Double Application of Translocator Protein Ligands and RAW Cells Inflammatory Milieu

Filomena Mattner

Original Research Paper

Ligands targeting the translocator protein exhibit a variety of anti-inflammatory and neuroprotective properties. A double application of eight translocator protein ligands to activated murine macrophage-like cells changed to a different extent the levels of secreted reactive nitrogen intermediates, pro- and anti-inflammatory cytokines. To our knowledge this is the first report suggesting that multiple applications of different translocator protein ligands may have selective effects on the macrophage inflammatory milieu that do not appear to be related to just the ligand affinity.

Keywords: Translocator Protein, Macrophages, Cytokines

Introduction

The translocator protein (18 kDa) (TSPO), in conjunction with the voltage dependent anion channel and the adenine nucleotide transporter, forms an integral component of the outer mitochondrial membrane, mediating steroidogenesis, heme biosynthesis, porphyrin and anion transport, apoptosis and cell proliferation (Gavish et al., 1999; Papadopoulos et al., 2006; Rupprecht et al., 2010). This broad spectrum of bioactivities makes it an attractive therapeutic drug target and a number of TSPO ligands has been synthesised and evaluated for their functional effects on TSPO and development of potential therapeutic agents (Szweczyk and Wojtczak, 2002; Galiegue et al., 2003; Karlstetter et al., 2014; Selvaraj et al., 2015). In addition, the development of radiolabelled TSPO ligands as molecular markers for imaging (Katsifis et al., 2000; Fookes et al., 2008; Pulli and Chen, 2014; Katsifis et al., 2004) helped localizing and monitoring the TSPO upregulation on activated microglia and/or astrocytes which is one of the hallmarks of neuroinflammation and neurodegeneration (Wilms et al., 2003; Girard et al., 2008; Mattner et al., 2011; Daugherty et al., 2013; Mattner et al., 2013).

In this study, the inflammatory milieu of non-activated and activated macrophage-like cells was tested after a single and double exposure to TSPO ligands. As the ligands affinity and selectivity is dependent on the substitution patterns on the 2-phenyl ring and the acetamide side chains, six novel TSPO ligands were selected to cover a spectrum of chemical structures. The ligands PK11195 and Ro5-4864, originally used in TSPO characterization, were also included.

Materials and Methods

TSPO Ligands

The selected TSPO ligands were synthesized as previously described (Katsifis et al., 2000; 2004; Homes et al., 2006; Fookes et al., 2008) and their chemical structures and affinity for TSPO and the central Benzodiazepine Receptor (CBR) are shown in the Fig. 1. The isoquinoline carboxamide PK11195 and the benzodiazepine Ro5-4864 were purchased from Sigma-Aldrich.

RAW 264.7 Cells

RAW cells (5×10^5) were left to adhere for 5 h in 8-well chambers (Nunc Lab-Tek Chamber Slide). Some wells were treated with Lипополисахарид (LPS, Calbiochem, La Jolla, CA, USA) and mouse Interferon-gamma (IFN-γ, R and D Systems, Minneapolis, USA) at final concentrations of 1000 and 10 ng mL^-1, respectively and incubated overnight. The culture medium (Staykova et al., 2008) was removed and a fresh medium containing the TSPO ligands at final concentrations of 100, 10 or 1 nM was added to non-treated or LPS-IFN-γ treated cells. After 12 h the medium was replaced with a fresh one containing the same ligands and at the same concentrations. Culture supernatants were collected 12 h later and assayed.
Fig. 1. TSPO ligand chemical structures and binding affinities for TSPO and CBR

**Reactive Nitrogen Intermediates (RNI)**

The nitrite was measured colorimetrically after addition of Griess reagent. Nitrate was first converted to nitrite by nitrate reductase in the presence of NADPH (Boehringer Mannheim, Germany) as previously described (Staykova et al., 2008).

**Cytokines**

The levels of Tumour Necrosis Factor (TNF), and interleukines (IL: IL-6, IL-10, IL-4) were measured in triplicates in culture supernatants of non-activated and (LPS+IFN-γ) activated RAW cells using ELISA kit (eBioscience, San Diego, Ca, USA) according to the manufacturer’s instructions.

**Results**

**RNI Levels**

There was no change in the RNI levels after a single exposure to any ligand at concentrations of 100 and 10 nM as well as after a double treatment of non-activated RAW cells. However, a double application of all ligands to activated RAW cells resulted in a decrease of the RNI values. At both doses, the strongest effect was observed with PBR103 (70±2.2 nM), PBR111 (72.6±2.8 nM) and PBR159 (59.7±3.2 nM) (Table 1 and Supplementary Table).

**Pro-Inflammatory (TNF and IL-6) and Anti-Inflammatory (IL-4 and IL-10) Cytokines**

At the three doses tested a single application of the TSPO ligands had no effect on the levels of these cytokines secreted by non-treated or activated RAW cells.

A double treatment of activated RAW cells with PBR103, PBR111, PBR121, PBR159 PBR200 and CLINDM also resulted in insignificant changes in TNF. In contrast, a double treatment with Ro5-4864 and PK11195 decreased the TNF levels to 2270±120 and 2160±120 pg mL⁻¹, respectively (Table 1 and Supplementary Table). Double exposure of activated RAW cells to all ligands reduced the IL-6 levels. Most effective was the dose of 1 nM for PBR103, PBR111 and PBR121 (1410±100 pg mL⁻¹).

In contrast, double treatment with all ligands resulted in an increase of both anti-inflammatory cytokines. For IL-4 an increase to 0.15±0.02 pg mL⁻¹ was observed at a dose of 1 nM for ligands PBR103, PBR111, PBR121 and PK11195, while the same dose of 1 nM increased the IL-10 values to 0.19±0.01 pg mL⁻¹ for PBR103, 0.17±0.01 pg mL⁻¹ for PBR111, 0.16±0.02 pg mL⁻¹ for PBR121, 0.17±0.02 pg mL⁻¹ for PBR159, 0.16±0.02 pg mL⁻¹ for CLINDM, 0.17±0.01 pg mL⁻¹ for PK11195 and 0.20±0.01 pg mL⁻¹ for PBR200. With respect to Ro5-4864, most efficient was the dose of 100 nM (0.17±0.01 pg mL⁻¹) (Table 1 and Supplementary Table).
Table 1. Effect of double application of TSPO ligands on RNI and cytokine levels in cultures of activated RAW cells

| Ligand   | TNF pg mL⁻¹ | IL-6 pg mL⁻¹ | IL-4 pg mL⁻¹ | IL-10 pg/mL⁻² |
|----------|--------------|--------------|--------------|---------------|
| PK11195  | ↑30 ±15      | ↑12 ±7       | ↑20 ±12      | ↑14 ±14       |
| Ro5-4864 | ↓20 ±12      | ↓20 ±20      | ↓36 ±18      | ↓7 ±14        |
| PBR200   | ↓40 ±15      | ↓8 ±0        | ↓25 ±14      | ↓18 ±15       |
| CLINDM   | ↓10 ±15      | ↓11 ±10      | ↓0 ±10       | ↓15 ±15       |
| PK11195  | ↓10 ±12      | ↓37 ±35      | ↓23 ±23      | ↓8 ±15        |

1% decrease and ↑% increase in the values in comparison to the ones for activated RAW cells. nt = not tested.

Supplementary Table. Effect of double application of eight TSPO ligands on RNI and cytokine levels in cultures of RAW cells

| Ligand | RNI nM | TNF pg mL⁻¹ | IL-6 pg mL⁻¹ | IL-4 pg mL⁻¹ | IL-10 pg/mL⁻² |
|--------|--------|-------------|-------------|-------------|---------------|
| PK11195| 70     | 4300 ±3800  | 2900 ±1400  | 1800 ±1400  | 1800 ±1400    |
| Ro5-4864| 72.6  | 3600 ±3500  | 3000 ±1600  | 1700 ±1400  | 1700 ±1400    |
| PBR103 | 77.6  | 3900 ±3700  | 3350 ±1600  | 1900 ±1400  | 1900 ±1400    |
| PBR111 | 59.7  | 3300 ±3500  | 2700 ±1800  | 1800 ±1400  | 1800 ±1400    |
| PBR159 | 89    | 3100 ±400   | 3600 ±2200  | 2200 ±1900  | 2200 ±1900    |
| PBR200 | 90    | 2260 ±3300  | 2900 ±1500  | 1500 ±1300  | 1500 ±1300    |
| CLINDM | 415   | 444 ±440    | 440 ±240    | 25 ±23       | 23 ±23        |
| PBR159 | 15    | 444 ±440    | 400 ±300    | 25 ±23       | 23 ±23        |
| PK11195| 15    | 444 ±440    | 400 ±300    | 25 ±23       | 23 ±23        |

Discussion

A number of reports show an increase in the number of TSPO binding sites in the central and peripheral nervous systems during inflammation as well as neuroprotective effects by TSPO ligands (Ferzaz et al., 2002; Wilms et al., 2003; Mattner et al., 2011; Girard et al., 2012; Daugherty et al., 2013; Mattner et al., 2013; Bae et al., 2014; Morato et al., 2014). Because the principal effector cells in neuroinflammatory and neuro-degenerative disorders are microglia, monocyte-derived macrophages, macrophages in the perivascular space, the choroid plexus and the meninges (Bogie et al., 2014), the use of the RAW cell line (derived from murine macrophages) was an appropriate choice for this study.

In a search of ways to decrease inflammation, and in particular-the neuroinflammation, the effects of two TSPO ligands, Ro5-4864 and PK11195, were extensively studied (Zavala et al., 1990; Ferzaz et al., 2002; Casellas et al., 2002; Cunningham, 2013; Bae et al., 2014; Morato et al., 2014). However, their anti-inflammatory properties were attributed mainly to changes in the steroid synthesis. Recently the TSPO specific ligand vinpocetine was shown to inhibit the production of nitric oxide, IL-1β, IL-6 and TNF-α in microglial cells treated with lipopolysaccharide or exposed to oxygen-glucose deprivation (Zhao et al., 2011). Our in vitro study supports the view that a spectrum of cytokine changes occurs also as a direct result of ligand binding to TSPO. On the other hand, it is difficult to compare our results about no change in RNI and cytokine levels after a single application of Ro5-4864 and PK11195 with the results for rat microglial cells (Choi et al., 2011) and the murine immortalized microglial cell line BV2 (Bae et al., 2014) because the cell activation and the ligands application were the other way round.

While there is a number of pharmacophores with high affinity binding and selectivity to TSPO, the
structure-affinity relationship models are unable to predict the therapeutic effects. Now we show that double application of nanomolar concentrations of eight TSPO ligands not only has a general inhibitory effect on the pro-inflammatory milieu of activated macrophage-like cells but, more importantly, that the effect do not appear to be related to just the ligand affinity for TSPO or CBR. We believe that these preliminary results on six novel TSPO ligands are encouraging and we intend to increase the number of TSPO ligands tested, as well as the number of applications to non-activated and activated macrophages in order to get a better picture for their potential selective use in regulation of inflammation.

Conclusion
To our knowledge this is the first report suggesting that multiple applications of different translocator protein ligands may have selective effects on the macrophage inflammatory milieu that do not appear to be related to just the ligand affinity.

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Author’s Contributions
Maria Staykova: Designed the experiment and performed the RNI tests.

David Linares: Carried out the tests for cytokines.

Filomena Mattner and Andrew Katsifis: Synthesized the six TSPO ligands.

Ethics
This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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