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PPAR-γ agonist GL516 reduces oxidative stress and apoptosis occurrence in a rat astrocyte cell line

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ABSTRACT

Aims: The worldwide increase in aging population is prevalently associated with the increase of neurodegenerative diseases. Peroxisome Proliferator-Activated Receptors (PPARs) are ligand-modulated transcriptional factors which belong to the nuclear hormone receptor superfamily which regulates peroxisome proliferation. The PPAR-γ is the most extensively studied among the three isoforms and the neuroprotective effects of PPAR-γ agonists have been recently demonstrated in a variety of preclinical models of neurological disorders. The aim of the study is to biologically evaluate the neuroprotective effects of new PPAR-γ selective agonists in an in vitro model.

Main methods: CTX-TNA2 rat astrocytes were treated with G3335, a PPAR-γ antagonist, to simulate the conditions of a neurological disorder. Newly synthetized PPAR-γ selective agonists were added to the cell culture. Cytotoxicity was assessed by MTT assay, catalase activity was investigated by a colorimetric assay, Reactive Oxygen Species (ROS) production and apoptosis occurrence were measured by flow cytometry. Western blotting were performed to measure the levels of protein involved in the apoptotic pathway.

Key findings: Four PPAR-γ agonists were selected. Among them, the GL516, a fibrate derivative, showed low cytotoxicity and proved effective in restoring the catalase activity, reducing ROS production and decreasing the apoptosis occurrence triggered by the G3335 administration. The effects of this molecule appear to be comparable to the reference compound rosiglitazone, a potent and selective PPAR-γ agonist, mainly at prolonged exposure times (96 h).

Significance: Based on recent evidence, hypofunctionality of the PPAR-γ in glial cells could be present in neurodegenerative diseases and could participate in pathological mechanisms through peroxisomal damage. The fibrate derivative PPAR-γ agonist GL516 emerged as the most promising molecule of the series and could have a role in preventing the pathophysiology of neurodegenerative disorders.

1. Introduction

The increase in the aging population is associated with the prevalent increase of neurodegenerative diseases, characterized by the progressive dysfunction, deterioration, and eventual loss of neurons in the nervous system.

The aetiology of different neurodegenerative diseases may vary, and many diseases exhibit a combination of both genetic and environmental aetiological factors (Sonntag, 2010). The complex manifestations of different diseases make it difficult to suggest a unifying mechanism for disease initiation and progression. However, there are recognized common features suggesting similar signaling cascades for these pathological conditions. Current hypotheses for the onset of neurodegenerative disorders include, in addition to genetics, selective vulnerabilities, aberrant protein structure, mitochondria dysfunction, oxidative stress, programmed cell death, inflammation/immune imbalance, and others (Haas, 2010; Jellinger 2010, 2012).

Peroxisome Proliferator-Activated Receptors (PPARs or NR1Cs) are ligand-modulated transcriptional factors belonging to the nuclear hormone receptor superfamily. There are three PPAR subtypes, namely PPAR-α, PPAR-β/δ and PPAR-γ, which have different tissue distribution, ligands and co-activators or co-repressors. The PPAR-α plays an important role in lipid metabolism; the PPAR-β/δ regulates fatty acid catabolism, insulin sensitivity, and energy homeostasis in muscle and adipose tissue; the PPAR-γ is involved in lipid metabolism, adipogenesis and inflammation (Abushouk et al., 2017).

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Aside from mediating peroxisome proliferation, and other metabolic effects, PPAR activation is also involved in other physiological events such as inflammation, oxidative stress, apoptosis and cell proliferation (Mandrekar-Colucci et al., 2013; Leporini el al. 2017).

These pleiotropic effects overlap with many of the hypotheses underlying the pathogenesis of neurodegeneration, ascribing to PPAR agonists a role as protective agents in neurological diseases. In fact, several studies demonstrated the potential use of the PPAR agonists as neuroprotective agents (Di Cesare Mannelli et al., 2014).

The PPAR-γ is the most extensively studied among the three isoforms; during the past decade, many studies have demonstrated the neuroprotective effects of its agonists in a variety of preclinical models of neurological disorders (Shie et al., 2009; Carta, 2013; Procaccio et al., 2014).

Since a common feature of several neurodegenerative diseases is inflammation occurrence (Hunter and Bing, 2007), the theoretical basis of a PPAR-γ therapeutic approach in neurodegenerative disorders is generally founded on the anti-inflammatory effect. A recent evidence has shown that the hypofunctionality of this receptor in glial cells could be present in neurodegenerative diseases and it could participate in pathological mechanisms through the peroxisomal damage (Di Cesare Mannelli et al., 2014).

Hydrogen peroxide (H_2O_2) is ascribed to Reactive Oxygen Species (ROS), and can be formed by the dismutation of $O_2^{\cdot -}$ via the hydroperoxyl radical. Although H_2O_2 is not harmful, its conversion, through the Fenton reaction catalyzed by metal ions, generates the hydroxyl radical (·OH), probably the most highly reactive and toxic form of oxygen (Moldovan and Moldovan, 2004). Catalase is a heme-containing peroxisomal enzyme that breaks down hydrogen peroxide to water and oxygen and is part of the main antioxidant defense molecular downstream (Powers and Jackson, 2008). De Duve and Baudhuin (1966) first described a respiratory pathway in peroxisomes in which electrons removed from various metabolites reduce O_2 to H_2O_2, which is further neutralized to H_2O. The high peroxisomal consumption of O_2 together with the demonstration of the production of H_2O_2, $O_2\cdot -$, ·OH, and recently of NO (Stolz et al., 2002), as well as the discovery of several ROS metabolizing enzymes in peroxisomes have supported the notion that these ubiquitous organelles play a key role in both the production and scavenging of ROS inside the cell (Schrader and Fahimi. 2006).

It is widely known that oxidative stress can lead to apoptosis occurrence. In this regard, PPAR-γ activation was demonstrated to be effective in inhibiting the apoptotic pathway in a rat model of Parkinson's disease (Tong et al., 2016).

In the search for new agonists, we previously reported the synthesis of fibrates and tyrosine derivatives capable of PPAR activation (Giampietro et al., 2012; De Filippis et al., 2015). Some of them showed a notable PPAR agonistic activity. In order to improve our knowledge of their pharmacological profile and being PPAR-γ agonists a novel class of drugs for treating neuroinflammatory diseases, we selected the best PPAR-γ agonists to evaluate their neuroprotective potential effect.

The cell viability, the catalase activity, the ROS production and the occurrence of apoptosis were investigated in an astrocyte cell line treated with these selected PPAR-γ agonists following the exposure to a PPAR-γ antagonist.

2. Materials and methods

2.1. Cell culture

CTX-TNA2 rat astrocyte cell line was purchased from the European Collection of Cell Cultures (cat. 98102213, ECACC, Sigma-Aldrich, St Louis, MO, USA) and maintained in DMEM (cat. ECM0728L) supplemented with 10% of FBS (cat. ECS0180L) and penicillin-streptomycin (100 μgmL^{-1}) (cat. ECB3001D, all from EuroCloneSpA Life-Sciences-Division, Milano, Italy) according to the EACC's instructions. Cell were grown at 37 °C in a humified atmosphere of 5% CO_2.

2.2. Cell viability assay

Cell viability was measured by MTT (3 [4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) growth assay (cat. M2128, Sigma-Aldrich) as previously described (Sancilio et al., 2016). Cell number was quantified by the amount of tetrazolium reduction in viable mitochondria. Cultured cells were seeded into 96-well plate at 5×10^4 cells/well and exposed to 30 μM concentration of the antagonist G3335 and to 100 μM of each of the agonist compounds for 48 and 96 h to compare the cytotoxicity of the four agonists. The reference concentrations were selected according to a previous paper about PPAR activation in primary astrocytes (Di Cesare Mannelli et al., 2014).

Afterwards, another set of MMT assay was performed at the same experimental points in order to select the best ligand concentrations in our experimental model. The optimazed concentrations were assessed at 110 μM for GL516 and at 40 μM for G3335. At each experimental time cells were processed according to the manufacturer's instructions and the absorbance of each sample was detected at 570 nm of wavelength. Percentage of viable cells was calculated using the equation A_s/A_o x 100 where A_s is the absorbance value obtained for a sample containing cells in the presence of a given concentration of agent, and A_0 is the absorbance value of vehicle treated control. Four independent experiments were performed under the same experimental conditions.

2.3. Catalase activity

The catalase activity was measured at the same experimental points seen for MTT assay by a colorimetric assay using the Amplex Red Catalase Assay kit (cat. A22180, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) and following the manufacturer's instructions. The absorbance of each experimental point was detected at 560 nm and subtracted of the value of the no-catalase control. The values of the catalase activity ($mUmL^{-1}$), calculated by means of the standard curve, were normalized to the total amount of protein (μgmL^{-1}) measured by QuantiPro BCA assay kit (cat. QPBCA, Sigma-Aldrich).

2.4. Flow cytometry analysis of reactive oxygen species (ROS) production

Reactive Oxygen Species production was determined by monitoring by flow cytometry the increase of green fluorescence after labeling the cells (5×10^5) with 5 $\mu molL^{-1}$ of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, cat. C6827, Molecular Probes, Invitrogen, Life-Sciences-Division, Milano, Italy) for 1 h at 37 °C, as already described (Cataldi et al., 2013). CM-H_2DCFDA passively diffused into cells, where its acetate groups were cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacted with intracellular glutathione and other thiols. When ROS were produced, CM-H_2DCFDA was oxidized and an increase in green fluorescence was detected by a Cytoflex cytometer with an FL1 detector in a log mode using the Cytoexpert software (Beckmann Coulter, FL, USA). The mean fluorescence intensity (MFI) ratio was obtained by histogram statistics and provided to quantify ROS production. Dead cells were excluded from analysis by Propidium Iodide (PI) staining (5 μgmL^{-1}) (cat. P4864, Sigma-Aldrich). At least 15000 events for each sample were acquired.

2.5. Annexin-V/PI detection of apoptotic cells in flow cytometry

To assess apoptosis, a commercial Annexin-V-FITC/PI Kit (cat. BMS500FI/300CEE, Bender Med System, Vienna, Austria) was used according to the manufacturer's instructions, as previously detailed (di Giacomo et al., 2013). Briefly, the cells were gently re-suspended in binding buffer and incubated for 10 min at room temperature in the dark with 5 μl of Annexin-V-FITC. Samples were then washed and supravitally stained with propidium iodide (PI) (5 μgmL^{-1}) (cat. P4864,

Sigma-Aldrich). Analyses were performed with a Cytoflex flow cytometer with the FL1 and FL3 detector in a log mode using the Cytoexpert analysis software (Beckmann Coulter). For each sample, 10000-20000 events were collected. Necrotic cells were Annexin-V^{neg}/PI^{pos}, while early and late apoptotic cells were Annexin-V^{pos} and PI^{neg} and PI^{pos}, respectively.

2.6. Western blotting analysis

Total cell lysates (60 μg) were electrophoresed on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred to nitrocellulose membrane. Nitrocellulose membranes, blocked in 5% non-fat milk, and BSA 10 $mmolL^{-1}$ Tris-HCl pH 7.5, 100 $mmolL^{-1}$ NaCl, 0.1% Tween-20, were probed with mouse anti-β actin monoclonal antibody (dilution 1:5000, cat. A5316, Sigma-Aldrich), as previously described (di Giacomo et al., 2010), mouse anti-Bax (1:200, cat. sc-7480), goat anti-Caspase 3 (1:200, cat. sc-1225) and rabbit polyclonal anti-PARP-1 (1:200, cat. Sc-7150, all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then incubated in the presence of specific enzyme-conjugated IgG horseradish peroxidase. Immunoreactive bands were detected by ECL detection system (cat. EMP013001, EuroCloneSpA) and analysed by densitometry. Densitometric values, expressed as Integrated Optical Intensity (I.O.I.), were estimated in a CHEMIDOC XRS system by the QuantiOne 1-D analysis software (BIORAD, Richmond, CA, USA). Values obtained were normalized basing on densitometric values of internal β actin.

2.7. Statistical analyses

Three independent experiments were performed under the same experimental conditions for each experimental setup with the exception of MTT assay where the independent replicates were four. Statistical analyses were performed using the analysis of variance (ANOVA). Results were expressed as mean ± SD. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. MTT assay

The metabolic activity of the CTX-TNA2 rat astrocyte cell line was evaluated by MTT assay following the exposure to four different PPAR-γ agonists (Fig. 1).

Either the two tyrosine based ligands DF1032 and DF1026, or the fibrate derivative GL535 affect cell metabolism at least after longer exposure periods (96 h), while the GL516 seems to have no effects. All PPAR-γ agonists were administered at a concentration of 100 μM, while the concentration of the antagonist G3335 was 30 μM (Fig. 2a). Since MTT assay can be considered an indirect measure of cytotoxicity, the GL516 was chosen to be further investigated because of its low cytotoxicity. Another set of MTT assays was performed administering the compounds in combination, in order to select the appropriate ligand concentrations, which were assessed at 110 μM for GL516 and at 40 μM for G3335. When administered at the concentration of 110 μM, GL516 is able to increase the cell viability, in a manner similar to the reference compound rosiglitazone (Fig. 2b).

3.2. Catalase activity

The influence of the four different PPAR-γ agonists on the catalase activity of CTX-TNA2 rat astrocytes was tested by an ELISA assay (Fig. 3a). All the new PPAR-γ ligands, administered at the concentration of 100 μM, proved to be effective in restoring the enzymatic activity disrupted by the treatment with 30 μM G3335 antagonist, after 96 h of exposure, in a measure similar to the reference compound, rosiglitazone (R).

The optimized GL516 concentration (110 μM), previously determined according to MTT data, was tested for its effects on catalase activity. GL516 seems to be ineffective as regard catalase activity after 48 h, whereas it appears to increase the enzymatic activity with respect to the sample exposed to G3335 (40 μM), if the exposure is prolonged for additional 48 h (Fig. 3b).

Based on the results of MTT and catalase activity assays, the following parameters were evaluated exposing the cells to the optimized concentrations, i. e. 110 μM for the agonist and 40 μM for the antagonist.

3.3. Oxidative stress and apoptosis

Reactive Oxygen Species (ROS) production shows no significative differences after a 24 h treatment both with G3335 alone and in co-treatment with PPAR-γ agonist GL516. After 72 h of exposure, the PPAR-γ antagonist G3335 generates oxidative stress into the cells, promptly reduced by GL516 (Fig. 4a).

AnnexinV/PI flow cytometry apoptosis assay reveals an increase in the number of early (Ann^{pos}/PI^{neg}) apoptotic cells when they are exposed to G3335 for 72 h and necrosis induction when the treatment is prolonged to 96 h. The fibrate derivative GL516 is able to revert the situation to values similar to controls (Fig. 4b).

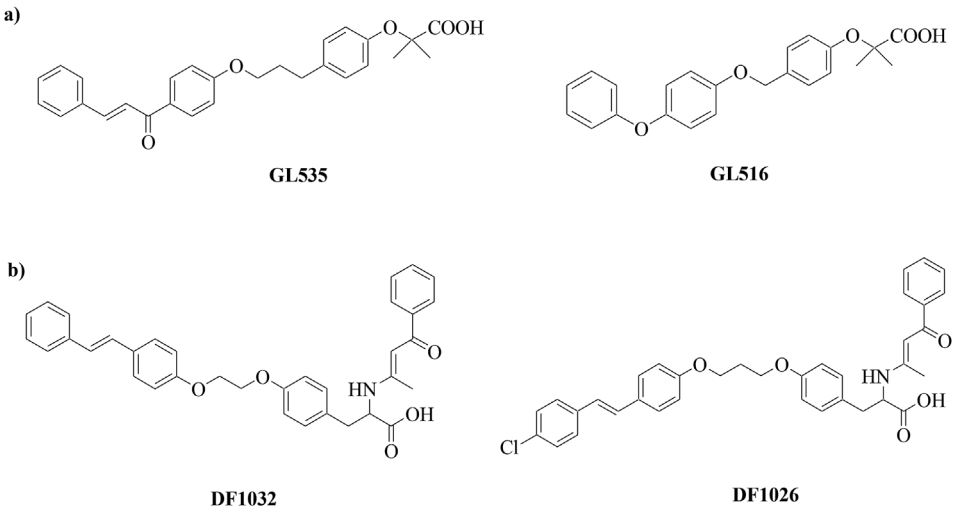

Fig. 1. Chemical structures of a) fibrates derivatives, b) tyrosine derivatives.

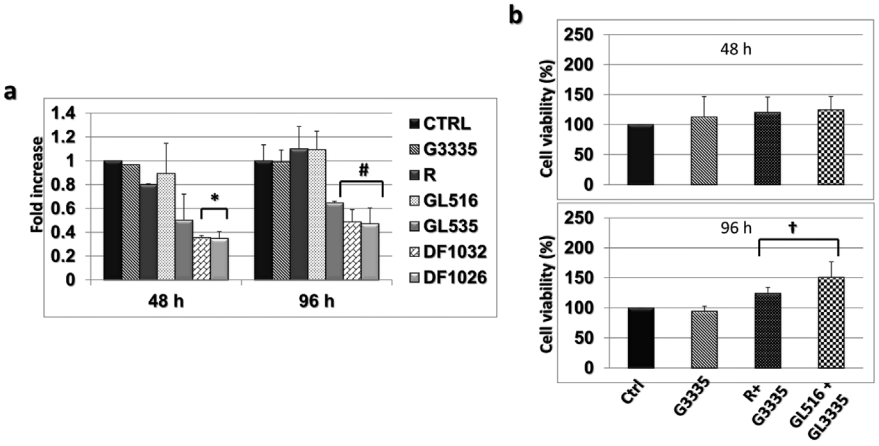

Fig. 2. MTT assay in CTX-TNA2 rat astrocyte cell line treated with a) four different PPAR-γ agonists (100 μM) and the antagonist G3335 (30 μM) b) GL516 (110 μM) in combination with the antagonist G3335 (40 μM) for 48 (above) and 96 h (below).
R: rosiglitazone
* DF1032 and DF1026 $p < 0.05$ vs Ctrl 48 h
# GL535, DF1032, DF1026 $p < 0.05$ vs Ctrl 96 h
† R + G3335, GL516 + G3335 $p < 0.05$ vs Ctrl

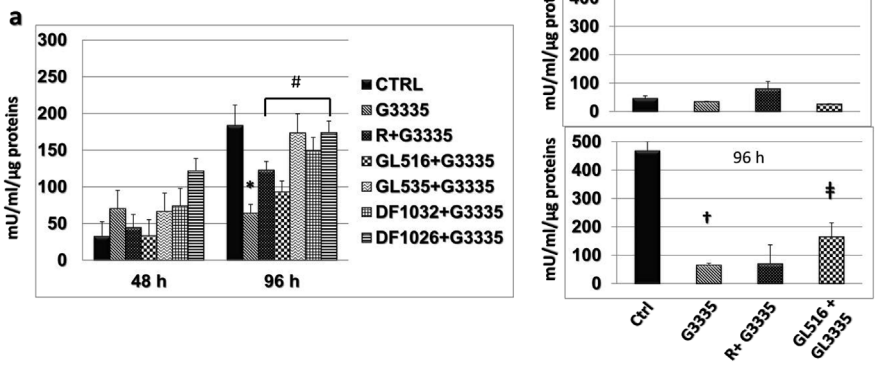

Fig. 3. Catalase activity of CTX-TNA2 rat astrocyte cell line when treated with a) four different PPAR-γ agonists (100 μM) and the antagonist G3335 (30 μM) b) GL516 (110 μM) in combination with the antagonist G3335 (40 μM) for 48 (above) and 96 h (below).
R: rosiglitazone
* G3335 $p < 0.05$ vs Ctrl 96 h
# $p < 0.05$ vs G3335 96 h
† G3335 $p < 0.05$ vs Ctrl
‡ GL516 + G3335 $p < 0.05$ vs G3335

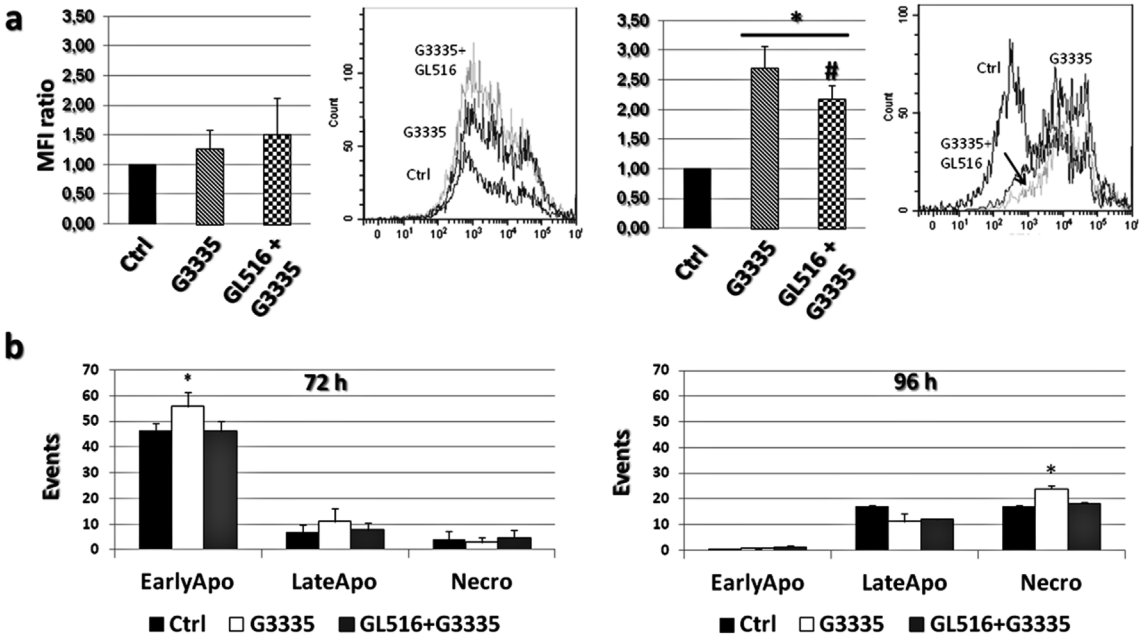

Fig. 4. Oxidative stress and apoptosis occurrence in CTX-TNA2 rat astrocyte cell line. a) Reactive Oxygen Species (ROS) production (at 24, left, and 72 h, right) MFI: Mean Fluorescence Intensity of the peaks
* G3335 and GL516 + G3335 $p < 0.05$ vs Ctrl
# GL516 + G3335 $p < 0.05$ vs G3335
b) AnnexinV/PI flow cytometry apoptosis assay (72 and 96 h)
*G3335 early apoptosis 72 h $p < 0.5$ vs Ctrl, GL516 + G3335 early apoptosis 72 h
* G3335 necrosis 96 h $p < 0.05$ vs Ctrl, GL516 + G3335 necrosis 96 h

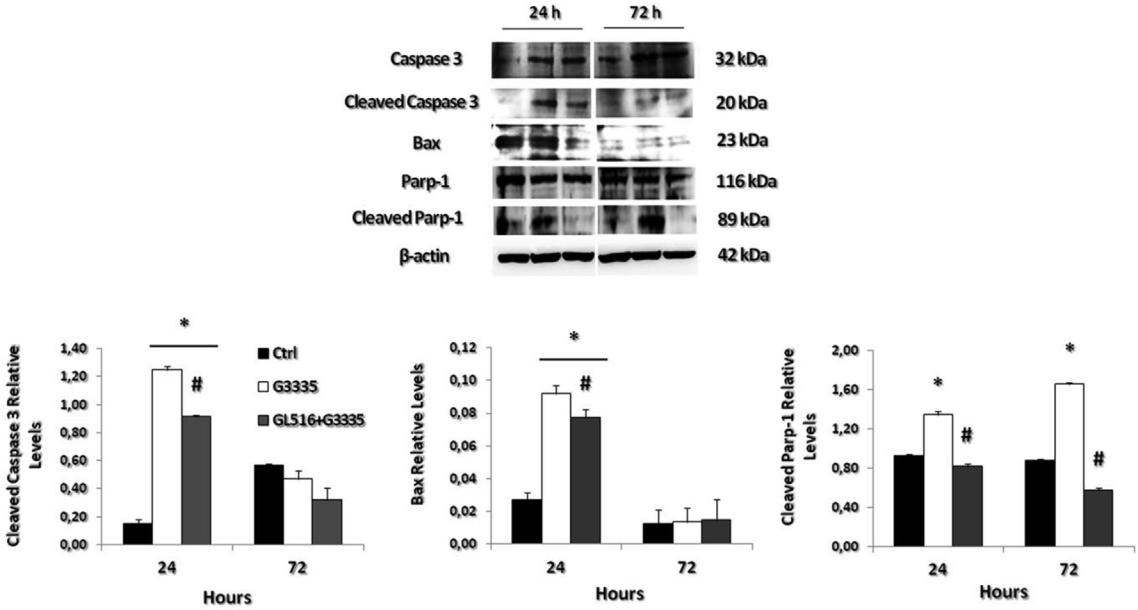

Fig. 5. Western blot analysis of Caspase 3, Bax and PARP-1 levels in CTX-TNA2 rat astrocyte cell line. Densitometric values (± SD) are expressed as Integrated Optical Intensity (IOI)
* treated cells $p < 0.05$ vs Ctrl
# GL516 + G3335 $p < 0.05$ vs G3335

3.4. Western blot analysis

After 24 h, the levels (Fig. 5) of all the three protein, namely cleaved Caspase 3, Bax and cleaved PARP-1are clearly augmented when cell are exposed to the PPAR-γ antagonist G3335, while the newly synthesized PPAR-γ agonist GL516 is able to induce a reduction in the levels of all the proteins taken under consideration. At longer experimental time, i. e. 72 h, no significant difference in protein levels is found for cleaved Caspase 3 and Bax, while cleaved PARP-1 level remains higher in the G3335 treated sample when compared to the other two experimental conditions.

4. Discussion

Neurodegenerative diseases are widely spread and affect a large percentage of the world population. Since a curative therapy is not available, novel therapeutic targets and/or active agents in neurodegenerative disease treatment are urgently needed. The nervous system is made of neurons and neuroglia cells. Among the latter, astrocytes are highly reactive cells which can cause neuron loss releasing damaging molecules (Bal-Price and Brown, 2001). The ability of astrocytes to promote inflammation and their responsiveness to PPAR agonists position these cells to play a critical role in the progression of neurological diseases and make them a suitable cell model for nervous abnormalities and a promising target for their treatment and prevention. It has been already demonstrated that PPAR agonists attenuate pathological astrocyte activation and modulate disease progression (Hong et al., 2012). Thus, four different newly synthesized PPAR-γ agonists were here tested on the astrocytes cell line CTX-TNA2, in comparison with the reference compound, rosiglitazone.

The new compounds appeared to restore the catalase activity when administered at the reference concentration of 100 μM after longer exposure times. The role of oxidative stress in the onset and progression of neurodegenerative diseases is widely known (Dai et al., 2017) and it due to the peculiar metabolism of brain cells which continuously generate peroxides. Brain cells, including astrocytes, are provided with high amounts of catalase and glutathione system for an efficient clearance of hydrogen peroxide (Dringen et al., 2005). Therefore, molecules able to regulate catalase, a key antioxidant enzyme, appear to have a critical role in the ongoing of these diseases. The results in the present paper are in alignment with data elsewhere reported by Di Cesare Mannelli and collaborators (2014) with a slight difference in time intervals. In the present experimental model the effect of PPAR-γ activation on G3335-induced catalase impairment is present mainly after four days of treatment, while in the other study (Di Cesare Mannelli et al., 2014) results were found at earlier exposure times. The reason for such dissimilarity could be found in the diverse metabolism of CTX-TNA2 astrocyte cell line and their primary counterpart. However, three of the new compounds, namely the two tyrosine derivatives DF1032 and DF1026, and the fibrate derivative GL535, showed an high cytotoxicity, reducing cell metabolic activity to less than 50%, even after only 48 h of exposure. Since many PPAR agonists are used in brain cancer treatment for their cytotoxicity (Ching et al., 2015; Wilk et al., 2015), we can suggest that such compounds have a functional group that, triggering antitumoral signaling, is able to overwhelm the PPAR-mediated catalase activity. In particular, the stilbene moiety present in the two tyrosine derivated compounds, DF1032 and DF1026, could be responsible for their cytotoxicity, as many stilbene containing compounds are known for having, among many biological properties, anticancer activity (De Filippis et al., 2017). As for the cytotoxicity exerted by GL535, the main difference in its chemical structure with respect to the non-toxic GL516, is the chalcone scaffold whose anticancer activity was proved to be exerted through the inhibition of various molecular targets (Mahapatra et al., 2015).

Anyway, being the cytotoxic potential of the compounds aimless for the present study, GL516 was selected for further analysis because of its low cytotoxicity and its ability to restore catalase activity as the reference compound rosiglitazone. The concentrations of both reversible antagonist G3335 and fibrate derivative agonist GL516 were slightly adjusted at the subtoxic dose as long as to obtain the best catalase activity restoration. The role of PPAR-γ receptors in modulating neurodegeneration is widely acknowledged, being the PPAR-γ agonists able to control inflammation and oxidative stress (Villapol, 2018). In the present in vitro model the G3335 antagonist seems somehow ineffective on the astrocyte cell line according to MTT data, but the increase in reactive oxygen species production, observed in the presence of the

PPAR-γ antagonist, demonstrated oxidative stress occurrence. The GL516 agonist was found able to reduce ROS production when administered with G3335, as similarly demonstrated for the PPAR-γ agonist pioglitazone in an in vitro model of ischemia (Xia et al., 2018). Being the MTT proliferation assay misleading in evidencing cell suffering from treatment with G3335, and, at the same time, having evidence of ROS production, a more accurate method for cell death detection was performed. The link between oxidative stress and apoptosis occurrence is well studied in many pathological conditions, including neurodegenerative diseases (Wu et al., 2014; Dai et al., 2016), therefore a flow cytometric apoptosis assay was performed. Not surprisingly, an increase in the early apoptotic cell percentage was found in the sample exposed to the antagonist G3335 for 72 h. When GL516 is added, CTX-TNA2 cells completely recovered their viability and the early apoptosis triggered by the G3335 exposure is completely absent. These data are consistent with findings already published in various disease models (Peng et al., 2017; Wang et al., 2015), including neurological conditions (Normando et al., 2016) where PPAR-γ agonists were found able to inhibit apoptosis. As for signaling molecules involved in the process, the levels of Bax and of the cleaved form of both Caspase 3 and PARP-1 found at early exposure times, are consistent with the results obtained in already investigated neuronal models (Chiang et al., 2016; Lim et al., 2003; Sharma et al., 2017) where the same molecules proved to be modulated by PPAR-γ activation.

At longer experimental times, i. e. 96 h, the cell treatment with G3335 results in necrosis occurrence in combination with a decrease in Caspase 3 cleavage whereas PARP-1 levels are persistent. These findings could be explained by the PARP-1 multifaceted roles in many cellular activities. While cleavage of PARP-1 by caspases (including Caspase 3) is considered to be a hallmark of apoptosis, the unchecked persistence of PARP-1 activity, due to an inadeguate caspase activation, could lead to necrotic cell death resulting from the prolonged ATP depletion (Chaitanya et al., 2010).

5. Conclusion

In conclusion, research in the last decade has uncovered the PPAR-γ receptors in most brain cell types, and has shown that their activation is involved in normal brain and cerebrovascular physiology, confering protection under pathological conditions. Notably, accumulating evidence has highlighted the therapeutic potential of PPAR-γ ligands in the treatment of brain disorders (Nikolakakis and Hamel, 2010; Swanson et al., 2011). Based on this evidence and on the results of the present study, GL516 appears to have a role in the prevention of neurodegenerative disorders. Further analysis will be focused on the study of the molecular mechanisms underlying the signaling cascade leading to cell recovery, and on feasible structure modifications able to optimize the PPAR-γ agonist neuroprotective effects.

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