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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.

Keywords: N,N-dialkyl-2-phenylindol-3-ylglyoxylamides, Pregnenolone, Aminoglutethimide, Oxidative stress, Inflammation, C6 glioma cells

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/GABAA 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 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 antiinflammatory 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, catalyzed 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 antiinflammatory 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, Na3VO4) 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% CO2 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 × 103 /well) were plated to a final volume of 150 µl and cultured for three days in a 96-well plate. After 24 h ofstarvation in serum free Dulbecco's modified Eagle's medium (DMEM) F-12, cells were stimulated with BSO (10 mM) alone or in presence of PIGA 795, or 796, or 823, or 1136 (10 µM) or PK11195 (3 µM). All compounds were dissolved in DMSO. The final DMSO percentage of 0.5% (v/v) allows the optimal solubilization of PIGAs, PK11195 and AMG in aqueous solutions and no effect on the measured parameters has been shown, through appropriate preliminary experiments performed with and without vehicle. Control and BSO wells received the same amount of DMSO. Cells were then incubated with 25 µl of MTT (5 mg/ml) for 3 h at 37 °C. Thereafter, cells were lysed with 100 µl of lysis buffer [50% (v/v) N,N-dimethylformamide, 20% (w/v) sodium dodecyl sulfate, pH 4.5]. After 20 h incubation at 37 °C, the optical densities (OD620) for the serial dilutions of compounds were compared with the OD of control wells to obtain a concentration-effect toxicity curve (data not shown) or BSO-challenged wells to assess cell viability. Cell viability percentage was calculated as drug treated OD/control OD × 100.

2.5 Malondialdehyde measurement

Cells (3 × 105 /P60 dish) were cultured for 3 days to confluence in complete medium, then were starved for 24 h in serum free DMEM/ F12. Then, cells were stimulated with BSO (10 mM) alone or in presence of PIGA 795, or 796, or 823, or 1136 (10 µM) or PK11195 (3 µM). In another set of experiments AMG (50 µM) was added 1 h prior to PIGA treatment. The concentration of these compounds was chosen on the basis of cell viability experiments (see Supplemental data). PIGAs and AMG were first dissolved in absolute DMSO and

then diluted with DMEM. The final DMSO concentration in wells was 0.5% (v/v). After 18 h MDA was evaluated in cell lysates, as a marker of lipid peroxidation, by the method described by (Draper and Hadley, 1990). MDA standards were prepared by using 1,1,3,3-tetramethoxypropane in PBS. Each sample (900 µl) was mixed with 10% TCA (2 ml), centrifuged at 1260g for 10 min, then 0.5% TBA was added (1.3 ml). After 20 min at 100 °C, fluorescence of the mixture was measured with a Perkin– Elmer spectrofluorimeter (model LS-5B; Perkin Elmer, Boston, MA, USA) at the wavelengths of 530 and 550 nm, respectively, for excitation and emission. The MDA quantities were calculated by linear regression analysis of the standard curve. Values were expressed as µmol MDA/mg proteins.

2.6 Western blot analysis

After 24 h of starvation in serum free DMEM/F12, cells (3 × 105 /P60 dish) cells were stimulated with lipopolysaccharide (LPS)/interferon (IFN)-γ (1 µg/ml and 100 U/ml, respectively) alone or in presence of PIGA 795, or 796, or 823, or 1136 (10 µM) or PK11195 (3 µM) (data not shown). Alternatively, AMG (50 µM) was added 1 h before PIGA treatment. After 24 h of PIGA incubation, cells were washed twice with ice cold phosphate-buffered saline (PBS), harvested, and resuspended in 20 mM Tris-HCl (pH 7.5), 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 mM Na3VO4, leupeptin (10 µg/ml) and trypsin inhibitor (10 µg/ml). After 40 min, whole cell lysates were obtained by centrifugation at 20,000g for 15 min at 4 °C. Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. In another set of experiments, cells were treated with PK11195 and PIGAs at 3 and 10 µM, respectively, for 24 h. After that, mitochondrial fractions were prepared and purified using Qproteome Mitochondria Isolation Kit from Qiagen (Ding et al., 2012). Equal amount of protein (mitochondrial fraction as well as whole cell lysates) was dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8%). Western blotting was performed by transferring proteins from slab gel to a sheet of nitrocellulose membrane at 240 mA for 1 h at room temperature. The filter was then blocked with 1× PBS and 5% non-fat dried milk for 40 min at room temperature and incubated with the specific anti-iNOS (1:1000; Transduction Laboratories, Lexington, KY, USA), or anti COX-2 (1:500; Cayman Chemical, Ann Arbor, Michigan, USA) or anti-StAR (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies in 1× PBS, 5% non-fat dried milk and 0.1% Tween 20 overnight at 4 °C. Thereafter, the filters were incubated with the secondary antibody (Jackson, West Grove, PA, USA) for 1 h at room temperature. Subsequently, the blot was extensively washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions, and the immune complex visualized by Imag Quant. The protein bands were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories, Milan, Italy). To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against the α-tubulin protein (Sigma-Aldrich, St Louis, MO, USA).

2.7 Pregnenolone assay

The pregnenolone production measurement in C6 cells, exposed to PIGA compounds (10 µM) or PK11195 (3 µM) alone or with AMG (50 µM), was performed by competitive enzyme-linked immunoassay (ELISA). Briefly, C6 cells were seeded in 96-well plates at a density of about 10,000 cells/well in a final volume of 100 µl and the pregnenolone secretion into the medium was performed as previously described (Selleri et al., 2005). The final concentration of DMSO and ethanol was constant for all the wells within each experiment and did not exceed 0.5% (v/v), a concentration that on its own had no effect on steroid production. At the end of the incubation period (2 h), the cell medium was used in an enzyme immunoassay for the direct quantitative determination of pregnenolone, under the conditions recommended by the supplier (Pregnenolone ELISA, the EiAsy Way, IBL Hamburg, Germany).

2.8. Data analysis

Data are reported as mean ± S.E.M. values of three independent experiments, which were done at least three times, each time with three or more independent observations. Data were subjected to analysis of variance with a computerised package for statistical analysis. Comparisons were made using the Bonferroni or, when appropriate, the Dunnett multiple comparison test. A P value <0.05 was considered significant.

3. Results

3.1 Protective effect of PIGAs and PK11195 on BSO-driven cell death and lipid peroxidation

To evaluate the protective effect of all compounds on oxidative stress, C6 cells were challenged with BSO in presence or not of PIGAs or the reference TSPO ligand PK11195, and then cell viability and MDA production were determined. PIGAs and PK11195 were used at a fixed 10 µM and 3 µM concentration, respectively, doses that produced no cytotoxic effects in preliminary experiments on cell viability (data not shown). As reported in Fig. 1A, cell viability findings evidenced a significant decrease in BSO challenged cells, which was prevented by all PIGAs (Fig. 1A). Conversely, PK11195, used as reference TSPO ligand, did not significantly reverse BSO induced cell death. Consistently, PIGAs were able to significantly reduce MDA production (Fig. 1B). A similar result was also observed with the reference drug PK11195 (Fig. 1B). The involvement of neurosteroids in the protective effect of PIGAs was indirectly demonstrated through its reversion by AMG, an inhibitor of P450scc enzyme. PIGAs inhibitory effect was significantly blunted by the pharmacological inhibition of neurosteroid synthesis. The AMG concentration was chosen on the basis of its efficacy in blocking the production of pregnenolone. No significant effect on MDA production was demonstrated by AMG in presence of BSO compared to that of BSO alone, even if a trend of increase was observed.

3.2. Effect of PIGAs on iNOS and COX-2 expression in C6 cells

The expression of iNOS and COX-2 was evaluated by Western blot analysis to determine the modulatory effect of PIGAs on the induction of these enzymes by combined LPS and IFN-γ. As shown in Fig. 2A and B, all PIGA compounds induce a significant decrease of iNOS expression at 10 µM, as well as the reference drug PK11195 used at 3 µM. Pre-treatment with AMG blunted the effect of PIGAs or PK11195 on iNOS expression (Fig. 2A and B). No significant effect was demonstrated by AMG alone. A similar effect was evidenced on COX-2 induced expression by LPS/ IFN-γ stimulation (Fig. 3A and B). Indeed, all PIGAs reduced COX-2 increased expression in challenged cells. When cells were pre-incubated with AMG, PIGA effect showed a full reversal of COX-2 induction, but not for PIGA 1136, since AMG only partially reverted its effect.

3.3. StAR modulation by PIGAs in the mitochondrial fraction of C6 cells

Positioned at the cytosolic site of the outer mitochondrial membrane, StAR is functional in cholesterol translocation and exhibit high activity as 37-kDa preprotein. After its cleavage in mitochondrial membrane contact site, the 30 kDa inactive intra-mitochondrial StAR is produced (Bose et al., 2002). Here, we showed that the stimulation of C6 cells with all tested PIGAs leads to an increase of the 30 kDa form of StAR after 24 h of incubation (Fig. 4), an indirect rate of cholesterol transfer into the mitochondria. A significant increase of the inactive StAR protein was also observed after incubation with PK11195. 3.4. PIGAs induce pregnenolone synthesis in C6 cell supernatant Among the de novo synthesized neurosteroids, we evaluated the amount of pregnenolone, the first precursor of all neurosteroids in C6 cell supernatant (Table 2). As expected, AMG treatment induced a decrease in pregnenolone synthesis of about 13%, whereas all PIGAs increased its synthesis compared to that of control cells. Among all compounds, PIGA 823 induced a two-fold increase in hormone production, being the most potent molecule. The reference drug PK11195 slightly increases pregnenolone synthesis, and, as expected, AMG pre-treatment reverted PIGA-induced increase in pregnenolone synthesis.

4. Discussion

In the present study, the pharmacological profile of a class of potent and selective TSPO ligands was evaluated performing in vitro experiments to address anti-oxidative and anti-inflammatory effects. Our results demonstrate that all the tested PIGAs protected astrocytes from oxidative damage and reduced the inflammatory response, by decreasing the induction of inducible isoforms of nitric oxide synthase and cyclooxygenase. Furthermore, these compounds increased the concentrations of pregnenolone, the precursor of all neurosteroids, in the conditioned medium from cultured C6 cells. Additionally, the blunting of neurosteroid de novo synthesis, through P450scc inhibition, reverted anti-oxidative and anti-inflammatory effects of the examined compounds. Collectively, these results indicate that these TSPO ligands may play a role in neuroprotection, through regulation of endogenous neurosteroid production which modulates the inflammatory response.

Actually, TSPO increased expression at central level has been shown in injured brain or in neurological disorders, suggesting its relevance as a therapeutic target (Da Pozzo et al., 2015). Indeed, its induction in inflammatory conditions has been interpreted as an attempt to counteract the overwhelming pro-inflammatory state, as a compensatory mechanism to brain injury.

Neuroendocrinological studies have demonstrated that a variety of neuronal and glial functions are influenced by the autocrine/paracrine actions of locally produced neurosteroids (Akk et al., 2007; Agis-Balboa et al., 2006). After brain injury, progesterone and its metabolites (i.e. 5α-dihydroprogesterone and allopregnanolone) can exert beneficial effects on neurons and glial cells, preventing thus cerebral oedema, necrosis, apoptosis, and inflammation, since they enhance at the same time different neuroprotective mechanisms (He et al., 2004; Djebaili et al., 2004; Shear et al., 2002; Hu et al., 2009; Guennoun et al., 2015). In addition, the levels of these neurosteroids have been shown to be altered in anxiety and major depression disorders, suggesting their involvement in the pathophysiology of such psychiatric disorders. Notably, the anxiolytic properties of the N,N-di-n-propyl-2-(4-methylphenyl)indol-3-ylglyoxylamide (MPIGA) have been associated to an increase in the production of progesterone and allopregnanolone, which are both synthetized from the pregnenolone (Costa et al., 2011), strengthening the hypothesis that the beneficial effects of this class of TSPO ligands are mediated by the pregnenolone and/or its metabolites.

A role for TSPO in dealing with reactive oxygen species (ROS)-in duced damage has been yet established (Casellas et al., 2002). In particular, the ROS-driven brain damage is one of the major risk factors in the development of several neurodegenerative disorders (i.e. aging and Alzheimer's disease) in which TSPO may play a role (Repalli, 2014).

TSPO ligands are used for wide variety of applications such as neuroimaging agents, therapeutic target for neurological and psychiatric disorders, brain damage and neuroinflammation (Rupprecht et al., 2010). PK11195 and Ro5-4864 are considered to be prototype compounds and several classes of compounds have been developed such as benzodiazepine derivatives, aryloxyanilide derivatives and isoquinoline carboxamide derivatives (Taliani et al., 2011).

In the present study, we used C6 cells as a model of astrocytes for glutathione (GSH) depletion-induced oxidative damage (Raso et al., 2011) to confirm the neuroprotective effect of PIGA compounds. The GSH and GSH peroxidase system plays a major role in controlling cellular redox states, being the primary defence mechanism for peroxide removal from the brain (Simonian and Coyle, 1996). All PIGAs no only reduced lipid peroxidation, as evaluated by the secondary reactive product of ROS, MDA, but also reduced BSO-induced cell death.

A pivotal role for NO and an excess of pro-oxidants in various brain areas has been recognized as responsible for both neuronal functional impairment and structural damage. Similarly, COX-2, another known source of oxidants, may account for stress-induced brain damage, as it is up-regulated in several brain diseases, such

as stroke, Alzheimer's dementia and seizures (Nogawa et al., 1997). The protective effects exerted by PIGAs are strengthened by biochemical evidences, in that these compounds not only reduced the increase of lipid peroxidation, but also iNOS and COX-2 expression, induced by LPS/IFNγ. Interestingly, inhibitors of COX-2 activity prevent the increase of membrane lipid peroxidation mediators and the depletion of the main tissue antioxidant (i.e. glutathione), suggesting that this enzyme isoform is involved in the accumulation of oxidative mediators in injured brain (Madrigal et al., 2006).

To evaluate the involvement of neurosteroids in PIGAs' effects, we used AMG, a specific inhibitor of P450scc. Here, we reported data indicating that PIGAs-induced reduction of oxidative stress, in the face of BSO induced damage, is related to neurosteroid biosynthesis. Indeed, the partial reversal of MDA reduction in the presence of the inhibitor AMG is suggestive of the capability of these molecules to induce their effects, at least in part, through the de novo synthesis of neurosteroids.

Actually, the rate-limiting step of steroidogenesis, previously thought to be the production of pregnenolone, is now also consideredthe cholesterol transfer from the outer to the inner mitochondrial membrane where the P450scc is located (Charalampopoulos et al., 2008; Keller et al., 2004; Belelli et al., 2006). It has been suggested that beyond TSPO, also steroidogenesis acute regulatory protein (StAR), localized in mitochondria, is a critical protein in the regulation of cholesterol availability in peripheral and central steroidogenic cells (Jung-Testas and Baulieu, 1998; Hauet et al., 2002; Sasso et al., 2010). A functional StAR/TSPO interaction plays a key role in the regulatory mechanism, underpinning cholesterol shuttling (Papadopoulos and Miller, 2012). StAR, synthesized as a 37-kDa preprotein, is rapidly imported into the mitochondria, and processed to the inactive mature 30-kDa form, found in the mitochondrial matrix. The N-terminal domain targets StAR in the mitochondria, and its cleavage effectively terminates the delivery of cholesterol to P450scc and consequently the synthesis of pregnenolone (Bose et al., 2002; Mathieu et al., 2002; Stocco, 2001; Thomson, 2003). Therefore, StAR moves cholesterol to the TSPO so that it reaches the inner mitochondrial membrane where it will be metabolized to pregnenolone by P450scc. Indeed, the 37-kDa StAR is not processed to the mature 30-kDa protein in TSPO-depleted cells or in the presence of TSPO antagonists (Hauet et al., 2005; Gazouli et al., 2002), suggesting that TSPO plays a direct role in the activity and import of StAR into mitochondria. In our experimental conditions, all PIGAs increased StAR (30-kDa) expression, suggesting an increase in mitochondrial cholesterol import after cell treatment. The increase in mature 30-kDa StAR protein after PIGAs treatment strengthened the relevance of TSPO and StAR interaction. Based on this evidence, the increase of StAR induced by PIGAs could act in a coordinated manner to induce steroidogenesis and, in turn, the increase of pregnenolone level induced by PIGAs in C6 cells. This latter event, and the reversal of PIGAs activity by AMG, clearly suggested a neurosteroid-mediated protective effect for these compounds, indicating the involvement of pregnenolone metabolites in PIGAs' effect. It is, indeed, conceivable to hypothesize that a converging protective effect through the neuroprotective neurosteroids can occur, also considering that the concentrations of pregnenolone metabolites in nervous tissues are not static, changing dynamically under different physiological and pathological states.

In conclusion, this study brings novel evidences characterizing the role of TSPO ligands in inflammatory-based conditions, illustrating a novel aspect in their mechanism of action, and could candidate these compounds as preferred drugs in brain injury.

Appendix A. Supplementary data Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejps.2016.04.006.

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.

References

Agis-Balboa, R.C., Pinna, G., Zhubi, A., Maloku, E., Veldic, M., Costa, E., Guidotti, A., 2006. Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. Proc. Natl. Acad. Sci. U. S. A. 103, 14602–14607.

Akk, G., Li, P., Manion, B.D., Evers, A.S., Steinbach, J.H., 2007. Ethanol modulates the interaction of the endogenous neurosteroid allopregnanolone with the alpha1beta2gamma2L GABAA receptor. Mol. Pharmacol. 71, 461–472.

Austin, C.J., Kahlert, J., Kassiou, M., Rendina, L.M., 2013. The translocator protein (TSPO): a novel target for cancer chemotherapy. Int. J. Biochem. Cell Biol. 45, 1212–1216.

Banati, R.B., 2002. Visualising microglial activation in vivo. Glia 40, 206–217.

Barresi, E., Bruno, A., Taliani, S., Cosconati, S., Da Pozzo, E., Salerno, S., Simorini, F., Daniele, S., Giacomelli, C., Marini, A.M., La Motta, C., Marinelli, L., Cosimelli, B., Novellino, E., Greco, G., Da Settimo, F., Martini, C., 2015. Deepening the Topology of the Translocator Protein Binding Site by Novel N, N-Dialkyl-2-arylindol-3-ylglyoxylamides. J. Med. Chem. 58, 6081–6092.

Barreto, G.E., Gonzalez, J., Torres, Y., Morales, L., 2011. Astrocytic-neuronal crosstalk: implications for neuroprotection from brain injury. Neurosci. Res. 71, 107–113.

Barron, A.M., Garcia-Segura, L.M., Caruso, D., Jayaraman, A., Lee, J.W., Melcangi, R.C., Pike, C.J., 2013. Ligand for translocator protein reverses pathology in a mouse model of Alzheimer's disease. J. Neurosci. 33, 8891–8897.

Batarseh, A., Papadopoulos, V., 2010. Regulation of translocator protein 18 kDa (TSPO) expression in health and disease states. Mol. Cell. Endocrinol. 327, 1–12.

Beinlich, A., Strohmeier, R., Kaufmann, M., Kuhl, H., 2000. Relation of cell proliferation to expression of peripheral benzodiazepine receptors in human breast cancer cell lines. Biochem. Pharmacol. 60, 397–402.

Belelli, D., Herd, M.B., Mitchell, E.A., Peden, D.R., Vardy, A.W., Gentet, L., Lambert, J.J., 2006. Neuroactive steroids and inhibitory neurotransmission: mechanisms of action and physiological relevance. Neuroscience 138, 821–829.

Bessler, H., Weizman, R., Gavish, M., Notti, I., Djaldetti, M., 1992. Immunomodulatory effect of peripheral benzodiazepine receptor ligands on human mononuclear cells. J. Neuroimmunol. 38, 19–25.

Biggio, G., Concas, A., Follesa, P., Sanna, E., Serra, M., 2007. Stress, ethanol, and neuroactive steroids. Pharmacol. Ther. 116, 140–171.

Borowicz, K.K., Piskorska, B., Banach, M., Czuczwar, S.J., 2011. Neuroprotective actions of neurosteroids. Front Endocrinol (Lausanne) 2, 50.

Bose, H.S., Lingappa, V.R., Miller, W.L., 2002. The steroidogenic acute regulatory protein, StAR, works only at the outer mitochondrial membrane. Endocr. Res. 28, 295–308.

Cagnin, A., Myers, R., Gunn, R.N., Lawrence, A.D., Stevens, T., Kreutzberg, G.W., Jones, T., Banati, R.B., 2001. In vivo visualization of activated glia by [11C] (R)-PK11195-PET following herpes encephalitis reveals projected neuronal damage beyond the primary focal lesion. Brain 124, 2014–2027.

Casellas, P., Galiegue, S., Basile, A.S., 2002. Peripheral benzodiazepine receptors and mitochondrial function. Neurochem. Int. 40, 475–486.

Charalampopoulos, I., Remboutsika, E., Margioris, A.N., Gravanis, A., 2008. Neurosteroids as modulators of neurogenesis and neuronal survival. Trends Endocrinol. Metab. 19, 300–307.

Chelli, B., Lena, A., Vanacore, R., et al., 2004. Peripheral benzodiazepine receptor ligands: mitochondrial transmembrane potential depolarization and apoptosis induction in rat C6 glioma cells. Biochem. Pharmacol. 68, 125–134.

Chelli, B., Rossi, L., Da Pozzo, E., et al., 2005. PIGA (N,N-Di-n-butyl-5-chloro-2-(4- chlorophenyl)indol-3-ylglyoxylamide), a new mitochondrial benzodiazepinereceptor ligand, induces apoptosis in C6 glioma cells. Chembiochem 6, 1082–1088.

Chen, M.K., Guilarte, T.R., 2008. Translocator protein 18 kDa (TSPO): molecular sensor of brain injury and repair. Pharmacol. Ther. 118, 1–17. Chen, C., Kuo, J., Wong, A., Micevych, P., 2014.

Estradiol modulates translocator protein (TSPO) and steroid acute regulatory protein (StAR) via protein kinase a (PKA) signaling in hypothalamic astrocytes. Endocrinology 155, 2976–2985.

Choi, H.B., Khoo, C., Ryu, J.K., van Breemen, E., Kim, S.U., McLarnon, J.G., 2002. Inhibition of lipopolysaccharide-induced cyclooxygenase-2, tumor necrosis factor-alpha and [Ca2+]i responses in human microglia by the peripheral benzodiazepine receptor ligand PK11195. J. Neurochem. 83, 546–555.

Costa, B., Da Pozzo, E., Chelli, B., et al., 2011. Anxiolytic properties of a 2- phenylindolglyoxylamide TSPO ligand: stimulation of in vitro neurosteroid production affecting GABAA receptor activity. Psychoneuroendocrinology 36, 463–472.

Da Pozzo, E., Giacomelli, C., Barresi, E., Costa, B., Taliani, S., Passetti Fda, S., Martini, C., 2015. Targeting the 18-kDa translocator protein: recent perspectives for neuroprotection. Biochem. Soc. Trans. 43, 559–565.

Da Settimo, F., Simorini, F., Taliani, S., et al., 2008. Anxiolytic-like effects of N,N-dialkyl-2- phenylindol-3-ylglyoxylamides by modulation of translocator protein promoting neurosteroid biosynthesis. J. Med. Chem. 51, 5798–5806. Ding, Y., Liu, Z., Desai, S., et al., 2012. Receptor tyrosine kinase ErbB2 translocates into mitochondria and regulates cellular metabolism. Nat. Commun. 3, 1271.

Djebaili, M., Hoffman, S.W., Stein, D.G., 2004. Allopregnanolone and progesterone decrease cell death and cognitive deficits after a contusion of the rat pre-frontal cortex. Neuroscience 123, 349–359.

Draper, H.H., Hadley, M., 1990. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol. 186, 421–431.

Esposito, E., Dal Toso, R., Pressi, G., Bramanti, P., Meli, R., Cuzzocrea, S., 2010. Protective effect of verbascoside in activated C6 glioma cells: possible molecular mechanisms. Naunyn Schmiedeberg's Arch. Pharmacol. 381, 93–105.

Gavish, M., Bachman, I., Shoukrun, R., Katz, Y., Veenman, L., Weisinger, G., Weizman, A., 1999. Enigma of the peripheral benzodiazepine receptor. Pharmacol. Rev. 51, 629–650.

Gazouli, M., Han, Z., Papadopoulos, V., 2002. Identification of a peptide antagonist to the peripheral-type benzodiazepine receptor that inhibits hormone-stimulated leydig cell steroid formation. J. Pharmacol. Exp. Ther. 303, 627–632.

Girard, C., Liu, S., Cadepond, F., et al., 2008. Etifoxine improves peripheral nerve regeneration and functional recovery. Proc. Natl. Acad. Sci. U. S. A. 105, 20505–20510.

Goya, L., Feng, P.T., Aliabadi, S., Timiras, P.S., 1996. Effect of growth factors on the in vitro growth and differentiation of early and late passage C6 glioma cells. International Journal of Developmental Neuroscience: The Official Journal of the International Society for Developmental Neuroscience 14, 409–417.

Guennoun, R., Labombarda, F., Gonzalez Deniselle, M.C., Liere, P., De Nicola, A.F., Schumacher, M., 2015. Progesterone and allopregnanolone in the central nervous system: response to injury and implication for neuroprotection. J. Steroid Biochem. Mol. Biol. 146, 48–61.

Hauet, T., Liu, J., Li, H., Gazouli, M., Culty, M., Papadopoulos, V., 2002. PBR, StAR, and PKA: partners in cholesterol transport in steroidogenic cells. Endocr. Res. 28, 395–401.

Hauet, T., Yao, Z.X., Bose, H.S., et al., 2005. Peripheral-type benzodiazepine receptor-mediated action of steroidogenic acute regulatory protein on cholesterol entry into leydig cell mitochondria. Mol. Endocrinol. 19, 540–554.

He, J., Hoffman, S.W., Stein, D.G., 2004. Allopregnanolone, a progesterone metabolite, enhances behavioral recovery and decreases neuronal loss after traumatic brain injury. Restor. Neurol. Neurosci. 22, 19–31.

Hu, Z., Li, Y., Fang, M., Wai, M.S., Yew, D.T., 2009. Exogenous progesterone: a potential therapeutic candidate in CNS injury and neurodegeneration. Curr. Med. Chem. 16, 1418–1425.

Jung-Testas, I., Baulieu, E.E., 1998. Steroid hormone receptors and steroid action in rat glial cells of the central and peripheral nervous system. J. Steroid Biochem. Mol. Biol. 65, 243–251.

Kannan, S., Balakrishnan, B., Muzik, O., Romero, R., Chugani, D., 2009. Positron emission tomography imaging of neuroinflammation. J. Child Neurol. 24, 1190–1199.

Keller, E.A., Zamparini, A., Borodinsky, L.N., Gravielle, M.C., Fiszman, M.L., 2004. Role of allopregnanolone on cerebellar granule cells neurogenesis. Brain Res. Dev. Brain Res. 153, 13–17.

Klegeris, A., McGeer, E.G., McGeer, P.L., 2000. Inhibitory action of 1-(2-chlorophenyl)-Nmethyl-N-(1-methylpropyl)-3-isoquinolinecarboxam ide (PK 11195) on some mononuclear phagocyte functions. Biochem. Pharmacol. 59, 1305–1314.

Kuhlmann, A.C., Guilarte, T.R., 2000. Cellular and subcellular localization of peripheral benzodiazepine receptors after trimethyltin neurotoxicity. J. Neurochem. 74, 1694–1704.

Lacapere, J.J., Papadopoulos, V., 2003. Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. Steroids 68, 569–585.

Leaver, K.R., Reynolds, A., Bodard, S., Guilloteau, D., Chalon, S., Kassiou, M., 2012. Effects of translocator protein (18 kDa) ligands on microglial activation and neuronal death in the quinolinic-acid-injected rat striatum. ACS Chem. Neurosci. 3, 114–119.

Li, W., Hardwick, M.J., Rosenthal, D., Culty, M., Papadopoulos, V., 2007. Peripheral-type benzodiazepine receptor overexpression and knockdown in human breast cancer cells indicate its prominent role in tumor cell proliferation. Biochem. Pharmacol. 73, 491–503.

Liu, G.J., Middleton, R.J., Hatty, C.R., et al., 2014. The 18 kDa translocator protein, microglia and neuroinflammation. Brain Pathol. 24, 631–653.

Madrigal, J.L., Garcia-Bueno, B., Caso, J.R., Perez-Nievas, B.G., Leza, J.C., 2006. Stress-induced oxidative changes in brain. CNS Neurol. Disord. Drug Targets 5, 561–568.

Maeda, J., Higuchi, M., Inaji, M., Ji, B., Haneda, E., Okauchi, T., Zhang, M.R., Suzuki, K., Suhara, T., 2007. Phase-dependent roles of reactive microglia and astrocytes in nervous system injury as delineated by imaging of peripheral benzodiazepine receptor. Brain Res. 1157, 100–111.

Mangoura, D., Sakellaridis, N., Jones, J., Vernadakis, A., 1989. Early and late passage C-6 glial cell growth: similarities with primary glial cells in culture. Neurochem. Res. 14, 941–947.

Maragakis, N.J., Rothstein, J.D., 2006. Mechanisms of disease: astrocytes in neurodegenerative disease. Nat. Clin. Pract. Neurol. 2, 679–689.

Mathieu, A.P., Fleury, A., Ducharme, L., Lavigne, P., LeHoux, J.G., 2002. Insights into steroidogenic acute regulatory protein (StAR)-dependent cholesterol transfer in mitochondria: evidence from molecular modeling and structure-based thermodynamics supporting the existence of partially unfolded states of StAR. J. Mol. Endocrinol. 29, 327–345.

Mattace Raso, G., Esposito, E., Iacono, A., Pacilio, M., Coppola, A., Bianco, G., Diano, S., Di Carlo, R., Meli, R., 2006. Leptin induces nitric oxide synthase type II in C6 glioma cells. Role for nuclear factor-kappaB in hormone effect. Neurosci. Lett. 396, 121–126.

Mattace Raso, G., Esposito, E., Vitiello, S., et al., 2011. Palmitoylethanolamide stimulation induces allopregnanolone synthesis in C6 cells and primary astrocytes: involvement of peroxisome-proliferator activated receptor-alpha. J. Neuroendocrinol. 23, 591–600.

Meli, R., Raso, G.M., Cicala, C., Esposito, E., Fiorino, F., Cirino, G., 2001. Thrombin and PAR-1 activating peptide increase iNOS expression in cytokine-stimulated C6 glioma cells. J. Neurochem. 79, 556–563.

Midzak, A., Zirkin, B., Papadopoulos, V., 2015. Translocator protein: pharmacology and steroidogenesis. Biochem. Soc. Trans. 43, 572–578.

Nogawa, S., Zhang, F., Ross, M.E., Iadecola, C., 1997. Cyclooxygenase-2 gene expression in neurons contributes to ischemic brain damage. J. Neurosci. 17, 2746–2755.

Papadopoulos, V., Miller, W.L., 2012. Role of mitochondria in steroidogenesis. Best Pract. Res. Clin. Endocrinol. Metab. 26, 771–790.

Papadopoulos, V., Amri, H., Boujrad, N., et al., 1997. Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. Steroids 62, 21–28.

Papadopoulos, V., Lecanu, L., Brown, R.C., Han, Z., Yao, Z.X., 2006. Peripheral-type benzodiazepine receptor in neurosteroid biosynthesis, neuropathology and neurological disorders. Neuroscience 138, 749–756.

Papadopoulos, V., Liu, J., Culty, M., 2007. Is there a mitochondrial signaling complex facilitating cholesterol import? Mol. Cell. Endocrinol. 265–266, 59–64.

Papadopoulos, V., Aghazadeh, Y., Fan, J., Campioli, E., Zirkin, B., Midzak, A., 2015. Translocator protein-mediated pharmacology of cholesterol transport and steroidogenesis. Mol. Cell. Endocrinol. 408, 90–98.

Parker, K.K., Norenberg, M.D., Vernadakis, A., 1980. "Transdifferentiation" of C6 glial cells in culture. Science 208, 179–181. Primofiore, G., Da Settimo, F., Taliani, S., et al., 2004. N,N-dialkyl-2-phenylindol-3-ylglyoxylamides. A new class of potent and selective ligands at the peripheral benzodiazepine receptor. J. Med. Chem. 47, 1852–1855.

Repalli, J., 2014. Translocator protein (TSPO) role in aging and Alzheimer's disease. Current Aging Science 7, 168–175.

Rupprecht, R., Papadopoulos, V., Rammes, G., Baghai, T.C., Fan, J., Akula, N., Groyer, G., Adams, D., Schumacher, M., 2010. Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nat. Rev. Drug Discov. 9, 971–988.

Ryu, J.K., Choi, H.B., McLarnon, J.G., 2005. Peripheral benzodiazepine receptor ligand PK11195 reduces microglial activation and neuronal death in quinolinic acidinjected rat striatum. Neurobiol. Dis. 20, 550–561.

Sasso, O., La Rana, G., Vitiello, S., et al., 2010. Palmitoylethanolamide modulates pentobarbital-evoked hypnotic effect in mice: involvement of allopregnanolone biosynthesis. Eur. Neuropsychopharmacol. 20, 195–206.

Selleri, S., Gratteri, P., Costagli, C., et al., 2005. Insight into 2-phenylpyrazolo[1.5- a]pyrimidin-3-yl acetamides as peripheral benzodiazepine receptor ligands: synthesis, biological evaluation and 3D-QSAR investigation. Bioorg. Med. Chem. 13, 4821–4834.

Shear, D.A., Galani, R., Hoffman, S.W., Stein, D.G., 2002. Progesterone protects against necrotic damage and behavioral abnormalities caused by traumatic brain injury. Exp. Neurol. 178, 59–67.

Simonian, N.A., Coyle, J.T., 1996. Oxidative stress in neurodegenerative diseases. Annu. Rev. Pharmacol. Toxicol. 36, 83–106. Stocco, D.M., 2001. StAR protein and the regulation of steroid hormone biosynthesis. Annu. Rev. Physiol. 63, 193–213.

Taliani, S., Simorini, F., Sergianni, V., et al., 2007. New fluorescent 2- phenylindolglyoxylamide derivatives as probes targeting the peripheral-type benzodiazepine receptor: design, synthesis, and biological evaluation. J. Med. Chem. 50, 404–407.

Taliani, S., Pugliesi, I., Da Settimo, F., 2011. Structural requirements to obtain highly potent and selective 18 kDa Translocator Protein (TSPO) Ligands. Curr. Top. Med. Chem. 11, 860–886.

Thomson, M., 2003. Does cholesterol use the mitochondrial contact site as a conduit to the steroidogenic pathway? BioEssays 25, 252–258.

Torres, S.R., Frode, T.S., Nardi, G.M., Vita, N., Reeb, R., Ferrara, P., Ribeiro-do-Valle, R.M., Farges, R.C., 2000. Anti-inflammatory effects of peripheral benzodiazepine receptor ligands in two mouse models of inflammation. Eur. J. Pharmacol. 408, 199–211.

Veenman, L., Gavish, M., 2012. The role of 18 kDa mitochondrial translocator protein (TSPO) in programmed cell death, and effects of steroids on TSPO expression. Curr. Mol. Med. 12, 398–412.

Veenman, L., Papadopoulos, V., Gavish, M., 2007. Channel-like functions of the 18-kDa translocator protein (TSPO): regulation of apoptosis and steroidogenesis as part of the host-defense response. Curr. Pharm. Des. 13, 2385–2405.

Veenman, L., Shandalov, Y., Gavish, M., 2008. VDAC activation by the 18 kDa translocator protein (TSPO), implications for apoptosis. J. Bioenerg. Biomembr. 40, 199–205.

Veiga, S., Azcoitia, I., Garcia-Segura, L.M., 2005. Ro5-4864, a peripheral benzodiazepine receptor ligand, reduces reactive gliosis and protects hippocampal hilar neurons from kainic acid excitotoxicity. J. Neurosci. Res. 80, 129–137.

Venneti, S., Lopresti, B.J., Wiley, C.A., 2006. The peripheral benzodiazepine receptor (translocator protein 18 kDa) in microglia: from pathology to imaging. Prog. Neurobiol. 80, 308–322.

Wilms, H., Claasen, J., Rohl, C., Sievers, J., Deuschl, G., Lucius, R., 2003. Involvement of benzodiazepine receptors in neuroinflammatory and neurodegenerative diseases: evidence from activated microglial cells in vitro. Neurobiol. Dis. 14, 417–424.

Figure Legends

Fig. 1. Effects of PIGAs and PK11195 on BSO-induced citotoxicity and lipid peroxidation.

Protective effect of PIGA 795, 796, 823, 1136 or PK 11195 on BSO damage was shown: cell viability (panel A) and MDA production (panel B) were determined. 18 h after BSO stimulation, cell viability or MDA content in cell lysates was evaluated. For the evaluation of MDA levels, aminoglutethimide (AMG, 50 μM) was added 1 h before PIGAs incubation (10 μM). Data were expressed as mean ± SEM of three independent experiments. ***P < 0.001 vs untreated cells; #P < 0.05, ##P < 0.01 and ###P < 0.001 vs BSO; °P < 0.05 and °°° P < 0.001 vs the respective PIGA alone.

Fig. 2. Effects of PIGAs and PK11195 on INFγ/LPS-induced iNOS expression in C6 cells.

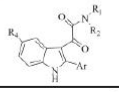
Shown are representative immunoblots for iNOS expression. C6 cells were stimulated with LPS/IFNγ in presence of PK11195 or PIGA 795 (panel A) or PIGA 796, PIGA 823 or PIGA 1136 (panel B). Neurosteroid involvement was assessed pre-incubating C6 cells with AMG. α-tubulin protein expression was used as a loading control. The densitometric quantification of all determinations of three independent experiments is also reported in lower panel. All data are expressed as mean ± SEM. ***P < 0.001 vs untreated control; # P < 0.05, ##P < 0.01 and ###P < 0.001 vs LPS/IFN-γ. °P < 0.05, °°P < 0.01, and °°° P < 0.001 vs the respective PIGA alone.

Fig. 3. Effects of PIGAs and PK11195 on INFγ/LPS-induced COX-2 expression in C6 cells. Shown are representative immunoblots for COX-2 expression. Cells were treated with LPS/IFNγ as described in Methods section in presence of PK11195 or PIGA 795 (panel A) or PIGA 796, PIGA 823 or PIGA 1136 (panel B). Neurosteroid involvement was assessed pre-incubating C6 cells with AMG. α-tubulin protein expression was used as a loading control. Respective densitometric analysis of protein bands is also reported in lower panel and obtained from three separated experiments. All data are expressed as mean ± SEM. ***P b 0.001 vs untreated control; #P < 0.05, ##P < 0.01 and ###P < 0.001 vs LPS/IFN-γ; °P < 0.05 and °°° P < 0.001 vs the respective PIGA alone.

Fig. 4. Effects of PIGAs and PK11195 on StAR protein expression in C6 cells.

Cells were treated with PIGAs (10 μM) and PK 11195 (3 μM) for 24 h in serum-free medium. A representative immunoblot is shown. The densitometric quantification of all determinations of three independent experiments is also reported. All data are expressed as mean ± SEM. Basal level of protein expression was also reported. *P b 0.05, **P b 0.01, and ***P b 0.001 vs untreated control cells.

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)

Table 2
Stimulatory effect of PIGA 795, 796, 823 and 1136 or PK 1195 on pregnenolone biosynthesis and its reversal by AMG. The pregnenolone production of untreated control cells was designated as 100%. Results are expressed as mean ± S.E.M. from at least three independent experiments.

Compounds	Increase in pregnenolone production vs control (%)
Vehicle	100 ± 3
AMG	87 ± 5
PIGA 795	128 ± 11
PIGA 795 + AMG	79 ± 8
PIGA 796	146 ± 10
PIGA 796 + AMG	55 ± 9
PIGA 823	197 ± 5
PIGA 823 + AMG	118 ± 6
PIGA 1136	161 ± 12
PIGA 1136 + AMG	119 ± 9
PK11195	114 ± 8
PK11195 + AMG	95 ± 9

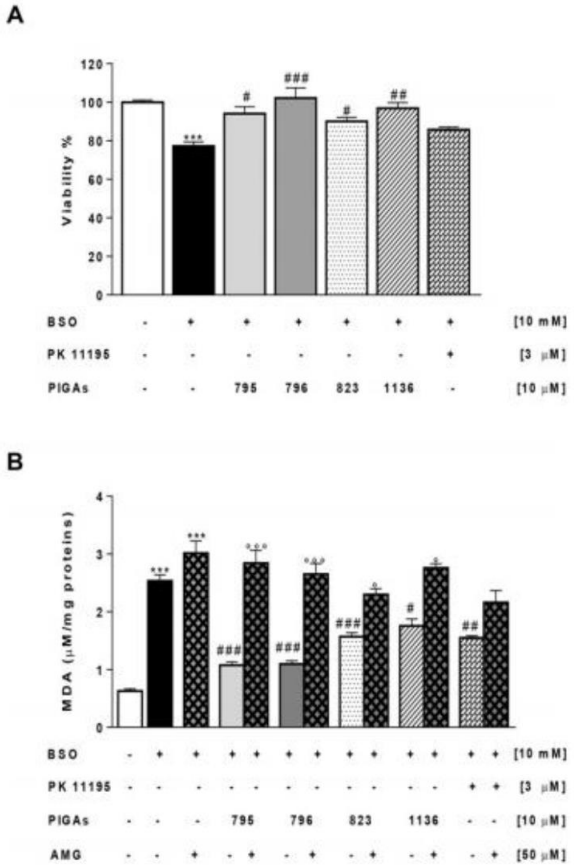


Fig.1

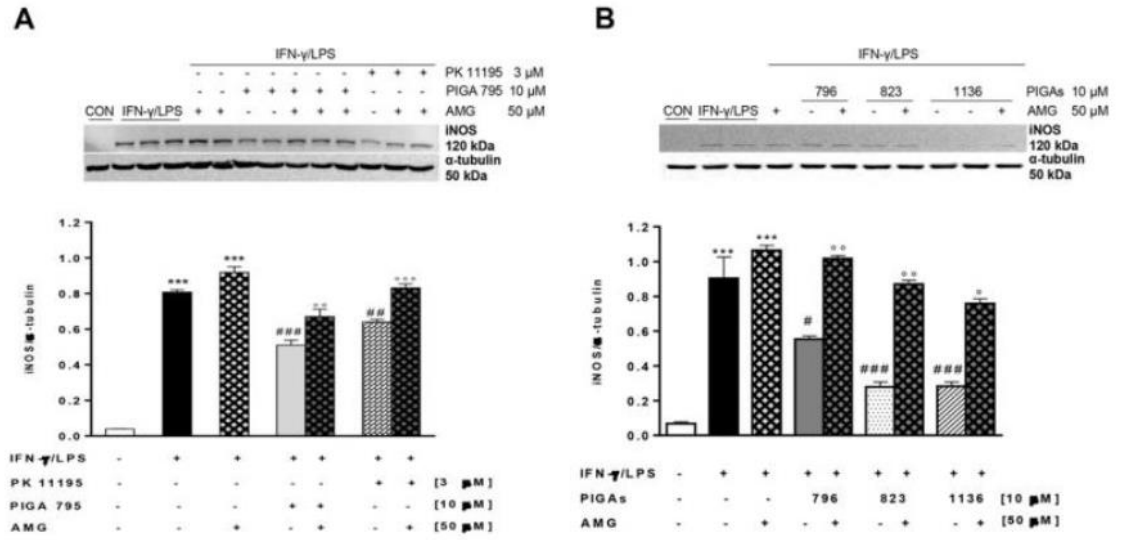

A
IFN-γ/LPS
PK 11195 3 μM
PIGA 795 10 μM
CON IFN-γ/LPS
AMG 50 μM
iNOS
120 kDa
α-tubulin
50 kDa
iNOS/α-tubulin
IFN-γ/LPS
PK 11195 [3 μM]
PIGA 795 [10 μM]
AMG [50 μM]
B
IFN-γ/LPS
796 823 1136 PIGAs 10 μM
CON IFN-γ/LPS AMG 50 μM
iNOS
120 kDa
α-tubulin
50 kDa
iNOS/α-tubulin
IFN-γ/LPS
PIGAs [10 μM]
AMG [50 μM]

Fig.2

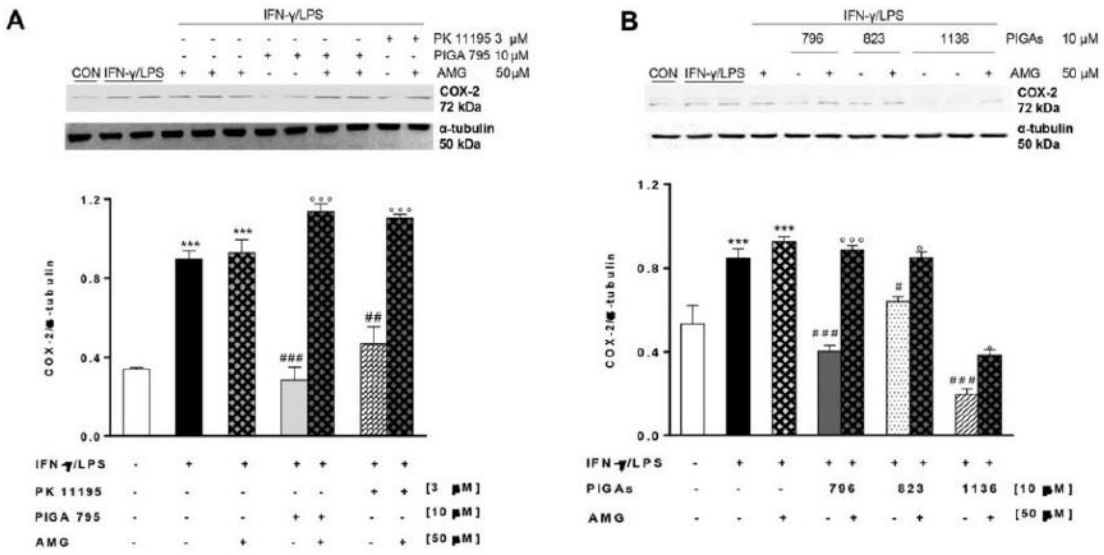

A
IFN-γ/LPS
PK 11195 3 μM
PIGA 795 10 μM
CON IFN-γ/LPS
AMG 50 μM
COX-2
72 kDa
α-tubulin
50 kDa
COX-2/α-tubulin
IFN-γ/LPS
PK 11195 [3 μM]
PIGA 795 [10 μM]
AMG [50 μM]
B
IFN-γ/LPS
796 823 1136 PIGAs 10 μM
CON IFN-γ/LPS AMG 50 μM
COX-2
72 kDa
α-tubulin
50 kDa
COX-2/α-tubulin
IFN-γ/LPS
PIGAs [10 μM]
AMG [50 μM]

Fig.3

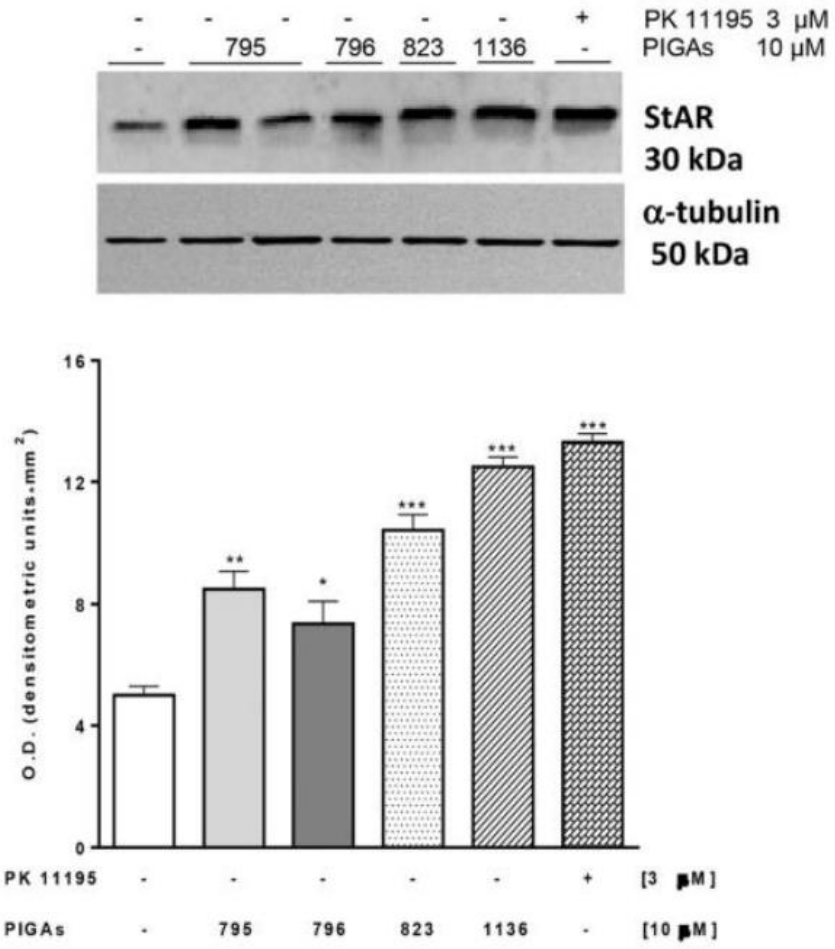

PK 11195 3 µM
PIGAs 10 µM
795
796
823
1136
StAR
30 kDa
α-tubulin
50 kDa
O.D. (densitometric units-mm²)
16
12
8
4
0
**
*
***
***
***
PK 11195
PIGAs
[3 µM]
[10 µM]

Fig.4