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New 4-thiazolidinone-based molecules Les-2769 and Les-3266 as possible PPARγ modulators

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ABSTRACT

Development of cancer drug-resistance is still an ongoing problem in the modern anticancer treatment. Therefore, there is a need to search for a new active substance, which may become a potential anticancer agent. 4-Thiazolidinones are well-described substances with cytotoxicity against cancer cells *in vitro*. Therefore, the aim of this study was to evaluate the effect of two 4-thiazolidinone-based derivatives (Les-2769 and Les-3266) on the PPARγ-dependent cytotoxicity in normal human skin fibroblasts (BJ) and squamous cell carcinoma (SCC-15) *in vitro*. The data obtained showed a cytotoxic effect of Les-2769 and Les-3266 used in micromolar concentrations on SCC-15 and BJ cells, manifesting by a decrease in the metabolic activity, an increase in the release of lactate dehydrogenase, and caspase-3 activity. The co-treatment of the cells with Les-3266 and an antagonist (GW9662) or an agonist (rosiglitazone) of the PPARγ receptor induced changes in the above-mentioned parameters in the BJ and SCC-15 cells, compared to the Les-3266 alone exposure; this was not found in the Les-2769-treated cells. The further analysis of the compounds indicated changes in the expression of the *PPARγ*, *KI67*, and *NF-κB* genes. Moreover, the tested compounds caused an increase in the level of *PPARγ* mRNA expression in a similar way to rosiglitazone in SCC-15, which may indicate the affinity of the compounds for PPARγ. Molecular docking is consistent with experimental *in vitro* data about the potential agonistic activity of Les-2769 and Les-3266 towards PPARγ receptors. Summarizing, the anticancer effect of both compounds was observed in the SCC-15 cells *in vitro*; moreover, the mechanism of action of Les-3266 in cells is mediated probably by interaction with the PPARγ receptor pathway, which needs in-depth study.

1. Introduction

Natural and synthetic heterocycles are widely used in medicine due to their chemical structure and activity [1]. Due to the important role of heterocycles in the treatment of diseases, synthetic heterocycles are included in Food and Drug Administration (FDA)-approved drugs, including anti-cancer medications [2]. Among heterocycles, various thiazolidinone derivatives are of special interest for modern medicinal chemistry [1,3,4]. The advantage of heterocycles is the improvement of the pharmacokinetic and pharmacodynamic properties of anti-cancer drugs, which is caused by an increase in the lipophilicity, polarity, and other physicochemical properties [5]. However, these chemical compounds are not without disadvantages, such as the lack of specific targeting [2]. Various types of TZD can be distinguished: 2-, 4-, and 5-thiazolidinones, 2-thioxo-4-thiazolidinones, or 2,4-thiazolidinediones [3]. The molecular mechanism of thiazolidinones is based on the

Abbreviations: BJ, human skin fibroblast line; COX, enzyme cyclooxygenase; EGFR, epidermal growth factor receptor; iNOS, nitric oxide synthase; Les-2769, ethyl rel-[(5aR,11bR)-10-bromo-2-oxo-5a,11b-dihydro-2H,5H-chromeno[4′,3′:4,5]thiopyrano[2,3-d][1,3]thiazol-3(6H)-yl]acetate; Les-3266, 7,8-dimethoxy-1-oxo-1H-isothiochromene-3-carboxylic acid (4-phenylthiazol-2-yl)-amide; MMPs, matrix metalloproteinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PPARγ, peroxisome proliferator-activated receptor gamma; ROSI, rosiglitazone; SCC-15, human squamous cell tumor line; TNF-α, tumor necrosis factor-α; TZD, thiazolidinone.

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inhibition of *inter alia* Raf kinase, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, cyclooxygenase (COX), histone deacetylase, alpha glucosidase, and protein tyrosine phosphatase type 1 as well as the effect on DNA intercalation and activation of peroxisome proliferator-activated receptors (PPARs), especially PPARγ [1,6]. Moreover, PPARγ ligands may influence cell apoptosis, proliferation, and differentiation [1,7]. The interest of the pharmaceutical industry in 4-thiazolidinones, which can be synthesized by [2 + 3]-cyclocondensation, synthesis from similar heterocycles, and ring conversion of some heterocycles to 4-thiazolidone rings, began at the beginning of the 20th century [8–11]. Moreover, the attempt to obtain chemically diverse 4-thiazolidinone-based heterocyclic derivatives in [2 + 4]- [12] and [3 + 3]-cycloadditions [13] and ring transformation reactions [14] proved to be promising. These compounds show a number of properties, e.g. antimicrobial [15–17], antioxidant [15], anticonvulsant [18], anti-inflammatory [19], and anti-cancer [20,21] activities.

Generally, PPARs are nuclear receptor transcription factors that play an important role in physiological and pathological conditions such as cancer, inflammation, cardiovascular disease, and infertility [22,23]. There are three types of PPARs: PPARα, PPARβ/δ, and PPARγ [22]. According to the literature, the role of PPARγ in neoplastic diseases at the transcriptional and protein levels is to inhibit cell proliferation, inhibit the cell cycle, promote apoptosis of cancer cells, and induce intercellular adhesion and inflammation in the tumor microenvironment [23]. A commonly used PPARγ agonist is rosiglitazone, which activated PPARγ leading to apoptosis of tumor cells in the human hepatocarcinoma (HepG2) cell line, while GW9662 (PPARγ antagonist) reversed the agonist-induced effect [24]. Similar induction of apoptosis as a result of PPARγ activation by rosiglitazone was found in bladder cancer cells [25].

According to the literature, the tested 4-thiazolidinones and 4-thiazolidinone-based heterocyclic derivatives: *rel*-N-(2,4-dichlorophenyl)-2-[(5a*R*,11b*R*)-2-oxo-5a,11b-dihydro-2*H*,5*H*-chromeno[4′,3′:4.5]thiopyrano[2,3-*d*][1,3]thiazol-3(6*H*)-yl]acetamide (Les-2194) [26], 5,10-dihydro-2*H*-benzo[6,7]thiochromeno[2,3-*d*][1,3]thiazole-2,5,10-trione (Les-3377) [27], and 3-{2-[5-(4-dimethylaminophenyl)-3-phenyl-4,5-dihydropyrazol-1-yl]-4-oxo-4,5-dihydro-1,3-thiazol-5-ylidene}-2,3-dihydro-1*H*-indol-2-one (Les-3640) [28] showed a cytotoxic effect in SCC-15 cells [28]. The cytotoxic effect of Les-2194, Les-3377, and Les-3640 was also found in lung cancer (A549), colon adenocarcinoma (CACO-2), and skin fibroblasts (BJ) cells [29]. As reported in some studies, the 4-thiazolidinones and 4-thiazolidinone-based heterocyclic derivatives listed above act as PPARγ agonists [29,30].

Therefore, the aim of this study was to analyze the cytotoxic effect of 4-thiazolidinone-based derivatives Les-2769 (ethyl *rel*-[(5a*R*,11b*R*)-10-bromo-2-oxo-5a,11b-dihydro-2*H*,5*H*-chromeno[4′,3′:4,5]thiopyrano [2,3-*d*][1,3]thiazol-3(6H)-yl]acetate) and Les-3266 (7,8-dimethoxy-1-oxo-1*H*-isothiochromene-3-carboxylic acid (4-phenylthiazol-2-yl)-amide) (resazurin reduction, lactate dehydrogenase (LDH) release, and caspase-3 activity) on both human healthy skin fibroblast cells (BJ) and the squamous carcinoma cell line (SCC-15) *in vitro*. Moreover, the *PPARγ*, *KI67*, and *NF-κB* mRNA expression was measured.

2. Methods

2.1. Materials and compounds

Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, DMEM/F12, and MEM without phenol red were purchased from Corning (Tewksbury, MA, USA). Trypsin, penicillin, streptomycin, hydrocortisone, sodium bicarbonate, L-glutamine, dimethyl sulfoxide (DMSO), resazurin, caspase-3 substrate (Ac-DEVD-pNA), hydroxyethyl piperazine ethanesulfonic acid (HEPES); sodium chloride (NaCl), 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate hydrate (CHAPS), ethylenediaminetetraacetic acid (EDTA), glycerol, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lactate dehydrogenase (LDH) release assay was purchased from Takara Bio (MK401) (Kusatsu, Japan). Fetal bovine serum (FBS), Universal RNA Purification Kit (E3598-02), and Fast Probe qPCR Master Mix were purchased from EURx (Gdańsk, Poland). The High Capacity cDNA – Reverse Transcription Kit and the TaqMan® probes corresponding to specific genes encoding *ACTB* (Hs01060665_g1), *GAPDH* (Hs02758991_g1), *PPARγ* (Hs00234592_m1), *NF-κB1* (Hs00765730_m1), and *KI67* (Hs04260396_g1) were purchased from Life Technologies (Forest City, CA, USA).

The synthesis and physicochemical data of the tested compounds were described previously: Les-2769 - ethyl *rel*-[(5a*R*,11b*R*)-10-bromo-2-oxo-5a,11b-dihydro-2*H*,5*H*-chromeno[4′,3′:4,5]thiopyrano[2,3-d][1,3]thiazol-3(6*H*)-yl]acetate [26] and Les-3266–7,8-dimethoxy-1-oxo-1*H*-isothiochromene-3-carboxylic acid (4-phenylthiazol-2-yl)-amide [14](Fig. 1).

2.2. In vitro evaluation of anticancer activity according to the DTP NCI protocol

The primary anticancer assay was performed on a panel of approximately sixty human tumor cell lines derived from nine neoplastic diseases in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda [31–34]. The tested compounds were added to the culture at concentrations ranging from 10^{-4} to 10^{-8} M. A 48-h continuous drug exposure protocol was followed and an SRB (sulforhodamine B) protein assay was used to estimate cell viability or growth.

Using absorbance measurements [time zero (Tz), control growth in the absence of the drug (C), and test growth in the presence of the drug (Ti)], the percentage growth was calculated for each drug concentration. Percentage growth inhibition was calculated as:

$$[(Ti - Tz)/(C - Tz)] \times 100 \text{ for concentrations for which } Ti \geq Tz,$$

$$[(Ti - Tz)/Tz] \times 100 \text{ for concentrations for which } Ti < Tz$$

Dose response parameters (GI_{50}, TGI, LC_{50}) were calculated for each compound. Growth inhibition of 50% (GI_{50}) was calculated from [(Ti - Tz)/(C - Tz)] × 100 = 50, which is the drug concentration resulting in a 50% lower net protein increase in the treated cells (measured by SRB staining), compared to the net protein increase seen in the control cells. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti = Tz. The LC_{50} (concentration of the drug resulting in a 50% reduction in the measured protein at the end of the drug treatment compared to that at the beginning) indicating a net loss of cells following the treatment was calculated from [(Ti − Tz)/Tz] × 100 = −50. The values were calculated for each of these parameters if the level of activity was reached; however, if the effect was not reached or was excessive, the value for the parameter was expressed as higher or lower than the maximum or minimum concentration tested. The lowest values were obtained with the most sensitive cell lines. Compounds having GI_{50} values ≤100 μM were declared to be active.

2.3. Cell culture and treatment

The human squamous cell line SCC-15 (ATCC CRL-1623) and the human skin fibroblast cell line BJ (ATCC CRL-2522) were supplied by the American Type Culture Collection (ATCC, distributor: LGC Standards, Łomianki, Poland). SCC-15 was cultured in DMEM/F12 medium without phenol red containing 2.5 mM L-glutamine, 1.2 g/L sodium bicarbonate, and 400 ng/mL hydrocortisone and supplemented with 0.1% antibiotic and 10% FBS. In turn, BJ was grown in nutrient MEM without phenol red with the addition of 2 mM L-glutamine and 10% FBS. The cell lines were maintained at 37 °C and a constant concentration of 5% CO_2. Confluent cells were plated at 5×10^3 per well in 96-well plates (for 24 h and 48 h treatments) and 10×10^4 per well in 12-well plates (for 24 h treatments). To evaluate the cytotoxic activity, the SCC-15 and

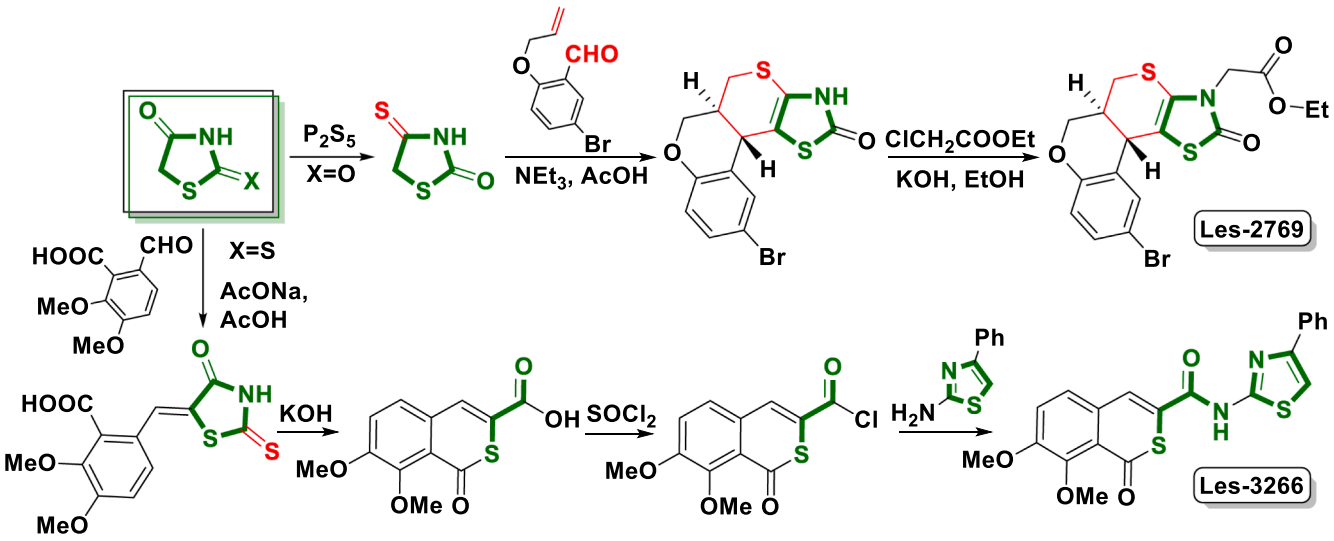

Fig. 1. Schemes for the synthesis of Les-2769 and Les-3266.

BJ cells were treated with the 4-thiazolidinone derivatives Les-2769 and Les-3266 at concentrations of 1, 10, 50, and 100 nM and 1, 10, 50, and 100 μM. Cells co-treated with the PPARγ antagonist and agonist were treated with a 1 μM concentration of GW9662 or a 1 μM concentration of rosiglitazone together with Les-2769 or Les-3266 for 24 h and 48 h. DMSO-treated cells served as controls; the amount of DMSO in the experiments was always the same and did not exceed 0.1%.

2.4. Cell viability test

Cell metabolism and viability were evaluated with the use of the resazurin reduction test according to a previously described protocol [28]. After 24 h or 48 h of treatment of the cells with the tested compounds, the medium was removed and replaced with the working solution of resazurin (10% v/v in the medium supplemented with 1% of FBS) after 1 h. Afterwards, the fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm on a microplate reader (FilterMax F5, Molecular Devices, Corp., Sunnyvale, CA, USA).

2.5. LDH cytotoxicity assay

The toxicity of the tested compounds was measured using the lactate dehydrogenase (LDH) release test. The protocol was performed according to the producer's manual (TakaBio). Briefly, after 24 h or 48 h, the culture medium was transferred to a new 96-well plate. Then a reaction mixture solution was added and incubated for 30 min at RT. The remaining cell plate was frozen and used to measure caspase activity. Absorbance was measured at a wavelength of 490 nm using a FilterMax F5 Multi-Mode microplate reader (Molecular Devices, Corp., Sunnyvale, CA, USA).

2.6. Caspase-3 activity assay

Caspase-3 activity is a well-established marker of apoptosis in cells. The analysis of caspase-3 activity was performed as in Nicholson et al. (1995) with modifications [35]. After 24- or 48-h treatment of the cells with the tested compounds, the culture medium was removed (for LDH release measurement) and the culture plates were frozen at −80 °C for further analysis. The cells were lysed by defrosting and incubation in lysis buffer (50 mM HEPES-pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM DTT) for 10 min at 4 °C. Next, the working mixture containing the caspase-3 (Ac-DEVD-pNA) substrate in lysis buffer (50 μL per well) was added. Absorbance was measured after 30 min at 405 nm using a microplate reader (FilterMax F5 Multi-Mode; Molecular Devices, Corp., Sunnyvale, CA, USA).

2.7. Real-Time PCR analysis of PPARγ, KI67, and NF-κB genes

The total RNA isolation was performed after 24-h treatment of the cells with the tested compounds (10 μM Les-2769, 10 μM Les-3266, 1 μM GW9662, 1 μM rosiglitazone and in cells co-treated with Les-2769 and GW9662 or rosiglitazone and cells co-treated with Les-3266 and GW9662 or rosiglitazone) according to the manufacturer's protocol (Universal RNA Purification Kit, EURx). Subsequently, the RNA quantity and quality were determined spectrophotometrically (260 nm and 280 nm) with the use of NanoDrop (ND/1000 UV/Vis; Thermo Fisher, USA). RT-PCR was performed by CFX Real Time (BioRad, USA), starting with the transcription of mRNA into cDNA (in a volume of 30 μL with 400 ng RNA template for BJ and 800 ng RNA template for SCC-15) according to the manufacturer's protocol (Thermo Fisher). Next, the quantitative polymerase chain reaction (qPCR) was performed using Fast Probe qPCR Master Mix (2x), Tag-Man probe, and primers for the *PPARγ*, *KI67*, *NF-κB*, *ACTB*, and *GADPH* genes in a volume of 20 μL with 1 μL of cDNA. The qPCR was performed with the following reaction parameters: 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C = 40 cycles, 1 min at 60 °C). The analyses were performed with the $\Delta\Delta_{Ct}$ method (the sample Ct threshold was established in the exponential phase). In order to assess the level of gene expression, two reference genes *ACTB* and *GAPDH* were selected and validated, and *ACTB* was selected as the most stable gene.

2.8. Statistical analysis

Data are presented as the mean ± standard deviation (SD) of six replications (n = 6). The statistical analysis was performed with GraphPad Prism 8 software using one-way analysis of variance (ANOVA) with Tukey's multiple post-hoc comparison test. The statistically significant differences between certain values were marked as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison with the control sample; #$p < 0.05$ within the compared group.

2.9. Molecular docking studies

In silico docking simulations were performed using AutoDock Tools 4.2.6. The crystal structures of the PPARγ receptors (PDB code: 5YCP [36], resolution of 2.0 Å) were prepared using the AutoDock Tools 4.2.6 [37]. First, the extra molecules such as co-crystallized ligands, water molecules, or additional subunits were deleted. All hydrogens and

charges were added. Then, the polar hydrogen atoms were added, non-polar hydrogen atoms were merged, Kollman charges were assigned to the protein structure and spread over the residues, and Gasteiger charges were added to the ligand molecules. Next, the torsions from the compounds were allowed to rotate during the docking study. Each grid was centered at the crystallographic coordinates (center_x = 23.439; center_y = − 21.172 and center_z = −1.173) of PPARγ. The grid dimensions were 50 × 50 × 50 points with a default spacing of PPARγ. The number of AutoDock 4.2.6 GA runs was increased from 10 to 50, and the population size was changed from 150 to 300. 3D structures of the Les-2769 and Les-3266 were made by energy minimization using the PM3 semi-empirical quantum technique by HyperChem 7.5. The 3D structure of the antagonist GW9662 was downloaded from the Pubchem portal (https://pubchem.ncbi.nlm.nih.gov/). The other docking parameters were used as default values. The molecular docking protocol was validated through re-docking of a co-crystallized ligand (rosiglitazone) into the binding site of PPARγ proteins. The conditions to reproduce the binding mode of the initial ligand were established and after the re-docking. We found that the root mean squares deviations (RMSD) between the co-crystal ligand and the re-docked structure were 1.25 Å to PPARγ, which indicated that the parameters in docking simulations were acceptable for reproducing orientation, conformation, and interactions in the original X-ray crystal structure. This RMSD value (≤2.0) confirms the relevant predictions for the compounds of interest [38]. Visualizations of the predicted ligand-enzyme complexes were performed by Discovery Studio Visualizer v.21.

3. Results

3.1. In vitro evaluation of the anticancer activity of Les-2769 and Les-3266

The derivatives were tested on a panel of 60 cancer cell lines at concentrations ranging from 10^{-4} to 10^{-8} M included in the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI, Bethesda, USA) [31–34]. In this study, 4-thiazolidinone-based derivatives Les-2769 and Les-3266 inhibited the growth of the majority of the cancer cell lines at micromolar concentrations (Table 1). The mean of three dose–response parameters GI_{50}, TGI, and LC_{50} were 14.6, 57.9, and 92.4 μM (Les-2769) and 16.5, 72.5, and 93.3 μM (Les-3266), respectively. Interestingly, Les-2769 inhibited the growth of some non-small cell lung cancer cell lines (EKVX, NCI-H460, NCI-H522), leukemia cell line SR, colon cancer cell lines HT-29 and SW-620, central nervous system (CNS) cancer cell line SF-295, melanoma cell line MDA-MB-435, ovarian cancer cell line IGROV1, and breast cancer cell line MCF-7 at concentrations <5.0 μM. The Les-3266 activity was an order of magnitude higher and the effect at the GI_{50} < 5.0 μM level was observed for 40 lines out of the 59 tested leukemia, melanoma, non-small cell lung cancer, colon cancer, CNS cancer, ovarian cancer, renal cancer, prostate cancer, and breast cancer lines.

The selectivity index (SI) obtained by dividing the full panel MG-MID (mM) of the tested compound by their individual subpanel MG-MID (mM) was considered as a measure of the selectivity of anticancer activity (Table 1). Ratios between 3 and 6 mean moderate selectivity, ratios greater than 6 indicate high selectivity toward the corresponding cell line, while compounds not meeting either of these criteria are rated non-selective [39]. Compound Les-3266 in the present study was found to be highly selective toward CNS, ovarian and renal cancer subpanels at GI_{50} levels (selectivity indices 7.19, 6.51, and 7.88 μM, respectively). In contrast, Les-2769 did not show significant selectivity for certain cancers.

3.2. Influence of the tested compounds on the metabolic activity of cells

To evaluate the metabolic activity of the SCC-15 and BJ cell lines after the treatment with Les-2769 and Les-3266, the resazurin reduction

Table 1
Influence of compounds Les-2769 and Les-3266 on the growth of individual tumor cell lines.

Cell line/ comp.	Les-2769				Les-3266[1]	
	GI_{50}^{2}, μM	TGI[3], μM	LC_{50}^{4}, μM	GI_{50}, μM	TGI, μM	LC_{50}, μM
Leukemia						
CCRF-CEM	17.4	84.7	>100	48.2/–	>100/ >100	>100/ >100
HL-60(TB)	15.7	>100	>100	38.3/–	>100/ >100	>100/ >100
K-562	–	>100	>100	–/–	>100/ >100	>100/ >100
MOLT-4	38.0	>100	>100	>100/ –	>100/ >100	>100/ >100
RPMI-8226	10.2	41.9	>100	1.80/–	–/–	>100/ –
SR	2.74	23.4	>100	–/2.73	>100/ –	>100/ >100
MG_MID/SI	16.8/ 0.87	70.5/ 0.82	>100/ 0.92	38.2/ 0.43	>100/ 0.73	>100/ 0.93
Non-Small Cell Lung Cancer						
A549/ATCC	15.0	60.8	>100	2.87/ 1.94	–/–	>100/ –
EKVX	3.46	23.6	87.5	>100/ –	>100/ –	>100/ >100
HOP-62	8.16	36.7	>100	3.80/ 1.97	>100/ –	>100/ –
HOP-92	17.4	66.2	>100	2.42/ 1.27	7.98/ 2.79	>100/ –
NCI-H226	20.2	57.9	>100	96.2/ 2.94	>100/ >100	>100 > 100
NCI-H23				7.51/ 2.81	>100/ –	>100/ >100
NCI-322M	24.4	79.5	>100	1.28/–	5.63/–	>100/ >100
NCI-H460	3.87	35.1	>100	3.86/ 2.06	>100/ –	>100/ >100
NCI-H522	3.83	18.7	92.9	–/–	>100/ >100	>100/ >100
MG_MID/SI	12.0/ 1.22	47.3/ 1.22	97.6/ 0.95	16.5/ 1.00	74.2/ 0.98	>100/ 0.93
Colon Cancer						
Colo 205	11.5	41.2	>100	–/3.10	>100/ >100	>100/ >100
HCC-2998	11.6	29.6	75.6	–/–	>100/ >100	>100/ >100
HCT-116	13.3	63.5	>100	3.76/ 1.81	>100/ –	>100/ –
HCT-15	20.9	92.2	>100	>100/ –	>100/ >100	>100/ >100
HT-29	3.44	18.8	>100	–/2.27	>100/ –	>100/ >100
KM12	5.68	35.5	>100	–/2.33	–/–	>100/ >100
SW-620	3.88	25.0	>100	–/2.22	>100/ –	>100/ >100
MG_MID/SI	10.0/ 1.46	43.7/ 1.32	96.5/ 0.96	16.5/ 1.00	>100/ 0.73	>100/ 0.93
CNS Cancer						
SF-268	14.6	60.5	>100	3.56/–	>100/ >100	>100/ >100
SF-295	3.79	19.4	>100	4.03/ 1.97	>100/ –	>100/ >100
SF-539	11.6	30.2	79.0			

(continued on next page)

Table 1 (*continued*)

Cell line/ comp.	Les-2769				Les-3266[1]	
	GI_{50}^{2}, μM	TGI^{3}, μM	LC_{50}^{4}, μM	GI_{50}, μM	TGI, μM	LC_{50}, μM
				1.77/ 1.72	3.63/ 3.38	7.44/ 6.64
SNB-19	16.3	99.5	>100	3.00/ 2.05	>100/ –	>100/ >100
SNB-75	13.6	>100	>100	1.54/ 1.59	3.56/ 3.07	8.27/ 5.90
U251	9.85	90.5	>100	2.01/ 2.00	4.46/ 3.57	–/6.36
MG_MID/SI	11.6/ 1.26	57.7/ 1.00	96.5/ 0.96	2.29/ 7.19	42.2/ 1.72	57.7/ 1.62
Melanoma						
LOX IMVI	13.6	37.4	>100	–/1.91	>100/ –	>100/ –
MALME-3M	18.5	60.0	>100	1.60/ 1.95	8.32/–	>100/ >100
M14	13.6	51.4	>100	>100/ –	>100/ >100	>100/ >100
MDA-MB-435	3.89	29.3	>100	–/–	>100/ –	>100/ >100
SK-MEL-2	9.64	28.1	79.8	>100/ >100	>100/ >100	>100/ >100
SK-MEL-28	24.6	>100	>100	–/–	>100/ –	>100/ –
SK-MEL-5	11.8	26.3	58.4	>100/ –	>100/ >100	>100/ >100
UACC-257	24.4	93.2	>100	>100/ –	>100/ >100	>100/ >100
UACC-62	15.1	38.5	98.0	–/2.76	>100/ >100	>100/ >100
MG_MID/SI	15.0/ 0.97	51.6/ 1.12	82.0/ 1.13	56.4/ 0.29	93.5/ 0.78	>100/ 0.93
Ovarian Cancer						
IGROV1	3.11	15.2	>100	2.57/ 1.80	>100/ 3.36	>100/ –
OVCAR-3	11.1	37.5	>100	1.89/ 1.80	3.72/–	–/–
OVCAR-4	32.7	>100	>100	1.88/ 1.87	–/–	–/–
OVCAR-5	25.9	89.5	>100	–/2.24	>100/ –	>100/ >100
OVCAR-8	22.4	>100	>100	4.03/ 2.41	>100/ –	>100/ >100
NCI/ADR-RES	16.5	80.0	>100	4.97/ 3.16	>100/ >100	>100/ >100
SK-OV-3	20.5	70.9	>100	2.39/ 1.89	7.44/ 3.87	>100/ –
MG_MID/SI	18.9/ 0.77	70.4/ 0.82	>100/ 0.92	2.53/ 6.51	57.6/ 1.26	>100/ 0.93
Renal Cancer						
786–0	14.1	47.4	>100	–/1.82	–/3.34	>100/ –
A498	19.5	62.3	>100	2.32/–	–/–	>100/ –
ACHN	27.6	>100	>100	2.01/ 2.04	–/3.56	>100/ –
CAKI-1	8.10	28.5	88.8	–/2.40	–/1.79	>100/ –
RXF 393	15.9	39.7	99.3	2.31/–	5.88/–	>100/ –
SN12C	22.0	57.2	>100	3.05/ 1.96	>100/ 4.34	>100/ –
TK-10	21.1	81.1	>100	1.43/ 2.08	3.23/ 3.82	7.30/ 5.68

Table 1 (*continued*)

Cell line/ comp.	Les-2769				Les-3266[1]	
	GI_{50}^{2}, μM	TGI^{3}, μM	LC_{50}^{4}, μM	GI_{50}, μM	TGI, μM	LC_{50}, μM
UO-31	15.1	40.4	>100	1.97/ 1.69	–/3.10	>100/ –
MG_MID/SI	17.9/ 0.82	57.1/ 1.01	98.5/ 0.94	2.09/ 7.88	14.3/ 5.05	79.2/ 1.18
Prostate Cancer						
PC-3	14.3	71.9	>100	–/1.64	>100/ –	>100/ –
DU-145	10.5	51.9	>100	–/–	>100/ –	>100/ >100
MG_MID/SI	12.4/ 1.18	61.9/ 0.94	>100/ 0.92	1.64/ 10.0	>100/ 0.73	>100/ 0.93
Breast Cancer						
MCF-7	3.68	>100	>100	>100/ –	>100/ >100	>100/ >100
MDA-MB-231/ ATCC	16.3	54.1	>100	3.53/ 2.08	>100/ 4.37	>100/ –
HS 578T	16.9	52.0	>100	2.61/ 2.43	–/–	>100/ >100
BT-549	11.4	33.2	97.1	5.41/–	78.1/ >100	>100/ >100
T-47D	27.8	>100	>100	>100/ 1.90	>100/ –	>100/ >100
MDA-MB-468				>100/ 2.57	>100/ –	>100/ >100
MG_MID/SI	15.2/ 0.96	67.9/ 0.85	99.4/ 0.93	22.1/ 0.74	85.3/ 0.85	>100/ 0.93
MG_MID (60 lines)	**14.6**	**57.9**	**92.4**	**16.5**	**72.5**	**93.3**

Comment: [1]Les-3266 was tested twice, [2]GI_{50} - drug concentration resulting in 50% decrease in total protein in the treated cells (measured by SRB staining), as compared to the protein amount in control cells, [3]TGI - drug concentration resulting in total (100%) growth inhibition, [4]LC_{50} - concentration of agent which is lethal to 50% of the treated cells.

test was performed. The results showed that Les-2769 decreased skin fibroblast metabolism more strongly than Les-3266 (Fig. 2).

The treatment of the BJ cells with Les-2769 for 24 h resulted in a decrease in the cell metabolism activity by 16.91 to 44.94% in the concentration range from 50 nM to 100 μM, compared to the control (Fig. 2A). After the 48-h exposure of the BJ cells to Les-2769, a decrease by 37.87 to 56.89% was found in the concentration range between 1 nM and 100 μM, compared to the control cells (Fig. 2A).

After the 24-h exposure of the BJ cells to Les-3266, the cell metabolic activity was reduced at the concentrations of 10 μM, 50 μM, and 100 μM (10.76, 38.83, and 33.52%, respectively, compared to the control). After the 48-h exposure of the BJ cells to 10 μM, 50 μM, and 100 μM of Les-3266, the cell metabolism decreased by 40.34, 51.72, and 52.01% respectively, compared to the control (Fig. 2B).

In the SCC-15 cell line, both derivatives had a similar effect as in the BJ cell line. After the 24-h exposure to Les-2769, only 10 μM, 50 μM, and 100 μM decreased the cell metabolism by 26.01, 34.92, and 42.73%, respectively, compared to the control (Fig. 2C). After the 48-h exposure to Les-2769, a similar decrease in the metabolic activity was observed in the range of 10–100 μM (by 48.61–63.91% in comparison to the control) (Fig. 2C).

After the 24-h exposure of the SCC-15 cell line to Les-3266, the decrease (by 54.90 and 51.66%) in the metabolic activity was noticed at

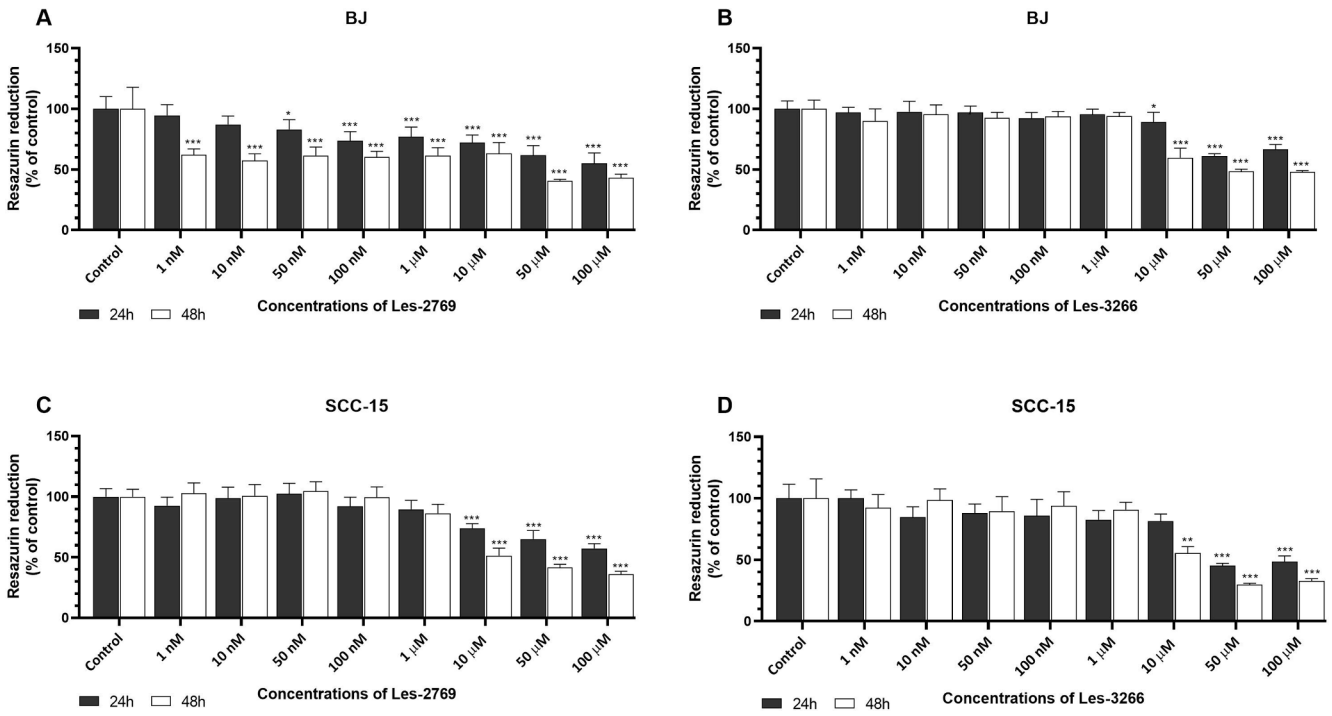

Fig. 2. Effect of increasing concentrations of Les-2769 and Les-3266 (1 nM-100 μM) on the metabolic activity in BJ (A, B) and SCC-15 (C, D) cell lines after the 24- and 48-h exposure. Data are expressed as a mean (n = 6) with standard deviation. Statistically significant values determined by Tukey's test for each study group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control cells.

the two highest μM concentrations (50 and 100 μM), compared to the control (Fig. 2D). In turn, after the 48-h exposure of the SCC-15 cells to Les-3266, only the 10 μM, 50 μM, and 100 μM concentrations caused a decrease in the metabolic activity by 44.70, 70.36, and 67.29%, respectively, compared to the control (Fig. 2D).

3.3. *Effect of the tested compounds on the LDH release level*

According to the results of the cytotoxicity assessment using the LDH test, compound Les-3266 had a greater effect on LDH release from both cell lines studied (Fig. 3).

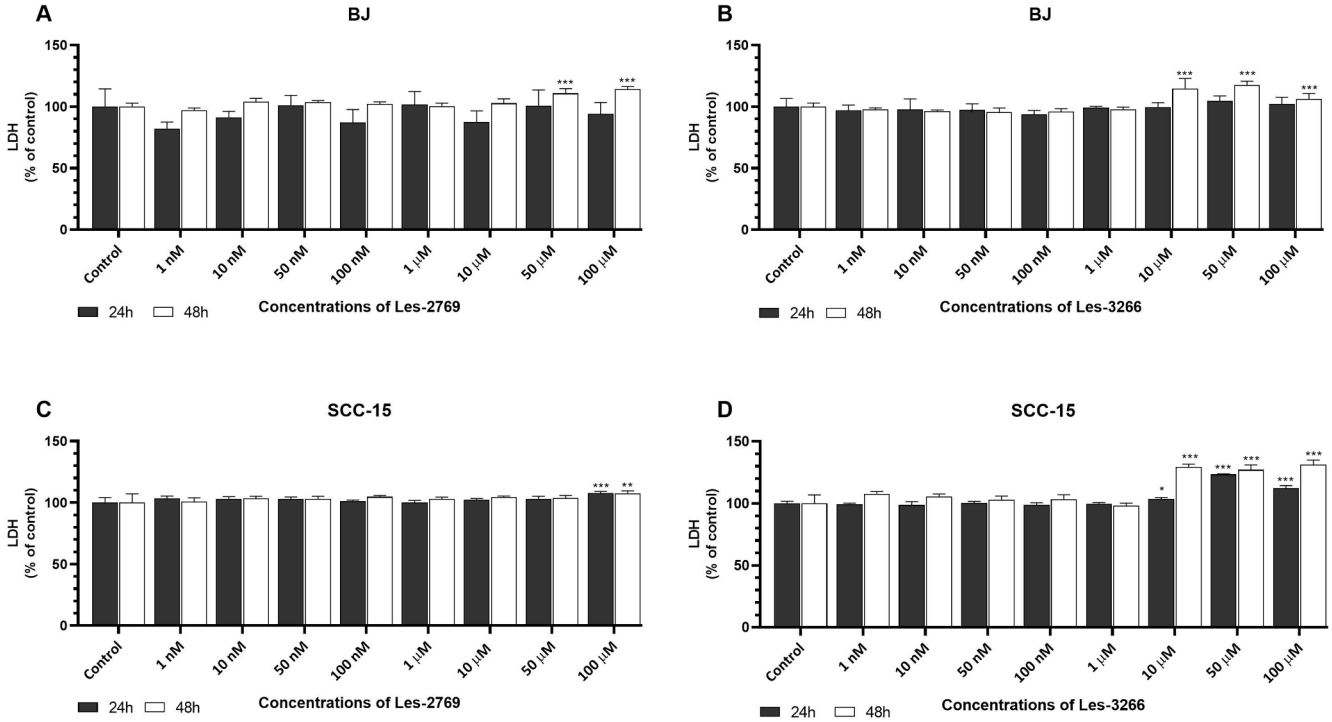

Fig. 3. Effect of increasing concentrations of Les-2769 and Les-3266 (1 nM-100 μM) on the level of LDH release in BJ (A, B) and SCC-15 (C, D) cell lines after the 24- and 48-h exposure. Data are expressed as a mean (n = 6) with standard deviation. Statistically significant values determined by Tukey's test for each study group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control cells.

The results showed that, after the 24-h treatment of the BJ cells with Les-2769, no increase in the LDH release was detected in contrast to the 48-h treatment, which caused an increase in the LDH release level at the 50 μM and 100 μM concentrations (by 10.68 and 14.14%, respectively), compared to the control (Fig. 3A).

After the 24-h of the BJ cells treatment with Les-3266, no changes in the LDH release were detected. However, after the 48-h exposure of the BJ cells to 10, 50, and 100 μM of Les-3266, the LDH release increased by 14.72, 17.69, and 6.13%, respectively, compared to the control (Fig. 3B).

In the SCC-15 cell line, after the 24- and 48-h exposure to Les-2769, an increase in the LDH release was observed only at the 100 μM concentration (a 7.82% increase after 24 h and a 7.46% increase after 48 h), compared to the control (Fig. 3C).

The 24-h exposure of the SCC-15 cell line to Les-3266 caused an increase in the LDH release at the 10, 50, and 100 μM concentrations (by 3.49, 23.46, and 12.36%, respectively). After the 48-h exposure, an increase in the LDH release was observed also at the 10, 50, and 100 μM concentrations (by 29.56, 27.15, and 31.45%, respectively, in comparison to the control) (Fig. 3D).

3.4. Influence of the tested compounds on caspase-3 activity

The analysis of caspase-3 activity showed a greater effect of Les-3266 on apoptosis in both cell lines; in addition, more cells underwent apoptosis after 24 h than after 48 h (Fig. 4).

The treatment of the BJ cells with Les-2769 resulted in an increase in caspase-3 activity at the 100 μM concentration (a 13.53% increase after 24 h and a 25.93% increase after 48 h), compared to the control (Fig. 4A).

After the 24-h exposure of the BJ cells to Les-3266, an increase in the caspase-3 activity at the concentrations of 50 μM and 100 μM was observed (increased by 60.75% and 86.18%, respectively, compared to the control). After the 48-h treatment of the BJ cells with 50 μM and 100 μM of Les-3266, the activity of caspase-3 increased by 50.64% and 78.36%, respectively, compared to the control (Fig. 4B).

In the SCC-15 cell line, the 24-h treatment with 50 μM and 100 μM of Les-2769 caused a 12.04% and 51.25% increase in the caspase-3 activity, respectively, compared to the control. After the 48-h exposure of the SCC-15 cells to Les-2769, only the 100 μM dose increased the caspase-3 activity (by 30.64%), compared to the control (Fig. 4C).

After the 24-h treatment of the SCC-15 cells with Les-3266, an increase in caspase-3 activity was observed at the concentrations of 10, 50, and 100 μM (by 12.99, 44.69, and 84.16%, respectively), compared to the control. After the 48-h treatment of the SCC-15 cells with Les-3266, the casapase-3 activity increased at the concentrations of 10, 50, and 100 μM by 9.59, 41.53, and 53.79%, respectively, compared to the control (Fig. 4D).

3.5. Cell metabolism after co–treatment with the agonist and antagonist of PPARγ

The experiments in the BJ cell line showed that the 24-h exposure to 1 μM of GW9662 and/or 1 μM of rosiglitazone did not change the metabolic activity. However, the use of 10 μM of Les-2769 alone, Les-2769 with GW9662, and Les-2769 with rosiglitazone decreased the metabolic activity in the BJ cells by 20.01, 13.66, and 20.98%, respectively, compared to the control (Fig. 5A). Similarly, after the 48-h exposure of the BJ cells to GW9662 and rosiglitazone alone, no changes in the cell metabolism were detected. In contrast, the treatment of the BJ cells with Les-2769 and the co-treatment with Les-2769 and GW9662 or Les-2769 and rosiglitazone were found to decrease the metabolic activity by 21.48, 28.05, and 30.13%, respectively, compared to the control (Fig. 5A).

In the studied BJ cell line, the 24-h exposure to GW9662 and rosiglitazone alone did not change the metabolic activity. The treatment of the BJ cells with Les-3266 and the co-treatment with Les-3266 with GW9662 and Les-3266 with rosiglitazone decreased the metabolic activity by 12.36, 42.37, and 13.57%, respectively, compared to the control (Fig. 5B). Moreover, the co-treatment of the cells with Les-3266 and

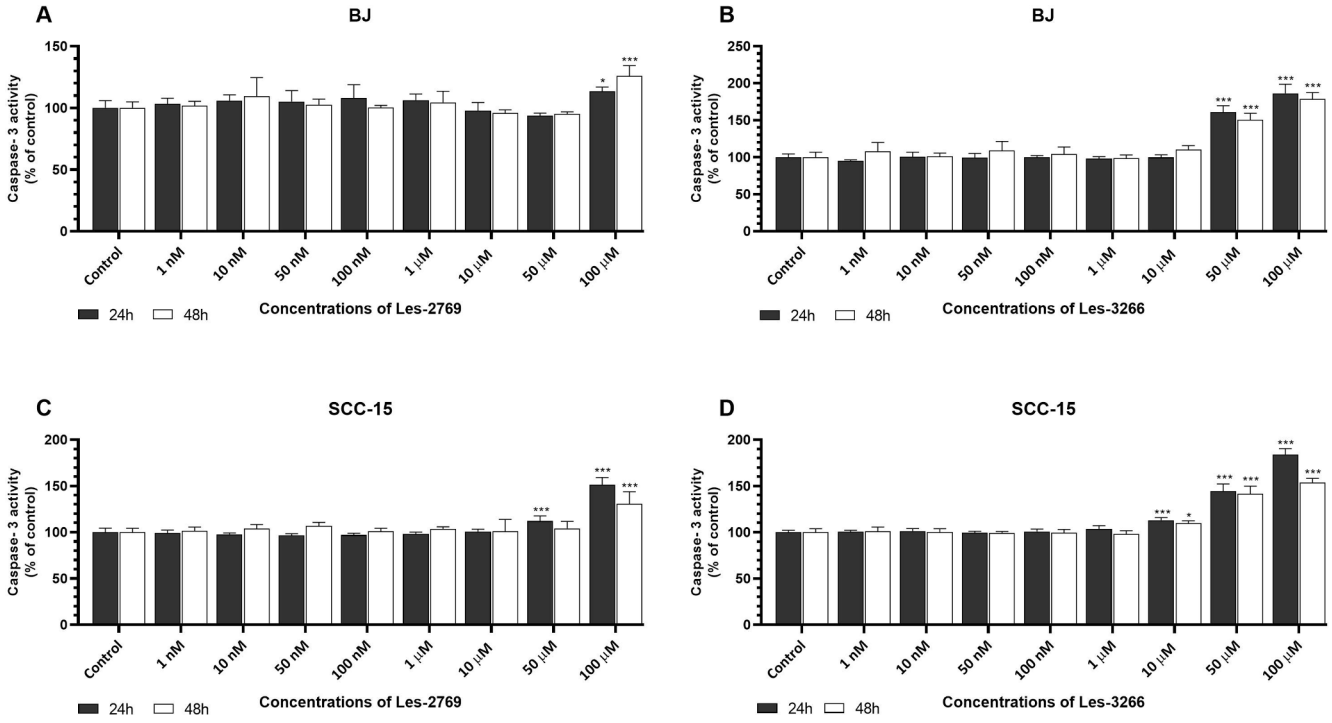

Fig. 4. Effect of increasing concentrations of Les-2769 and Les-3266 (1 nM-100 μM) on caspase-3 activity in BJ (A, B) and SCC-15 (C, D) cell lines after the 24- and 48-h exposure. Data are expressed as a mean (n = 6) with standard deviation. Statistically significant values determined by Tukey's test for each study group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control cells.

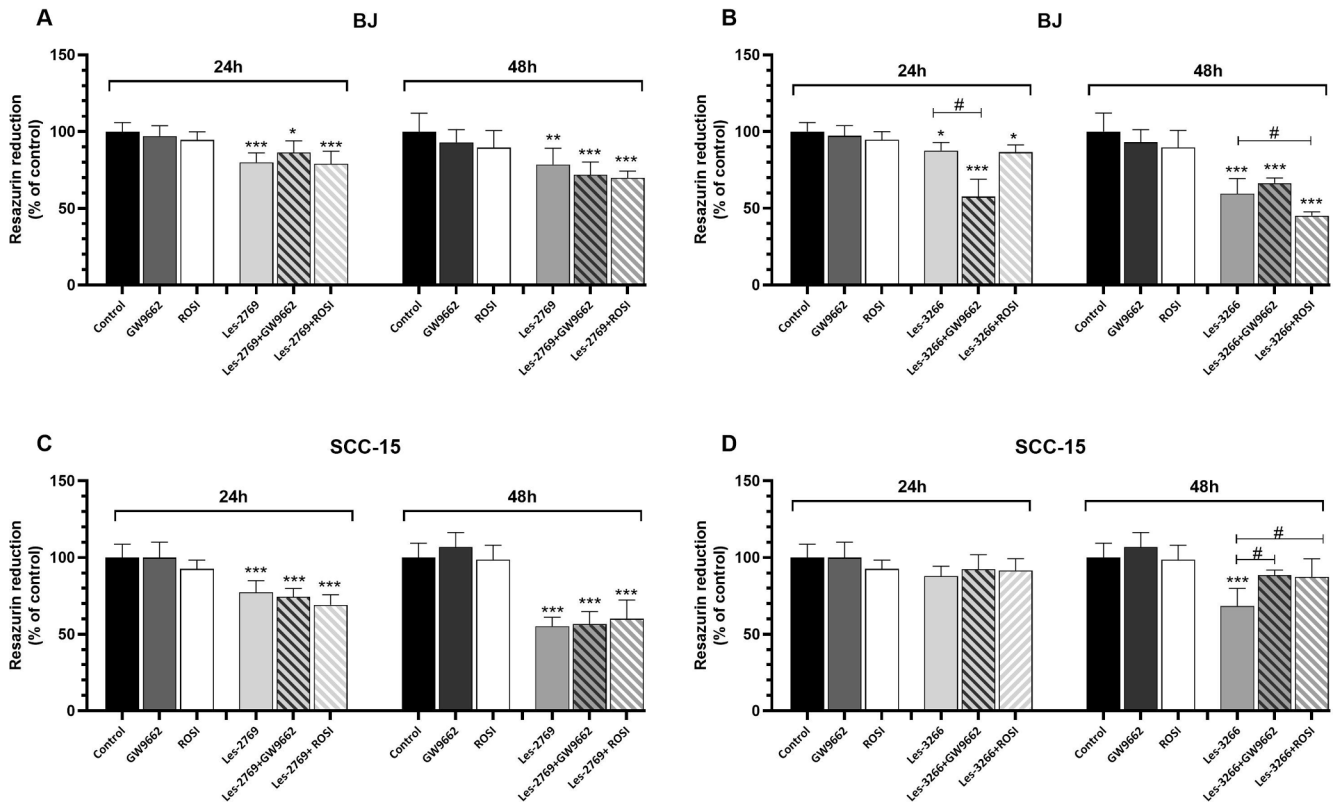

Fig. 5. Effect of 10 μM Les-2769, 10 μM Les-3266, 1 μM GW9662, and 1 μM rosiglitazone (ROSI) or concomitant treatment with Les-2769 with GW9662, Les-2769 with rosiglitazone, Les-3266 with GW9662, and Les-3266 with rosiglitazone on metabolic activity in BJ (A, B) and SCC-15 (C, D) cell lines after the 24- and 48-h exposure to the tested compounds. Data are expressed as a mean (n = 6) with standard deviation. Statistically significant values determined by Tukey's test for each study group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control cells and # $p < 0.05$ compared to cells treated with Les-2769 or Les-3266 alone.

GW9662 decreased the metabolic activity of the BJ cells by 30.01%, compared to cells treated with Les-3266 alone (Fig. 5B). After the 48-h exposure of the BJ cells to Les-3266, Les-3266 with GW9662, and Les-3266 with rosiglitazone, a decrease in the cell metabolism by 40.63, 33.70, and 54.93%, respectively, was observed, compared to the control (Fig. 5B). Moreover, after the co-treatment with Les-3266 and

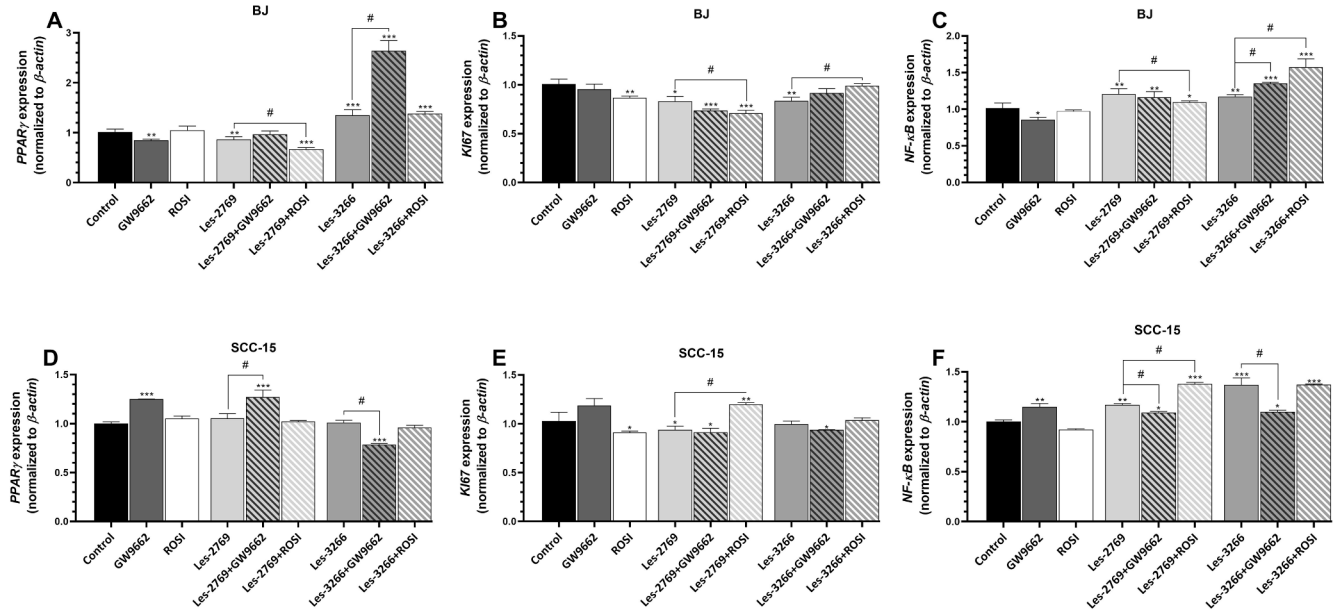

Fig. 6. Effect of 10 μM Les-2769, 10 μM Les-3266, 1 μM GW9662, and 1 μM rosiglitazone or concomitant treatment with Les-2769 with GW9662, Les-2769 with rosiglitazone, Les-3266 with GW9662, and Les-3266 with rosiglitazone on *PPARγ*, *KI67*, and *NF-κB* mRNA expression in BJ (A, B, C) and SCC-15 (D, E, F) cell lines after the 24-h exposure to the tested compounds. Data are expressed as a mean (n = 9) with standard deviation. Statistically significant values determined by Tukey's test for each study group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control cells and # $p < 0.05$ compared to cells treated with Les-2769 or Les-3266 alone.

rosiglitazone, the metabolic activity decreased by 14.30%, compared to the treatment with Les-3266 alone (Fig. 5B).

In the SCC-15 cell line, no changes in the resazurin reduction were observed after the 24-h and 48-h treatment with GW9662 and rosiglitazone (Fig. 5C). After the 24-h and 48-h treatment of the cells with Les-2769 or in the co-treatment with Les-2769 with GW9662 and Les-2769 with rosiglitazone, the metabolic activity of SCC-15 decreased, compared to the control (by 22.71, 25.67, and 30.92%, respectively in the 24-h exposure and by 44.93, 43.47, and 39.92%, respectively, in the 48-h exposure variant) (Fig. 5C).

In the experiment on the SCC-15 cells, after the 24-h exposure to Les-3266, there were no changes in the metabolism of the cells (Fig. 5D). After 48 h, only Les-3266 reduced the metabolic activity of the SCC-15 cells by 31.69%, compared to the control. Moreover, the co-treatment of the SCC-15 cells with Les-3266 with GW9662 and Les-3266 with rosiglitazone increased cell metabolism by 20.18 and 19.07%, respectively, compared to the treatment with Les-3266 alone (Fig. 5D).

3.6. Real-time PCR analysis of mRNA specific for genes encoding PPARγ, KI67, and NF-κB

The experiments showed that the *PPARγ* mRNA expression in the BJ cell line was decreased by 15.20 and 13.71% after the 24-h exposure to 1 μM of GW9662 and 10 μM of Les-2769, respectively, compared to the control (Fig. 6A). However, 10 μM of Les-3266 increased the *PPARγ* mRNA expression by 35.15%, compared to the control (Fig. 6A). Cells co-treated with Les-2769 and rosiglitazone were characterized by a 19.79% decrease in *PPARγ* mRNA expression, compared to the Les-2769-treated group (Fig. 6A). The co-treatment of the cells with Les-3266 and GW9662 resulted in a 128.37% increase in *PPARγ* mRNA expression, compared to the Les-3266 group. The Les-3266 and rosiglitazone co-treated cells exhibited a 38.32% increase in the *PPARγ* mRNA expression, compared to the control, and this result did not differ from that recorded in the Les-3266 alone variant (Fig. 6A).

After the 24-h exposure of the BJ cell line to rosiglitazone, Les-2769, and Les-3266, a decrease in the *KI67* mRNA expression was observed (by 13.23, 17.01, and 16.50%, respectively, compared to the control). The Les-2769 and GW9662 co-treatment resulted in a 26.37% decrease in the *KI67* mRNA level, compared to the control (Fig. 6B). Similarly, the cells co-treated with Les-2769 and rosiglitazone exhibited a 12.07% decrease in the *KI67* mRNA level, compared to the Les-2769 alone exposure (Fig. 6B). Interestingly, a 15.45% increase in the *KI67* mRNA level was observed in the cells co-treated with Les-3266 and rosiglitazone, compared to the Les-3266 alone treatment (Fig. 6B).

The experimental data revealed that, after the 24-h exposure of the BJ cell line to GW9662, the *NF-κB* mRNA expression increased by 14.57%, compared to the control (Fig. 6C). However, Les-2769 and Les-3266 increased the *NF-κB* mRNA expression by 20.60 and 17.30% respectively, compared to the control (Fig. 6C). The cell co-treatment with Les-2769 and GW9662 resulted in increased *NF-κB* mRNA expression by 16.25%, compared to the control (Fig. 6C). In turn, the co-treatment with Les-2769 and rosiglitazone resulted in an 11.17% decrease in the *NF-κB* mRNA level, compared to the Les-2769 alone variant (Fig. 6C). The co-treatment of the BJ cells with Les-3266 and GW9662 as well as Les-3266 and rosiglitazone caused an increase in *NF-κB* gene expression by 17.79 and 39.86%, respectively, compared to the Les-3266 alone exposure (Fig. 6C).

The experiments showed a significant increase in the *PPARγ* mRNA expression in the SCC-15 cell line after the 24-h exposure to GW9662 (by 24.96%, compared to the control) (Fig. 6D). The cell treatment with rosiglitazone, Les-2769, and Les-3266 did not change the *PPARγ* mRNA expression. The co-treatment of the SCC-15 cells with Les-2769 and GW9662 increased the *PPARγ* mRNA expression by 21.86%, compared to the cells treated with Les-2769 alone (Fig. 6D). In contrast, the SCC-15 cell co-treatment with Les-3266 and GW9662 caused a 22.56% decrease in the *PPARγ* mRNA expression, compared to the Les-3266 alone treatment (Fig. 6D).

The 24-h exposure of the SCC-15 cell line to rosiglitazone and Les-2769 alone decreased the *KI67* mRNA gene expression by 9.31 and 6.21%, respectively, compared to the control (Fig. 6E). The co-treatment of the SCC-15 cells with Les-2769 with rosiglitazone increased in the expression of the *KI67* mRNA gene by 25.78%, compared to the Les-2769 alone variant. After the exposure of the cells to Les-3266 with GW9662, a 6.20% decrease in the *KI67* mRNA expression was observed, compared to the control (Fig. 6E).

The SCC-15 cell line exposed to GW9662, Les-2769, and Les3266 for 24 h exhibited a significant increase in the *NF-κB* mRNA expression (by 14.73, 16.86, and 36.94%, respectively), compared to the control (Fig. 6F). The co-treatment of the SCC-15 cells with Les-2769 and GW9662 resulted in a 7.75% decrease in the *NF-κB* mRNA expression, compared to the cell treatment with Les-2769 alone. Similarly, the Les-3266 and GW9662 co-treated cells exhibited a 26.88% decrease in the *NF-κB* mRNA expression, compared to the Les-3266 alone exposure (Fig. 6F). In turn, the cell co-treatment with Les-2769 and rosiglitazone increased the *NF-κB* mRNA expression by 20.92%, compared to the Les-2769 alone variant. However, no significant differences were found in the group co-treated with Les-3266 and rosiglitazone, compared to the Les-3266 alone variant (Fig. 6F).

3.7. Molecular docking simulations

The docking simulations were performed to explore the interaction of Les-2769 and Les-3266 with the PPARγ receptors. We used either the agonist rosiglitazone or the antagonist GW9662 to compare the position of the docked ligands and the features of the Les-2769 and Les-3266 complexes. The predicted binding energies and the estimated inhibition constants are highlighted in Table 2.

The docking simulations confirm the good affinity of Les-2769 and especially Les-3266 to the PPARγ receptors. Les-2769 connects with the receptors by four hydrogen bonds with His323, Tyr473, and Tyr327. In addition, the molecule forms a number of different lipophilic interactions inside the allosteric center. Oxygens from the carboxylic group and the thiazolidine ring mimic the “head” of the rosiglitazone molecule, and another part imitates the “tail” of the reference agonist (Fig. 7). Les-3266 demonstrated the best docking score, higher than the reference rosiglitazone and GW9662. The compound forms only 2 hydrogen bonds, compared to Les-2769, but the tail shape is fitted to the lipophilic pocket formed by Tyr473, Leu469, Leu453, His449, and Phe363. Also, the Pi donor hydrogen bonds with His449 and Tyr473 give additional energy to the whole complex (Fig. 8).

Les-2769 and Les-3266 interact with Arg288, which is important for the agonistic activity of the ligands. The simple replacement of the phenyl-to-pyridine ring in GW9662 converts a transcriptionally neutral antagonist (GW9662) into a repressive inverse agonist (T0070907) relative to basal cellular activity [40]. As a result, Les-2769 and Les-3266 would possess agonistic activity towards PPARγ receptors.

3.8. In silico evaluation of the ADMET properties of compounds Les-2769, Les-3266 rosiglitazone, and GW9662 using admetSAR

Predictions of a drug-likeness model score of the studied compounds Les-2769, Les-3266, and the reference molecules rosiglitazone and GW9662 were performed using the admetSAR portal (http://lmmd.ecus

Table 2
Binding energies and inhibition constant of the docked compounds.

Compound	Binding energies kcal/mol	Inhibition constants, Ki nM
Les-2769	−8.23	926.69
Les-3266	−9.19	183.74
Rosiglitazone	−8.95	277.24
GW9662	−8.59	503.86

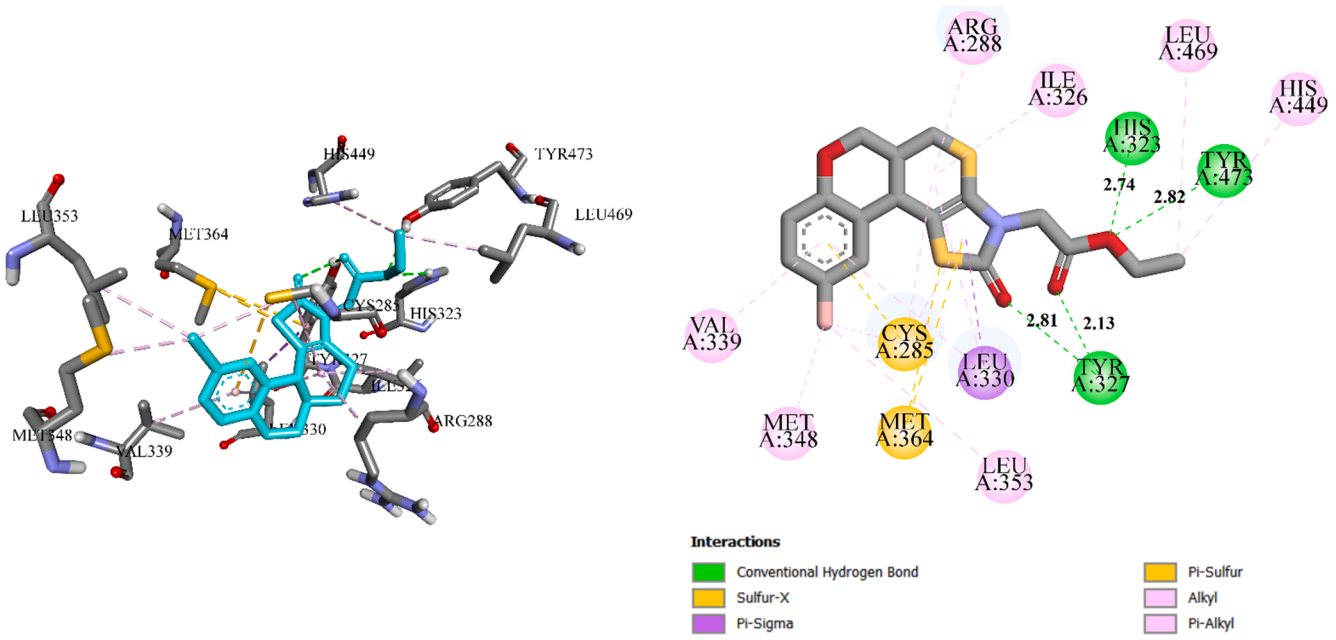

Fig. 7. Les-2769 interactions with the PPARγ receptor (PDB code: 5YCP).

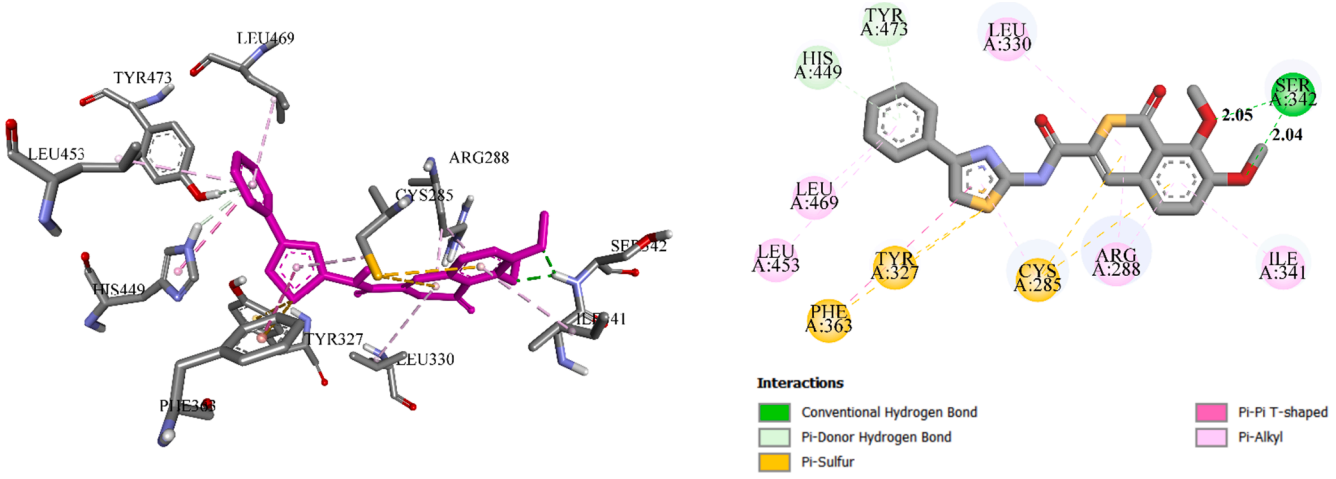

Fig. 8. Les-3266 interactions with the PPARγ receptor (PDB code: 5YCP).

t.edu.cn/admetsar2/). The calculated prognostic ADMET parameters are presented in Table 3. The results indicate that all the synthesized compounds, i.e. Les-2769, Les-3266, and the reference molecules, theoretically could be active upon oral administration and possess high cytochrome (CYP) inhibitory promiscuity. The acute oral toxicity prediction indicates that all studied molecules may belong to the III category of toxicity ("slightly toxic compounds" with LD_{50} values in the range of 500–5000 mg/kg). Interestingly, the predicted profiles of Les-2769 and Les-3266 are similar to rosiglitazone, whereas there are differences in terms of Caco-2 permeability and carcinogenicity with

Table 3
Calculations of ADMET profiles for Les-2769, Les-3266, and reference molecules rosiglitazone and GW9662.

Compound	BBB[a]	Hum In Ab[b]	Caco-2 *P*[c]	LogS[d]	CYP Inh Prom[e]	Carcino-genicity	AOT[f]
Les-2769	+	+	−/0.537	−4.350	High	Non	III
Les-3266	+	+	−/0.529	−3.886	High	Non	III
Rosiglitazone	+	+	−/0.545	−3.238	High	Non	III
GW9662	+	+	+/0.632	−4.526	High	Carcinogens	III

[a] Blood-Brain Barier.
[b] Human Intestinal Absorption.
[c] Caco-2 Permeability LogPapp, cm/s.
[d] Aqueous solubility.
[e] CYP Inhibitory Promiscuity.
[f] Acute Oral Toxicity.

GW9662.

4. Discussion

Cancer diseases have been a serious problem for society for centuries. Due to drug resistance in cancer treatment, it is invariably important to develop new strategies [41]. Given their scaffolding properties and the interaction of pharmacophoric substituents with the biological system, 4-thiazolidinones and 4-thiazolidinone-based derivatives are of great interest nowadays [11]. A confirmation of the progress in the field of 4-thiazolidinones intended for cancer treatment are patents granted by the World Intellectual Proprietary Organization (WIPO) and the United State Patent Trademark Office (USPTO) [11]. According to research, 4-thiazolidinones and conjugates based on 4-thiazolidinones have anti-tumor activity [8,42–49].

In the first part of our study, we analyzed the cytotoxic effect of two 4-thiazolidinone-based compounds, namely the chromeno[4′,3′:4,5] thiopyrano[2,3-*d*][1,3]thiazole derivative (Les-2769) and the isothiocoumarin-3-carboxylic acid derivative (Les-3266) on the human cancer SCC-15 cell line and healthy BJ cells. These compounds were selected on the basis of their identified micromolar cytotoxic activity towards leukemia, colon cancer, CNS, and ovarian cancer cell lines following the NCI 60-line protocol. Our study showed that Les-2769 (in a wide range of concentrations) and Les-3266 (only in the micromolar concentrations) decreased the metabolic activity in the BJ cells after 24 and 48 h. On the other hand, a decrease in the metabolic activity of Les-2769 and Les-3266 was observed in a concentration range of 10–100 μM in the SCC-15 cell line. To date, Skóra et al. (2022) reported a decrease in the metabolic activity of BJ cells induced by other 4-TZD-based derivatives such as 2-{2-[3-(benzothiazol-2-ylamino)-4-oxo-2-thioxothiazolidin-5-ylidenemethyl]-4-chlorophenoxy}-N-(4-methoxyphenyl)-acetamide (Les-3166), 5-fluoro-3-[2-(4-hydroxyphenylamino)-4-oxo-4*H*-thiazol-5-ylidenemethyl]-1*H*-indole-2-carboxylic acid methyl ester (Les-6166), 7-oxa-10-thia-8-aza-cyclopenta[*b*] phenanthren-9-one (Les-5935), and 5-fluoro-3-(4-oxo-2-thioxothiazolidin-5-ylidenemethyl)-1*H*-indole-2-carboxylic acid methyl ester (Les-6009) [50] (Fig. 9). Szychowski et al. (2021) proved that, among the tested 4-TZD-derivatives (Les-2194, Les-3377, and Les-3640), only Les-3377 (in a concentration of 10 μM) decreased the metabolic activity in the BJ cell line, which is comparable to the decrease in the metabolic activity caused by compound Les-2769 in this study [29]. Interestingly, Finiuk et al. (2017), described the cytotoxicity of the 4-TZD derivative 5-bromo-3-{2-[5-(4-methoxyphenyl)-3-naphthalen-2-yl-4,5-dihydropyrazol-1-yl]-4-oxo-4,5-dihydro-1,3-thiazol-5-ylidene}-2,3-dihydro-1*H*-indol-2-one (Les-3833) in human keratinocytes (HaCaT) was lower (IC_{50} = 3.16 μg/mL) than that of doxorubicin (IC_{50} = 0.47 μg/mL) [42]. Reduction of metabolic activity and an increase in cytotoxicity (IC_{50}) by 4-TZD has been demonstrated in a wide variety of human cancer cell lines [42,50,51]. Treatment with such 4-TZD-derivatives as 5Z-(4-fluorobenzylidene)-2-(4-hydroxyphenylamino)-thiazol-4-one (Les-236) reduced SCC-15 cell metabolism (by 50%) at micromolar (1-100 μM) concentrations [52]. In the SCC-15

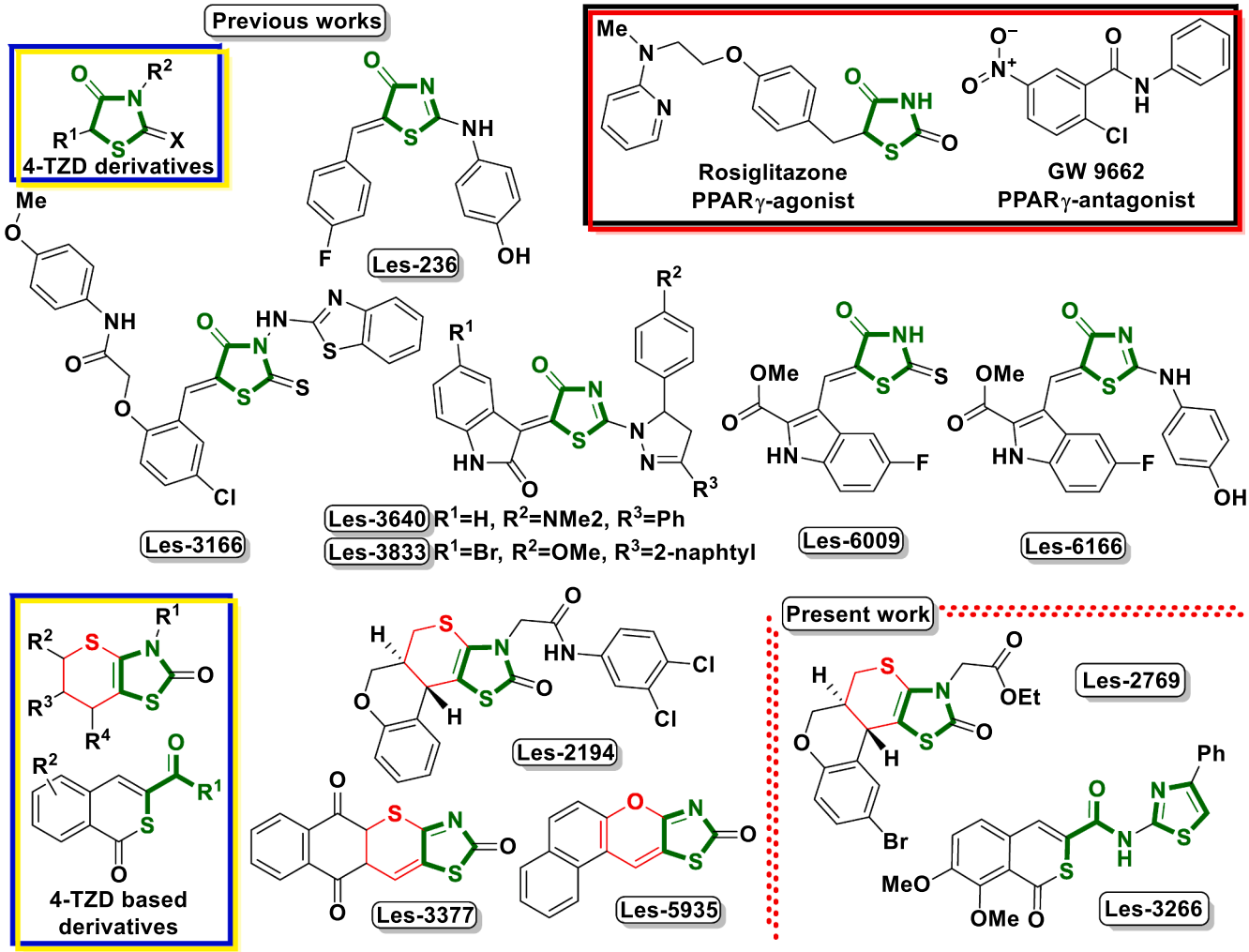

Fig. 9. Structure of 4-TZD and 4-TZD-based derivatives used in the present study.

cell line Les-2194, Les-3377, and Les-3640 compounds in concentrations of 50–100 μM decreased the cell metabolism after 24-h exposure [28]. The use of lower concentrations of Les-2194 and Les-3640 in the range of 1-10 μM did not affect the metabolism of SCC-15 cells [28,53].

Our experiments in the BJ cells showed an increase in LDH release only after the 48-h exposure to the highest concentrations of Les-2769 (50–100 μM) and Les-3266 (10–100 μM) (Fig. 10). However, in the SCC-15 line, Les-3266 and Les-2769 induced LDH release at the high micromolar concentrations of 10–100 μM (for Les-3266) and 100 μM (for Les-2769). To date, an increase in the LDH release of 4-TZD-derivatives on healthy BJ cells was observed for Les-236 (10–100 μM) [52]. However, Les-236 was much more toxic and caused LDH release in SCC-15 cells (1-100 μM), compared to normal cells [52]. An μM-range increase in LDH release in SCC-15 cells was observed after exposure to Les-2194, Les-3640, and Les-3377 [28]. Other studies reported that TZD-derivatives induced LDH release from murine glioma (GL261), rat glioma (C6), human colorectal adenocarcinoma (HT29), and human colon carcinoma (COLO-205) cells [54,55].

To assess the mode of cell death, caspase-3 activity as a well-established marker of apoptosis was chosen. Activation of caspase-3 was only observed at the highest concentrations of both compounds in BJ (after 48 h); however, a stronger impact on this parameter was observed in the Les-3266-treated cells. A similar relationship was also reported by e.g. Skóra et al. and Szychowski et al. in the same cell model treated with other 4-TZD-derivatives (Les-3166, Les-6166, Les-6009, Les-2194, and Les-3377) [29,50]. In turn, the same effect on caspase-3 activity was observed in normal mouse fibroblasts (3 T3-L1), which were characterized by an increase in this parameter after treatment with Les-3640 and Les-6166 [56]. This shows the ability of the new 4-TZD-based derivatives to induce active apoptosis in normal cells, which may limit the usefulness of such compounds in anticancer treatment. In our study, the SCC-15 cells were characterized by the same dose- and time-dependent correlation in the caspase-3 activity induction. An increase in this parameter was noticed only at the 10–100 μM concentrations of the compounds, with a stronger increase in caspase-3 activity after the cell treatment with Les-3266 (after the 24-h exposure). As reported by Szychowski et al. (2017), the Les-2194, Les-3640, and Les-3377 4-TZD compounds tested in the SCC-15 cells line at high micromolar concentrations showed an increase in caspase-3 activity, but only Les-3640 was able to induce apoptosis at lower concentrations [29,30]. The tendency to induce apoptosis by Les-236 over time was consistent with the results obtained in the present study [52]. Moreover, as described in the literature, the effect of certain new 4-TZD derivatives may be ROS-dependent or ROS-independent and may be dependent on caspase-8 and -9, which clearly shows that each new 4-TZD derivative should be tested comprehensively to fully elucidate its mechanism of action and potential usefulness in anticancer therapy [42,50,57]. As shown above, both Les-2769 and Les-3266 are able to induce cytotoxicity in cancer cells *in vitro* and trigger apoptosis; however, since the effect was observed only at the micromolar concentrations, we decided to determine the potential PPARγ-dependent effect at the gene level in the further parts of this study.

Our molecular docking simulations show the potential interaction between the studied compounds (Les-2769 and Les-3266) and the PPARγ receptor. The analyses of Les-2769 and Les-3266 show that these compounds are characterized by similar affinity for PPARγ as rosiglitazone (positive control during the analysis; agonist). Based on literature reports, induction of the activity of PPARγ by rosiglitazone can lead to a decrease in *inter alia* oxidative stress by translocation of this receptor to the nucleus and, in consequence, induction of certain genes (*HO-1, SOD, CAT, GPx*, and others) [58]. Therefore, we suppose that both Les-2769 and Les-3266 will decrease the expression of certain genes, analogically to rosiglitazone. Therefore, the further analysis focused on elucidation of the PPARγ-related mechanism of action of Les-2769 and Les-3266 with the agonist (rosiglitazone) and antagonist of this receptor (GW9662) using the resazurin reduction assay. The results revealed a possible interaction of Les-3266 with the PPARγ receptor in both BJ and SCC-15 cell lines. In our study, Les-3266 was characterized by higher cytotoxicity to the BJ and SCC-15 cells than the well-known PPARγ agonist (rosiglitazone). In turn, the cell co-treatment with GW9662 (antagonist) with Les-3266 resulted in an increase in the metabolic activity of the cells. This is consistent with the results described by Szychowski et al. (2017), who proved the influence of such 4-TZD-derivatives as Les-2194, Les-3377, and Les-3640 acting through the PPARγ pathway [30]. In addition, the researchers concluded that the tested 4-TZD-derivatives acted as PPARγ agonists, which is consistent with our results [30]. However, since cell metabolism increases after the application of GW9662, we cannot exclude that Les-3266 also acts through other molecular pathways.

As revealed by the gene expression analysis, 10 μM Les-3266 increases the *PPARγ* gene expression in BJ. This is in line with the results obtained by Szychowski et al. (2019), who demonstrated an increase in *PPARγ* mRNA expression in BJ cells after treatment with Les-236 [52]. This was most likely a result of post-activation degradation of the PPARγ receptor. In turn, an increase in the *PPARγ* expression in the case of SCC-15 was induced by both 4-TZD derivatives tested by our team. The analyzed Les-2769 and Les-3266 most likely interacted with the PPARγ pathway. An increase in PPARγ protein expression was demonstrated by Szychowski et al. (2021); it was induced by the interaction of 10 μM Les-3377 and Les-3640 in SCC-15 cells (after 24 h) [29]. A reverse effect, i.e. reduced *PPARγ* mRNA expression, was observed after treatment of SCC-15 with 10 μM of Les-236 (after 6 h) and 10 μM of Les-2194, Les-3377,

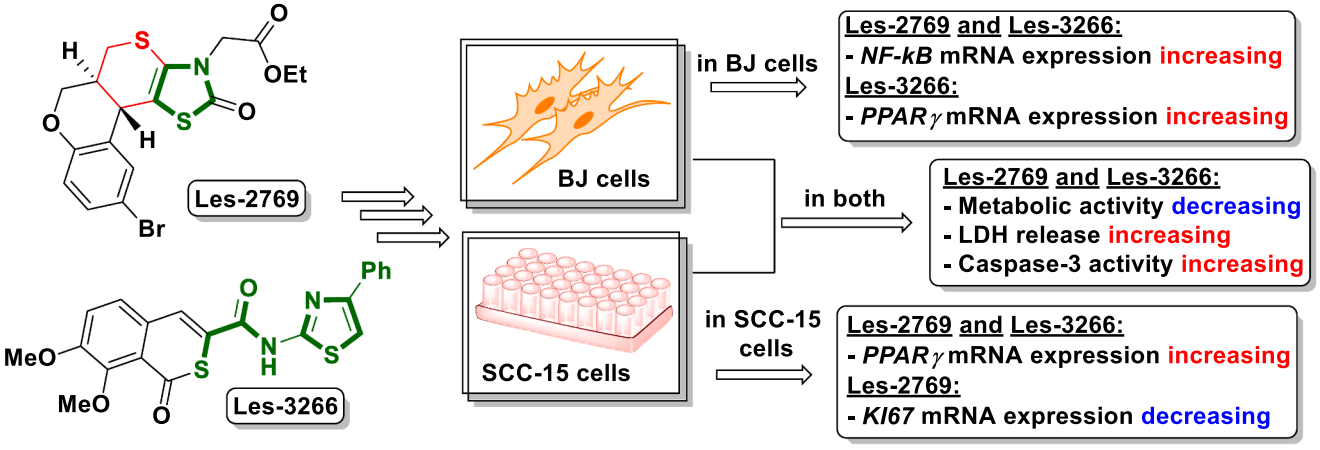

Fig. 10. Summary of the evaluation of the cytotoxic effect and mechanisms of action of Les-2769 and Les-3266.

and Les-3640 (after 4 h) [30,52]. This was most likely related to the shorter time of cell exposure to the tested compounds and the different cellular effects of the action of the 4-TZD derivatives. In our study, the co-treatment of the BJ cells with Les-2769 and rosiglitazone decreased the PPARγ mRNA expression, in comparison to cells treated with Les-2769 alone. However, the co-treatment of the BJ cells with Les-3266 and GW9662 resulted in an increase in the *PPARγ* mRNA expression, in comparison to Les-3266. In contrast, an opposite effect of the Les-2769 and Les-3266 exposure was found in the SCC-15 cell line *versus* the BJ cell line. Thus, the cell co-treatment with Les-2769 and GW9662 increased the *PPARγ* mRNA expression, compared to the Les-2769 alone variant, and the cell co-treatment with Les-3266 and GW9662 decreased the *PPARγ* mRNA expression, compared to the Les-3266 alone exposure. These data suggest that both compounds (Les-2769 and Les-3266) acts through the PPARγ pathway.

The present analyses revealed changes in the expression of the *KI67* gene in the BJ cells, i.e. rosiglitazone, Les-2769, and Les-3266 alone produced a decrease in *KI67* mRNA. Moreover, the co-treatment of the BJ cells with Les-2769 and rosiglitazone led to a decrease in the *KI67* mRNA expression, in comparison to the Les-2769 alone variant. Interestingly, the co-treatment of the BJ cells with Les-3266 and rosiglitazone and Les-3266 with GW9662 did not change the *KI67* mRNA expression, in comparison to the control. As reported by Szychowski et al. (2019), the analyzed 4-TZD (Les-236) reduced the expression of protein *KI67* in BJ [52]. The decrease in KI67 protein expression in BJ after co-treatment of the cells with both Les-236 with rosiglitazone and Les-236 with GW9662 [52] is consistent with the present data. In the SCC-15 line, Les-2769 induced a decrease in the expression of the *KI67* gene. Moreover, the co-treatment with rosiglitazone and Les-2769 caused an increase in the *KI67* mRNA expression in SCC-15. However, rosiglitazone did not affect the KI67 mRNA expression in cells co-treated with rosiglitazone and Les-3266, in comparison to cells treated with Les-32966 alone. To date, Szychowski et al. (2019) have described that cell co-treatment with Les-236 and rosiglitazone increased the KI67 protein expression, compared to the control and the Les-236 alone treatment, in SCC-15 [52]. This suggests similar mechanisms of action.

Our study shows that both Les-2769 and Les-3266 increased the *NF-κB* mRNA expression in the healthy BJ cells and the SCC-15 tumor cells. According to the literature, *NF-κB* can promote or inhibit the process of cell apoptosis, which depends on the cell type and the inducer [59]. In addition, by interacting with such a transcription factor as *NF-κB* through the mechanism of inhibition of the *NF-κB* pathway (*NF-κB* antagonism), *PPARγ* consequently has pro-apoptotic effects [60]. A study conducted by Neri et al. (2011) showed that the increase in *NF-κB* activation by microparticles (MP) in epithelial lung carcinoma (A549) was inhibited by *PPARγ* agonists, such as rosiglitazone, and the effect was reversed by such a *PPARγ* receptor antagonist as GW9662 [61]. In our study, the co-treatment of the BJ cell line with Les-2769 and rosiglitazone led to a decrease in *NF-κB* mRNA, compared to the Les-2769 alone exposure, which was similar to the *PPARγ* mRNA expression. In turn, in the SCC-15 cells, the antagonist decreased *NF-κB* but the agonist increased *NF-κB* mRNA expression, compared to the Les-2769 alone variant, which is a mirror image of the *PPARγ* mRNA expression and is consistent with the theory about the antagonistic interaction between PPARγ and NF-κB pathways. Finally, in the BJ cells, both the agonist and the antagonist of PPARγ potentiated the *NF-κB* mRNA expression initiated by Les-3266. However, the *NF-κB* mRNA expression in the SCC-15 cells stimulated by Les-3266 was inhibited only by the PPARγ agonist – GW9662.

5. Conclusion

Our results showed a cytotoxic effect of both 4-TZD-based derivatives (Les-2769 and Les-3266) on the BJ and SCC-15 cells measured with the use of the metabolic activity, LDH release, and caspase-3 activity assays. Les-2769 increased the expression of the *NF-κB* gene (in the BJ and SCC-15 cell lines) but decreased the expression of *PPARγ* (in the BJ cell line) and *KI67* (in the BJ and SCC-15 cell lines). In turn, Les-3266 increased the expression of *PPARγ* (in the BJ cell line) and *NF-κB* mRNA (in the BJ and SCC-15 cell lines) but decreased the *KI67* mRNA expression only in the BJ cell line. Moreover, the data showed that both compounds could potentially interact with the PPARγ receptor pathway. The molecular docking simulations confirmed the experimental *in vitro* data about the potential agonistic activity of Les-2769 and Les-3266 towards the PPARγ receptors. The ADMET profiles of Les-2769 and Les-3266 predicted using the admetSAR portal are similar to that of rosiglitazone, whereas there are differences in terms of Caco-2 permeability and carcinogenicity with GW9662. For full elucidation of the mechanism of action of Les-2769 and Les-3266, more studies are needed, including *in vivo* conditions. Although the cytotoxic effect of the tested 4-TZD derivatives on the SCC-15 line was observed only at the highest micromolar concentrations, which may indicate its limited potential for medical use, it would be useful to investigate the effect of Les-2769 and Les-3266 applied at lower concentrations on other types of tumor cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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