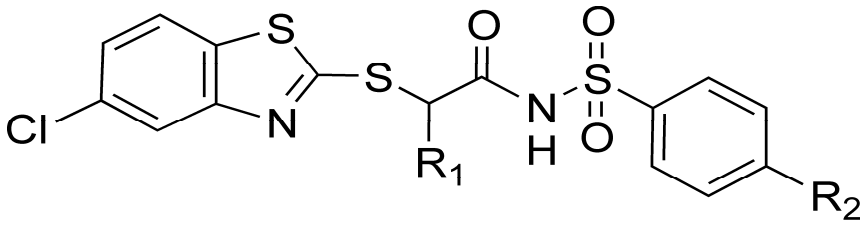
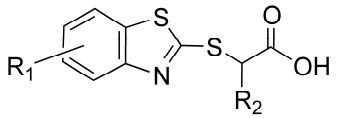


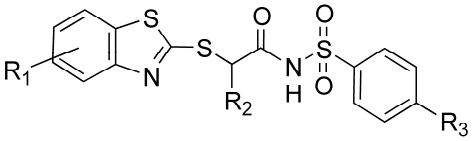
1a R_1= Ph; R_2 = H
1b R_1= *n*Pr; R_2 = OMe
1c R_1= *n*Pr; R_2 = NO_2
1d R_1= Ph; R_2 = NO_2

PPARα agonists



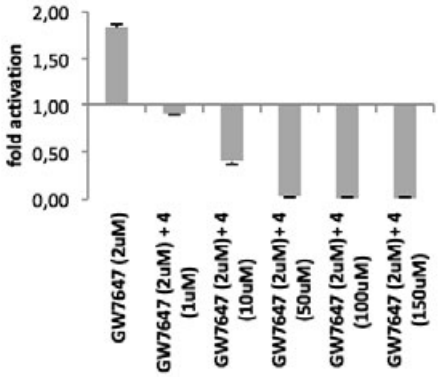
R_1 monosubstitution in 5 or 6
R_2 alkyl or phenyl

PPARα antagonists



R_1 monosubstitution in 5 or 6
R_2 alkyl or phenyl
R_3 amide groups well tolerated

A)



	FA
GW7647 (2uM)	1.83
GW7647 (2uM) + 4 (1uM)	0.91
GW7647 (2uM) + 4 (10uM)	0.39
GW7647 (2uM) + 4 (50uM)	0.02
GW7647 (2uM) + 4 (100uM)	0.02
GW7647 (2uM) + 4 (150uM)	0.01

B)