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TSPO-ligands prevent oxidative damage and inflammatory response in C6 glioma cells by neurosteroid synthesis

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ABSTRACT

Translocator protein 18 kDa (TSPO) is predominantly located in the mitochondrial outer membrane, playing an important role in steroidogenesis, inflammation, cell survival and proliferation. Its expression in central nervous system, mainly in glial cells, has been found to be upregulated in neuropathology, and brain injury.
In this study, we investigated the anti-oxidative and anti-inflammatory effects of a group of TSPO ligands from the *N,N*-dialkyl-2-phenylindol-3-ylglyoxylamide class (PIGAs), highlighting the involvement of neurosteroids in their pharmacological effects. To this aim we used a well-known in vitro model of neurosteroidogenesis: the astrocytic C6 glioma cell line, where TSPO expression and localization, as well as cell response to TSPO ligand treatment, have been established. All PIGAs reduced L-buthionine-(S,R)-sulfoximine (BSO)-driven cell cytotoxicity and lipid peroxidation. Moreover, an anti-inflammatory effect was observed due to the reduction of inducible nitric oxide synthase and cyclooxygenase-2 induction in LPS/IFNγ challenged cells. Both effects were blunted by aminoglutethimide (AMG), an inhibitor of pregnenolone synthesis, suggesting neurosteroids' involvement in PIGA protective mechanism. Finally, pregnenolone evaluation in PIGA exposed cells revealed an increase in its synthesis, which was prevented by AMG pre-treatment.
These findings indicate that these TSPO ligands reduce oxidative stress and pro-inflammatory enzymes in glial cells through the *de novo* synthesis of neurosteroids, suggesting that these compounds could be potential new therapeutic tools for the treatment of inflammatory-based neuropathologies with beneficial effects possibly comparable to steroids, but potentially avoiding the negative side effects of long-term therapies with steroid hormones.

1. Introduction

Translocator protein (TSPO) 18 kDa (Papadopoulos et al., 2006) has been originally described as a peripheral binding site for diazepam and named peripheral-type benzodiazepine receptor (PBR) to distinguish it from the central benzodiazepine/$GABA_A$ receptor. TSPO, located in the outer mitochondrial membrane, has been involved in numerous functions, such as steroidogenesis (Casellas et al., 2002; Lacapere and Papadopoulos, 2003; Papadopoulos et al., 1997; Midzak et al., 2015), inflammation (Torres et al., 2000; Wilms et al., 2003; Liu et al., 2014), stress adaptation (Biggio et al., 2007), apoptosis (Veenman et al., 2007; Veenman et al., 2008), and cell proliferation (Beinlich et al., 2000; Li et al., 2007; Austin et al., 2013). At central level, TSPO is mainly expressed in microglia (Casellas et al., 2002; Gavish et al., 1999) as well as in reactive astrocytes (Kuhlmann and Guilarte, 2000; Maeda et al., 2007), where its constitutive expression has been found to be upregulated in sites of acute brain injury and in different neuropathologies (Banati, 2002; Chen and Guilarte, 2008; Kannan et al., 2009; Batarseh and Papadopoulos, 2010). Therefore, TSPO is recognized to be a marker of neuroinflammation and neurodegeneration (Cagnin et al., 2001; Venneti et al., 2006; Liu et al., 2014). Currently, it is also considered an attractive therapeutic target, as TSPO ligands have shown to have protective effects in several in vitro and animal models of

Abbreviations: TSPO, Translocator protein; PIGAs, *N,N*-dialkyl-2-phenylindol-3-ylglyoxylamides; BSO, L-buthionine-(S,R)-sulfoximine; AMG, aminoglutethimide; PBR, peripheral-type benzodiazepine receptor; StAR, steroidogenic acute regulatory protein; P450scc, P450 side chain cleavage enzyme; PK11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide; Ro5-4864, 4′-chlorodiazepam; NO, nitric oxide; TNF, tumor necrosis factor; COX, cyclooxygenase; LPS, lipopolysaccharide; GSH, glutathione; iNOS, inducible nitric oxide synthase; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylendiaminetetraacetic acid; MDA, malondialdehyde; TBA, thiobarbituric acid; TCA, trichloroacetic acid; DMEM, Dulbecco's modified eagle medium; IFN, interferon.

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neurodegenerative/neurologic diseases with inflammation-related features (Barron et al., 2013; Leaver et al., 2012; Girard et al., 2008; Da Pozzo et al., 2015). Several studies have shown that some TSPO ligands reduce the activation of astrocytes and microglia after neurodegenerative insult *in vivo* (Ryu et al., 2005; Veiga et al., 2005). Their anti-inflammatory and neuroprotective effects have been ascribed to multiple mitochondrial functions, including the modulation of neurosteroids' synthesis (Veenman and Gavish, 2012). TSPO binds cholesterol with high affinity, and, in concerted action with steroidogenic acute regulatory protein (StAR) (Chen et al., 2014; Papadopoulos et al., 2015), plays a key role in cholesterol translocation from the outer to the inner mitochondrial membrane (Papadopoulos et al., 2006; Papadopoulos et al., 2007). The first and rate-limiting-step in the biosynthesis of all steroid hormones is the conversion of cholesterol to pregnenolone, which is accomplished by the cleavage of the cholesterol side chain, catalysed by the P450 side chain cleavage (P450scc) enzyme. Indeed, pregnenolone is the precursor of all steroids/neurosteroids (Lacapere and Papadopoulos, 2003), many of which are known to exert neuroprotective effects (Borowicz et al., 2011).

Classical synthetic TSPO ligands are the isoquinoline carboxamide PK11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide) and Ro5–4864 (4′-chlorodiazepam) (Rupprecht et al., 2010). In particular, PK11195 has shown to bind exclusively to TSPO, differently from Ro5-4864, which requires other mitochondrial protein components to show its binding capacity. PK11195 inhibited the secretion of pro-inflammatory cytokines (Klegeris et al., 2000), the proliferation of monocytes (Bessler et al., 1992) and reduced the production of nitric oxide (NO), the expression of cyclooxygenase (COX)-2, and the amount of tumor necrosis factor (TNF)-α after lipopolysaccharide (LPS) stimulation of cultured rodent and human microglia (Wilms et al., 2003; Choi et al., 2002). PK11195 has also shown to possess anti-proliferative effects in C6 rat glioma cells, where TSPO expression and sub-cellular localization has been established, as well as cell response to TSPO ligand treatment (Chelli et al., 2004; Chelli et al., 2005). Therefore, in the present study we used C6 cells, which provide a pure source of astroglial-derived cells, where the combination of LPS plus cytokines can induce the transcription of pro-inflammatory enzymes (Meli et al., 2001; Mattace Raso et al., 2006). Here, the capability of some synthetic *N,N*-dialkyl-2-phenylindol-3-ylglyoxylamides (PIGAs), a class of potent and selective TSPO ligands (Da Settimo et al., 2008) Table 1) to reduce lipid peroxidation and inflammatory response was evaluated. Lipid peroxidation, as a result of oxidative stress, was induced by cell glutathione (GSH) depletion through L-buthionine–(S,R)-sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase (Raso et al., 2011). The anti-inflammatory effect of PIGAs was assessed evaluating the modulation of pro-inflammatory enzymes (inducible nitric oxide synthase [iNOS] and COX-2) expression induced by LPS/IFN-γ. Furthermore, the involvement of neurosteroids in the protective effect of these compounds against oxidative stress or inflammatory insults was assessed by using aminoglutethimide (AMG), a well-known inhibitor of the P450scc enzyme.

2. Materials and methods

2.1. Drugs and materials

Fetal bovine serum (FBS), cell culture media and supplements were purchased from Cambrex Bio Science Verviers (B-800; Verviers, Belgium). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DL-aminoglutethimide (AMG), L-buthionine-(S,R)-sulfoximine (BSO), ethylenediaminetetraacetic acid (EDTA) solution, malondialdehyde (MDA), thiobarbituric acid (TBA) and trichloroacetic acid (TCA), bovine serum albumin, protease and phosphatase inhibitors (leupeptin, trypsin inhibitor, phenylmethylsulfonylfluoride, PMSF, sodium orthovanadate, Na_3VO_4) were purchased from Sigma-Aldrich (St Louis, MO, USA). 1-(2-chlorophenyl-*N*-methyl-1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) was obtained from Tocris Bioscience (Ellisville, MO, USA).

Synthetic *N,N*-dialkyl-2-phenylindol-3-ylglyoxylamides, PIGA 795, 796, 823 and 1136 are reported in Table 1.

2.2. Synthesis of PIGA derivatives

PIGA compounds were synthesized essentially following experimental procedures already reported (Primofiore et al., 2004; Taliani et al., 2007). Briefly, the appropriate 2-arylindoles, commercially available or simply obtained with a one-step Fischer indole synthesis, were acylated with oxalyl chloride, in anhydrous diethyl ether, at room temperature, to yield the corresponding 2-arylindolylglyoxylyl chlorides. These last derivatives were then allowed to react with the appropriate dialkylamine, in the presence of triethylamine, in dry toluene solution, at room temperature, to give the target PIGAs (Primofiore et al., 2004; Taliani et al., 2007).

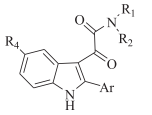
2.3. Cell culture

The rat C6 cells, originated from a rat brain glioma, were purchased from the American Type Culture Collection (ATCC). C6 cells were maintained in Dulbecco's modified essential medium (DMEM) containing 10% FBS supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified incubator under 5% CO_2 and 95% air. Cells were passaged at confluence by using a solution of 0.025% trypsin and 0.01% EDTA and they were used between passages 54–58. C6 cells at such passage number have predominantly an astrocytic phenotype (Parker et al., 1980; Goya et al., 1996; Mangoura et al., 1989), a cell population playing a crucial role in brain antioxidant defence and in many housekeeping functions (Maragakis and Rothstein, 2006, Barreto et al., 2011).

2.4. Cell viability

Cell viability was determined by MTT assay (Esposito et al., 2010). Briefly, C6 cells (2.5×10^3/well) were plated to a final volume of 150 μl and cultured for three days in a 96-well plate. After 24 h of

Table 1
General structure of the *N,N*-dialkyl-2-phenylindol-3-ylglyoxylamides PIGAs.



Compound	R_1	R_2	R_4	Ar	Ref.
PIGA 795	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	H	4-F-C_6H_4	Primofiore et al. (2004)
PIGA 796	$(CH_2)_5CH_3$	$(CH_2)_5CH_3$	H	4-Cl-C_6H_4	Primofiore et al. (2004)
PIGA 823	$CH_2C_6H_5$	CH_2CH_3	Cl	4-Cl-C_6H_4	Da Settimo et al. (2008)
PIGA 1136	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	H	naphth-2-yl	Barresi et al. (2015)