Neuroscience 302 (2015) 23–35

NEUROPROTECTIVE AND ANTI-INFLAMMATORY PROPERTIES OF A NOVEL NON-THIAZOLIDINEDIONE PPARγ AGONIST *IN VITRO* AND IN MPTP-TREATED MICE

D. LECCA,[a†] D. K. NEVIN,[b†] G. MULAS,[a] M. A. CASU,[c] A. DIANA,[a] D. ROSSI,[d] G. SACCHETTI[d] AND A. R. CARTA[a*]

[a] *Department of Biomedical Sciences, University of Cagliari, Italy*

[b] *School of Biochemistry & Immunology, Trinity College, Dublin, Ireland*

[c] *CNR-Institute of Translational Pharmacology, U.O.S. of Cagliari, Italy*

[d] *Department of Life Science and Biotechnology, University of Ferrara, Italy*

Abstract—Peroxisome proliferator-activated receptor (PPAR)γ is a potential pharmacological target for disease-modification in Parkinson's disease (PD), mainly acting by modulating the neuroinflammatory response. However, currently available agonists thiazolidinediones (TZDs) present limitations due to safety concerns. We evaluated a novel thiobarbituric-like compound MDG548, which acts as a functional PPARγ agonist displaying higher and selective binding affinity as compared to TZDs. Neuroprotection by MDG548 was tested *in vitro* and in a mouse MPTP model of PD, and neuroinflammation was investigated as a putative underlying mechanism. Viability assay on rat cortical neurons showed lack of cytotoxic effect in the dose-range of 100 nM–10 μM, which was therefore used for testing *in vitro* protection against H_2O_2 and MPP+ neurotoxicity. MDG548 dose-dependently increased cell viability of rat cortical neurons co-treated with H_2O_2 or pre-exposed to MDG548 prior to H_2O_2. Moreover, MDG548 induced neuroprotection in MPP+-treated PC12 cells. NF-kB activation was investigated to assess anti-inflammatory activity. MDG548 dose-dependently decreased NF-kB activation induced by LPS (100 ng/100 ml) in HEK-Blue-hTLR4 cells. Given the supposed cancer risk of other PPARγ agonists, Ames test for genotoxicity was performed in *Salmonella typhimurium* TA100 and TA98 strains, showing that MDG548 was not genotoxic. *In vivo*, BL/6J mice were treated with MPTP (20 mg/kg i.p. once/day for 4 days) in association with saline or MDG548 (2, 5, 10 mg/kg i.p.). Stereological counting showed that MDG548 prevented the MPTP-induced reduction in TH-positive cells in the substantia nigra compacta (SNc) at all doses tested. Moreover, MDG548 reduced reactive microglia and iNOS induction in the SNc. MDG548, being a non-TZD compound with high PPARγ affinity, void of genotoxicity, and with *in vitro* as well as *in vivo* neuroprotective properties, provides a promising alternative in the search for safer PPARγ agonists to be tested as potential disease-modifying drugs in PD.

This article is part of a Special Issue entitled: Inflammation in Nervous System Disorders.

Key words: neuroinflammation, microglia, MPTP, PPAR, Parkinson.

INTRODUCTION

Chronic neuroinflammation contributes to degeneration of nigral dopaminergic neurons in Parkinson's disease (PD) (Long-Smith et al., 2009; Ransohoff and Cardona, 2010; Tansey and Goldberg, 2010; Mosley et al., 2012) and targeting the inflammatory response and microglia reactivity may offer a valid disease-modifying therapeutic approach (Hirsch and Hunot, 2009; Nolan et al., 2013; Schapira et al., 2014).

Peroxisome proliferator-activated receptor (PPAR)γ is a nuclear receptor which modulates the transcription of insulin-responsive genes involved in the control of glucose and lipid metabolism, as well as genes involved in inflammatory responses and in oxidative stress pathways (Desvergne and Wahli, 1999; Varga et al., 2011; Zhao et al., 2014). Following nuclear translocation, PPARγ modulates the expression of genes involved in inflammation by regulating the activity of inflammation-related transcription factors such as nuclear factor (NF)-kB (Moraes et al., 2006; Straus and Glass, 2007). PPARγ is widely expressed in all CNS cell types, displaying high levels in immune cells including microglia (Moreno et al., 2004; Bernardo and Minghetti, 2008). PPARγ agonists thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are marketed as antidiabetic drugs which display neuroprotective properties in preclinical PD models both in rodents and primates, and a number of studies have pointed to neuroinflammation and microgliosis as a primary target of PPARγ-mediated neuroprotection in PD (Dehmer et al., 2004; Sundararajan et al., 2006; Swanson et al., 2011; Carta, 2013; Pisanu et al., 2014).

Recently, concerns have been raised relating to potential negative effects on cardiovascular function

*Corresponding author.

† Authors equally contributed.

Abbreviations: ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; IR, immunoreactivity; LPS, lipopolysaccharide; NBM, neurobasal medium; PBS, phosphate-buffered saline; PD, Parkinson's disease; PPAR, peroxisome proliferator-activated receptor; SEAP, Secreted Embryonic Alkaline Phosphatase; SNc, substantia nigra compacta; TH, tyrosine hydroxylase; TZDs, thiazolidinediones.

http://dx.doi.org/10.1016/j.neuroscience.2015.04.026

and increased bladder cancer risk, respectively for rosiglitazone and pioglitazone (Home et al., 2009; Tseng and Tseng, 2012; Kostapanos et al., 2013; Carta and Simuni, 2014; Turner et al., 2014). This limitation has prompted for the search of novel non-TZD compounds void of such side-effects.

MDG548 is a novel thiobarbituric-like compound recently identified by computational integrated virtual screening, displaying specific PPARγ binding with an affinity approximately double that of rosiglitazone (Nevin et al., 2012). Here, we investigated MDG548 by *in vitro* and *in vivo* tests with the final goal of evaluating the neuroprotective potential in experimental PD and assessed the activity on the inflammatory response as an underlying mechanism.

First, cytotoxicity against rat cortical neurons was determined and a non cytotoxic concentration range was assessed for subsequent *in vitro* assays. Second, neuroprotective effect of MDG548 against H_2O_2 or MPP+ insult was assayed in neuronal cultures. Third, the potential inhibitory effect of MDG548 on lipopolysaccharide (LPS)-stimulated NF-kB activation was tested in HEK-BlueTH cells. Fourth, the genotoxic potential of MDG548 was tested by the Ames assay. Finally, the neuroprotective potential and anti-inflammatory activity of MDG548 *in vivo* was tested in a MPTP mouse model of PD, by mean of unbiased stereological analysis of TH-positive neurons in the substantia nigra compacta (SNc) and evaluation of CD11b and iNOS as markers of inflammation in the same area.

EXPERIMENTAL PROCEDURES

Drugs

MPTP-HCl (Sigma, Italy) was dissolved in saline. For *in vitro* studies MDG548 (Specs ID number: AN-698/15136006; 5-[4-(benzyloxy)-3-chlorobenzylidene]-2-thioxodihydropyrimidine-4,6(1H,5H)-dione; Mol. Weight: 372.83) and rosiglitazone (Santa Cruz) were dissolved in 0.1% dimethyl sulfoxide (DMSO). For *in vivo* tests MDG548 was suspended in 30% DMSO, 10% polyethylene glycol (PEG) and water.

Primary culture of cerebral cortical neurons

Primary cerebral cortical neurones were obtained from postnatal 1-day-old Wistar rats. Cortical tissue was incubated with 2 ml sterile phosphate-buffered saline (PBS) containing trypsin (0.3%; Sigma–Aldrich, Dorset, UK) in a humidified chamber for 25 min at 37 °C, followed by PBS containing soyabean trypsin inhibitor (0.1%), DNAse (0.2 mg/ml) and $MgSO_4$ (0.1 M) (all from Sigma–Aldrich, Dorset, UK). The cell suspension was gently filtered and centrifuged at 2000×*g* for 3 min at 20 °C. The pellet was resuspended in neurobasal medium (NBM, Invitrogen, Paisley, UK), supplemented with heat-inactivated horse serum (10%) penicillin (100 U/ml), streptomycin (100 U/ml) and glutamine (2 mM) (all from Gibco BRL, Maryland, USA). Resuspended neurons were placed on coverslips at a density of 0.25×10^6 and incubated with NBM containing 5 ng/ml cytosine-arabino-furanoside (ARA-C; Sigma–Aldrich, Dorset, UK) for 24 h to prevent proliferation of non-neuronal cells. Cells were grown in NBM (400 µl/well) media for up to 5 days post ARA-C treatment.

CalceinAM cell viability assay

Cells were treated with MDG548, rosiglitazone or vehicle (0.1% DMSO) and allowed to incubate for 24 or 48 h prior to the addition of calceinAM (2 µM) at the experimental endpoint. After incubation (30 min at 37 °C), fluorescence intensity (Ex: 495 nm, Em: 515 nm) derived from cleaved calcein retained within viable cells was read on a Spectramax Gemini fluorometric plate reader using SOFTmax Pro (V4.0, Molecular Devices) software package. Data were presented in relative fluorescence units (RFU).

PrestoBlue™ cell viability assay

For the PrestoBlue™ assay, cortical neurons were analyzed in two experimental conditions: (A) pre-treatment with MDG548 or rosiglitazone for 24 h prior to exposure to H_2O_2 (50 µM), and (B) co-treatment with MDG548 or rosiglitazone and H_2O_2 (50 µM) at the same time point. In both instances, cortical neurons were incubated for a further 24 h prior to measurement of cellular viability. MDG548 or rosiglitazone were added at increasing concentrations (100 nM to 10 µM). Based on results of MDG548-mediated neuroprotection, in a following experiment PPARγ antagonist GW9662 was added 1 h prior to MDG548 and the experimental condition A was followed. PrestoBlue™ reagent is reduced to a highly fluorescent compound by the reducing the environment of the viable cell. Fluorescence intensity was assessed as a directly proportional measure of the amount of viable cells.

Neutral red uptake assay for cell viability/cytotoxicity

PC12 cells, the rat adrenal pheochromocytoma cell line, were purchased from American Type Culture Collection. Cells were grown on poly-L-lysin-coated flasks (100 µg/ml) in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin (all from Sigma–Aldrich). All cells were cultured at 37 °C with 5% CO_2. In order to ensure the expression of dopaminergic features, 10^4 cells per well were seeded in 24 well plates and immediately treated with NGF (50 ng/ml) every second day so that neuronal differentiation was achieved after 10 days. At that time, the complete medium was replaced by the same medium but with lower (1%) concentration of FBS to avoid the risk of any possible protein interference on the assay sensitivity. GW9662 (1 µM) or vehicle was added 1 h prior MDG548 (500 nM and 5 µM) plus MPP+ (500 µg/ml). After the scheduled treatment, cells were briefly washed with PBS and incubated with neutral red solution (40 µg/ml) for 3 h at 37 °C in