Original Research Paper

Double Application of Translocator Protein Ligands and RAW Cells Inflammatory Milieu

[1,2]**Maria Staykova**, [3]**Filomena Mattner**, [2]**David Linares and** [3,4]**Andrew Katsifis**

[1]*The John Curtin School of Medical Research, Australian National University, Australia*
[2]*Neurosciences Research Unit, Canberra Hospital, Australia*
[3]*Department of PET and Nuclear Medicine, Royal Prince Alfred Hospital, Sydney, Australia*
[4]*School of Pharmacy, University of Sydney, Australia*

Abstract: 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 bio-activities makes it an attractive theurapeutic 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 (Szewczyk 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 Lipopolysaccaride (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.
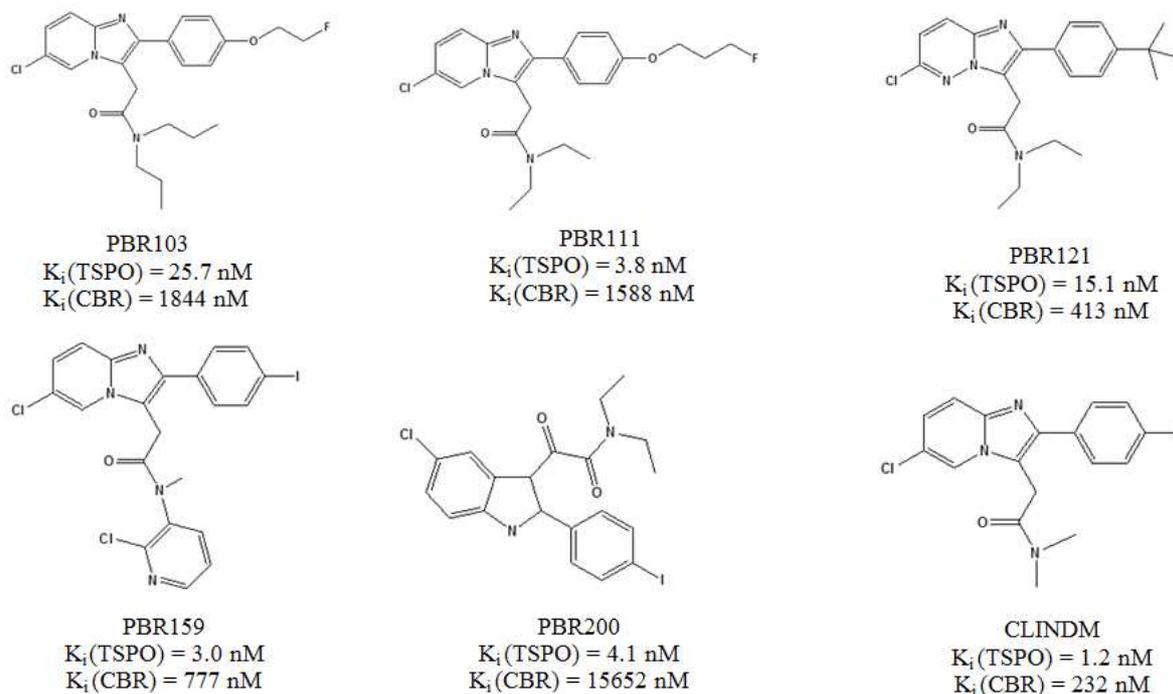
Fig. 1. TSPO ligand chemical structures and binding affinities for TSPO and CBR

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

	RNI		TNF			IL-6			IL-4			IL-10		
Ligand nM	100	10	100	10	1	100	10	1	100	10	1	100	10	1
PBR103	↓30	↓30	↑12	↑7	↓20	↓20	↓20	↓36	↑14	↑14	↑27	↑90	nt	↑90
PBR111	↓27	↓27	0	0	↓13	↓23	↓28	↓36	↑8	↑8	↑27	↑70	nt	↑70
PBR121	↓22	↓20	↑9	↑4	↓7	↓14	↓28	↓35	↑8	↑15	↑27	↑56	nt	↑60
PBR159	↓40	↓37	↓8	0	↓25	↓20	↓20	↓20	↑18	↑15	↑15	↑44	↑56	↑67
PBR200	↓10	↓10	↓13	↑13	0	0	0	↓14	↑15	↑15	↑15	↑56	↑90	↑100
CLINDM	↓10	↓15	↓37	↓7	↓20	↓28	↓22	↓28	↑8	↑8	↑15	↑33	↑45	↑56
Ro5-4864	↓15	↓15	↓44	↓44	↓40	↓25	↓23	↓23	0	↑8	↑15	↑67	↑56	↑33
PK11195	↓10	↓12	↓37	↓35	↓42	↓23	nt	↓23	↑8	↑15	↑27	↑44	↑70	↑70

↓% 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

	RNI nM		TNF pg mL^{-1}			IL-6 pg mL^{-1}			IL-4 pg mL^{-1}			IL-10 pg/mL^{-1}		
Non treated	5.3±0.5		380±100			18±3			0.09±0.03			0.06±0.007		
Activated	99.5±3.8		3600±400			2200±200			0.12±0.02			0.10±0.01		
Ligand nM	100	10	100	10	1	100	10	1	100	10	1	100	10	1
PBR103	70	70.1	4300	3800	2900	1800	1800	1410	0.14	0.14	0.15	0.19	nt	0.19
	±2	±2	±300	±400	±500	±300	±300	±200	±.02	±.02	±.02	±.01		±.01
PBR111	72.6	72.6	3600	3500	3000	1700	1600	1410	0.13	0.13	0.15	0.17	nt	0.17
	±3	±3	±300	±400	±500	±300	±400	±200	±.02	±.02	±.01	±.01		±.01
PBR121	77.6	79.6	3900	3750	3350	1900	1600	1410	0.13	0.14	0.15	0.16	nt	0.16
	±3	±3	±400	±500	±500	±300	±300	±100	±.02	±.02	±.02	±.02		±.02
PBR159	59.7	63	3300	3500	2700	1800	1800	1800	0.14	0.14	0.14	0.14	0.16	0.17
	±3	±3	±400	±400	±500	±200	±300	±300	±.02	±.02	±.02	±.02	±.02	±.02
PBR200	89	89.2	3100	4070	3600	2200	2200	1900	0.14	0.14	0.14	0.16	0.19	0.2
	±4	±3	±300	±500	±400	±300	±200	±300	±.02	±.02	±.02	±.01	±.02	±.01
CLINDM	90	84.1	2260	3300	2900	1500	1700	1500	0.13	0.16	0.14	0.14	0.15	0.16
	±5	±3	±200	±500	±400	±300	±400	±300	±.02	±.02	±.02	±.02	±.02	±.02
Ro5-4864	84.6	84.5	2270	2270	2160	1600	1700	1700	0.12	0.14	0.14	0.17	0.16	0.13
	±4	±5	±120	±150	±120	±300	±300	±500	±.02	±.02	±.02	±.01	±.02	±.02
PK11195	90	87.1	2260	2340	2090	1700	nt	1700	0.13	0.14	0.15	0.14	0.17	0.17
	±5	±5	±350	±300	±210	±300		±300	±.02	±.02	±.02	±.02	±.02	±.01

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 milleu 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.

Funding Information

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.

References

Bae, K.R., H.J. Shim, D. Balu, S.R. Kim and S.W. Yu, 2014. Translocator protein 18 kDa negatively regulates inflammation in microglia. J. Neuroimmune. Pharmacol., 9: 424-437. PMID: 24687172

Bogie, J.F., P. Stinissen and J.J. Hendriks, 2014. Macrophage subsets and microglia in multiple sclerosis. Acta Neuropathol., 128: 191-213. DOI: 10.1007/s00401-014-1310-2

Casellas, P., S. Galiegue and A.S. Basile, 2002. Peripheral benzodiazepine receptors and mitochondrial function. Neurochem. Int., 40: 475-486. DOI: 10.1016/S0197-0186(01)00118-8

Choi, J., M. Ifuku, M. Noda and T.R. Guilarte, 2011. Translocator protein (18 kDa)/peripheral benzodiazepine receptor specific ligands induce microglia functions consistent with an activated state. Glia, 59: 219-230. DOI: 10.1002/glia.21091

Cunningham, C., 2013. Microglia and neurodegeneration: The role of systemic inflammation. Glia, 61: 71-90. DOI: 10.1002/glia.22350

Daugherty, D.J., V. Selvaraj, O.V. Chechneva, X.B. Liu and D.E. Pleasure *et al.*, 2013. A TSPO ligand is protective in a mouse model of multiple sclerosis. EMBO Mol. Med., 5: 891-903. DOI: 10.1002/emmm.201202124

Ferzaz, B., E. Brault, G. Bourliaud, J.P. Robert and G. Poughon *et al.*, 2002. SSR180575 (7-Chloro-N,N,5-trimethyl-4-oxo-3-phenyl-3,5-dihydro-4H-pyridazino[4,5-b]indole-1-acetamide), a peripheral benzodiazepine receptor ligand, promotes neuronal survival and repair. J. Pharmacol. Exp. Ther., 301: 1067-1078. DOI: 10.1124/jpet.301.3.1067

Fookes, C.J., T.Q. Pham, F. Mattner, I. Greguric and C. Loc'h *et al.*, 2008. Synthesis and biological evaluation of substituted [^{18}F]Imidazo[1,2-a]pyridines and [^{18}F]Pyrazolo[1,5-a]pyrimidines for the study of the peripheral benzodiazepine receptor using positron emission tomography. J. Med. Chem., 51: 3700-3712. DOI: 10.1021/jm7014556

Galiegue, S., N. Tinel and P. Casellas, 2003. The peripheral benzodiazepine receptor: A promising therapeutic drug target. Curr. Med. Chem., 10: 1563-1572. PMID: 12871127

Gavish, M., I. Bachman, R. Shoukrun, Y. Katz and L. Veenman *et al.*, 1999. Enigma of the peripheral benzodiazepine receptor. Pharmacol. Rev., 51: 629-650. PMID: 10581326

Girard, C., S. Liu, D. Adams and G. Groyer, 2012. Axonal regeneration and neuroinflammation: Roles for the translocator protein 18 kDa. J. Neuroendocrinol., 24: 71-81. DOI: 10.1111/j.1365-2826.2011.02215.x

Girard, C., S. Liu, F. Cadepond, D. Adams and C. Lacroix *et al.*, 2008. Etifoxine improves peripheral nerve regeneration and functional recovery. PNAS, 105: 20505-20510. DOI: 10.1073/pnas.0811201106

Homes, T.P., F. Mattner, P.A. Keller and A. Katsifis, 2006. Synthesis and *in vitro* binding of *N,N*-dialkyl-2-phenylindol-3-yl-glyoxylamides for the peripheral benzodiazepine binding sites. Bioorg. Med. Chem., 14: 3938-3946. DOI: 10.1016/j.bmc.2006.01.039

Karlstetter, M., C. Nothdurfter, A. Aslanidis, K. Moeller and F. Horn *et al.*, 2014. Translocator protein (18 kDa) (TSPO) is expressed in reactive retinal microglia and modulates microglial inflammation and phagocytosis. J. Neuroinflammat., 11: 3-3. DOI: 10.1186/1742-2094-11-3

Katsifis, A., F. Mattner, B. Dikic and V. Papazian, 2000. Synthesis of substituted [123I]imidazo[1,2-α]pyridines as potential probes for the study of the peripheral benzodiazepine receptors using SPECT. Radiochim Acta, 88: 229-232. DOI: 10.1524/ract.2000.88.3-4.229

Katsifis, A., G. Barlin, F. Mattner and B. Dikic, 2004. Synthesis of [123I]iodine labelled imidazo[1,2-b] pyridazines as potential probes for the study of peripheral benzodiazepine receptors using SPECT. Radiochimica Acta, 92: 305-309. DOI: 10.1524/ract.92.4.305.35581

Mattner, F., D. Bandin, M. Staykova, P. Berghofer and M.C. Gregoire *et al.*, 2011. Evaluation of [123I]-CLINDE as a potent SPECT radiotracer to assess the degree of astroglia activation in cuprizone-induced neuroinflammation. Eur. J. Nucl. Med. Mol. Imag., 38: 1516-1528. DOI: 10.1007/s00259-011-1784-2

Mattner, F., M. Staykova, P. Berghofer, H.J. Wong and S. Fordham *et al.*, 2013. Central nervous system expression and pet imaging of the translocator protein in relapsing–remitting experimental autoimmune encephalomyelitis. J. Nucl. Med., 54: 291-298. DOI: 10.2967/jnumed.112.108894.

Morato, L., E. Bertini, D. Verrigni, M. Ruiz and I. Ferrer *et al.*, 2014. Mitochondrial dysfunction in central nervous system white matter disorders. Glia, 62: 1878-1894. PMID: 24865954

Papadopoulos, V., M. Baraldi, T.R. Guilarte, T.B. Knudsen and J.J. Lacapère *et al.*, 2006. Translocator protein (18 kDa): New nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol. Sci., 27: 402-409. DOI: 10.1016/j.tips.2006.06.005

Pulli, B. and J.W. Chen, 2014. Imaging neuroinflammation-from bench to bedside. J. Clin. Cell Immunol., 5: 266-266. DOI: 10.4172/2155-9899.1000226

Rupprecht, R., V. Papadopoulos, G. Rammes, T.C. Baghai and J. Fan *et al.*, 2010. Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nat. Rev. Drug. Discov., 9: 971-988. DOI: 10.1038/nrd3295

Selvaraj, V., D.M. Stocco and L.N. Tu, 2015. Minireview: Translocator Protein (TSPO) and steroidogenesis: A reappraisal. Mol. Endocrinol., 29: 490-501. PMID: 25730708

Staykova, M., D. Linares, S. Fordham, J. Paridaen and D.O. Willenborg, 2008. The innate immune response to adjuvants dictates the adaptive immune response to autoantigens. J. Neuropathol. Exp. Neurol., 67: 543-554. DOI: 10.1097/NEN.0b013e31817713cc

Szewczyk, A. and L. Wojtczak, 2002. Mitochondria as a pharmacological target. Pharmacol. Rev., 54: 101-127. PMID: 11870261

Wilms, H., J. Claasen, C. Rohl, J. Sievers and G. Deuschl *et al.*, 2003. Involvement of benzodiazepine receptors in neuroinflammatory and neurodegenerative diseases: Evidence from activated microglial cells *in vitro*. Neurobiol. Dis., 14: 417-424. DOI: 10.1016/j.nbd.2003.07.002

Zavala, F., V. Taupin and B. Descamps-Latscha, 1990. *In vivo* treatment with benzodiazepines inhibits murine phagocyte oxidative metabolism and production of interleukin 1, tumor necrosis factor and interleukin-6. J. Pharmacol. Exp. Ther., 255: 442-450. J. Pharmacol. Exp. Ther., 255: 442-450. PMID: 1978727

Zhao, Y., J. Yu, Q. Li, C. Ma and C. Lu *et al.*, 2011. TSPO-specific ligand vinpocetine exerts a neuroprotective effect by suppressing microglial inflammation. Neuron Glia Biol., 7: 187-197. DOI: 10.1017/S1740925X12000129