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# TSPO as a target for glioblastoma therapeutics

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## Abstract

**The translocator protein (TSPO) is an 18-kDa five-transmembrane protein, which is primarily found in the outer mitochondrial membrane. Levels of this protein are up-regulated in the most aggressive and common glioma, glioblastoma multiforme (GM). Levels of TSPO also correlate with GM clinical outcome, suggesting that TSPO may be a novel GM diagnostic imaging agent. Therapeutically, targeting the TSPO may provide a mechanism to abrogate the apoptotic-resistant, invasive and aggressive nature of GM and may also provide a way of targeting other anti-cancer treatments to GM sites. This review highlights recent progress in research on TSPO-based diagnostic imaging and therapeutics for GM.**

## The challenge of glioblastoma multiforme

Gliomas are a family of highly aggressive brain tumours formed from brain glial cells or their precursors. The most common glioma in adults is glioblastoma multiforme (GM), comprising 60 %–70 % of all primary brain tumour diagnoses [1]. GM is also the most malignant glioma [2] and even with the standard treatment of surgery paired with radiotherapy and temozolomide chemotherapy, median survival rate is only 14.2 months [1].

Numerous features of the disease contribute to the challenge of GM treatment. Firstly, as the name implies, it is heterogeneous with each tumour containing multiple cell types, pathologies and genetic mutations [2]. Secondly, it is very invasive, resulting in a topographically diffuse distribution that is difficult to completely resect with surgery [2]. Thirdly, GM shows resistance to many anti-cancer treatments, partly due to the difficulties with effectively activating intracellular death pathways [3]. Also, anti-GM agents must not only show effectiveness in treating cancer, but must also be able to effectively cross the blood–brain barrier. There is evidence that targeting the translocator protein (TSPO) may be able to circumvent these challenging aspects of GM. As such, it has recently attracted attention as both a potential GM diagnostic marker and an anti-GM therapeutic target.

## Translocator protein

The TSPO is a highly-conserved 18 kDa protein with five transmembrane $\alpha$-helices, which form a hydrophobic pocket [4]. TSPO is most commonly located on the outer mitochondrial membrane that positions it for key roles in regulating mitochondrial functions, such as $Ca^{2+}$ homoeostasis and energy production [5,6]. *In vitro* studies suggest the TSPO is involved in cholesterol translocation into the mitochondria, which is the rate-limiting step in steroidogenesis. TSPO can also bind some iron-free dicarboxylate porphyrin molecules like protoporphyrin IX and can transport them into the mitochondria, which is a major site of heme synthesis [7]. TSPO is also up-regulated in neuroinflammation, and has been suggested as a therapeutic target in neurodegenerative diseases such as Alzheimer's disease [8]. The critical role of TSPO in these functions was evidenced by an early TSPO knockout study in which animals showed an embryonic lethal phenotype [9]. However, more recent knockout and conditional knockdown studies produce some viable mice with normal cholesterol transport, no defects in steroidogenesis and normal protoporphyrin IX metabolism [10,11]. Although the authors acknowledge that

**Key words:** apoptosis, drug design, glioblastoma, translocator protein.

**Abbreviations:** Bcl-2, B-cell lymphoma 2; BNCT, boron neutron capture therapy; BPA, BPA-fr (L-boronophenylalanine–D-fructose complex); BSH, borocaptate ion $[B_{12}H_{11}SH]^{2-}$; DPA-714, N,N-diethyl-2-(2-[4(2-fluoroethoxy)phenyl]-5,7-dimethylpryazolo[1,5-a]pyrimidine-3-yl)acetamide; GM, glioblastoma multiforme; MPTP, mitochondrial permeability transition pore; TSPO, translocator protein.

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it is possible that other systems may have partly compensated for TSPO knockout [12], these recent studies suggest the function of TSPO may be more complex than originally proposed.

In addition to the aforementioned functions, the TSPO has been implicated in regulation of apoptosis and cellular proliferation, as detailed in section 'TSPO as a Glioblastoma Therapeutic Target'. The evidence implicating the TSPO in these functions has led to it becoming a potential diagnostic imaging and drug target in GM.

## TSPO as a glioblastoma diagnostic marker

Identification of GM imaging biomarkers may assist in clinical diagnosis and outcome prediction. Oncology studies often use 2-deoxy-2-[18F]fluoroglucose as a diagnostic marker, but the high use of glucose by normal brain tissue results in poor tumour-to-background contrast in GM [13]. Hence, there is a requirement for further development of diagnostic markers that show high expression within the cancer relative to non-cancerous brain tissue, as well as a correlation between marker level and clinical outcome.

Imaging the TSPO meets both these criteria. Immunocytochemistry reveals undetectable to low TSPO levels in normal human brain tissue, but high levels in human glioma (15× that of normal brain tissue) [14–16]. *In vitro*, levels of TSPO correlate with tumorogenicity, for example, they are highest in the highly proliferative C6 glioma cells and lowest in the less-aggressive T98G glioblastoma cells [17]. The level of TSPO in sections of human glioma strongly correlates with malignancy level, proliferative index and life expectancy [14–16]. In pre-clinical trials, positron emission tomography using radiolabelled high-affinity TSPO ligands in glioma-bearing rats showed good specific tumour uptake, monitoring and quantification of progression, which corresponded with subsequent immunohistochemical analysis of the tumours [13,18–20]. Ligands could be displaced by high concentrations of unlabelled TSPO ligands, further suggesting specific binding to TSPO sites on tumours [18,19].

Two clinical trials have examined the ability of the high-affinity TSPO ligand $^{11}$C-PK 11195 to imaging human GM. An early study found increased radioactivity in glioma in 80% of patients [21], with a 2-fold higher signal in glioma tissue compared with normal grey matter [22]. A more recent trial found low-grade astrocytomas could be differentiated from GM-like gliomas on the basis of the kinetics of tissue time–activity curves [23]. Although it has high affinity to the TSPO, PK 11195 shows highly variable kinetic behaviour and low bioavailability *in vivo* [24,25], suggesting it may not be an ideal ligand to use for clinical GM imaging. Further clinical trials utilizing ligands such as DPA-714 (N,N-diethyl-2-(2-[4(2-fluoroethoxy)phenyl]-5,7-dimethylpryazolo[1,5-a]pyrimidine-3-yl)acetamide) that show good bioavailability and stability in humans [26] may further reveal the usefulness of TSPO ligands for GM diagnostic imaging.

## TSPO as a glioblastoma therapeutic target

The up-regulation of TSPO in GM and the correlation between TSPO levels, GM malignancy and clinical outcome has sparked investigation of TSPO as a GM therapeutic target. Several lines of evidence have revealed that the TSPO may play a functional role in GM. Investigation has focused on the ability of TSPO modulation to influence the characteristics of GM which make it difficult to treat: resistance to apoptosis, invasiveness and aggressiveness.

### Resistance to apoptosis

Apoptosis or programmed cell death is a necessary process through which damaged cells are eliminated. Through up-regulation of anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2), cancer cells develop a resistance to apoptosis during tumorogenesis, as shown in Figure 1 [27]. This resistance to apoptosis is a major cause of GM treatment failure. Recently, drugs that can activate cancer cell death through mitochondrial membrane permeabilization have shown use in overcoming GM resistance to apoptosis [28]. Mitochondrial membrane permeabilization can involve opening of the mitochondrial permeability transition pore (MPTP), resulting in cytochrome *c* release into the cytoplasm, subsequent activation of caspase 9, then caspase 3 that initiates the caspase cascade, resulting in apoptosis [29] (Figure 1). TSPO may form part of the MPTP as it co-precipitates with adenine nucleotide transporter (ANT) and the voltage-dependent anion carrier (VDAC), which are key members of the pore [30,31]. Given this, the TSPO is ideally situated to regulate mitochondrial membrane permeabilization and apoptosis, potentially allowing a means to bypass the controlling effect of the Bcl-2 family of anti-apoptotic proteins in GM [28] (Figure 1).

The necessity of the TSPO to apoptosis in GM cells is reinforced by the fact that numerous agents that show pro-apoptotic activity in GM require the TSPO to exert these effects. Ligands to the TSPO co-applied with erucylphosphohomocholine or cobalt chloride block the pro-apoptotic effects of these agents, presumably by competing at the TSPO site [3,32]. The pro-apoptotic effect of glutamate in C6 cells is also abrogated when TSPO levels are knocked down [33]. Furthermore, there is a wealth of experimental evidence suggesting direct binding of the TSPO can induce apoptosis in GM cells. PK 11195 can greatly reduce the anti-apoptotic effect of Bcl-2 family proteins [28]. Various TSPO ligands dissipate mitochondrial membrane permeability, release cytochrome *c*, lead to DNA fragmentation, induce apoptosis and decrease viability in seven different glioma and glioblastoma cell lines [28,32,34–37]. It should be noted, though, that the TSPO ligand Ro 5-4864 does not induce apoptosis and in fact has been reported to be either inactive or to decrease apoptosis in GM cells [17,32,38]. Ro 5-4864 binds the TSPO at slightly different residues to PK 11195 [39], therefore pro-apoptotic effects of TSPO ligands may depend on binding site. This is an aspect of TSPO pharmacology that has been largely ignored. In

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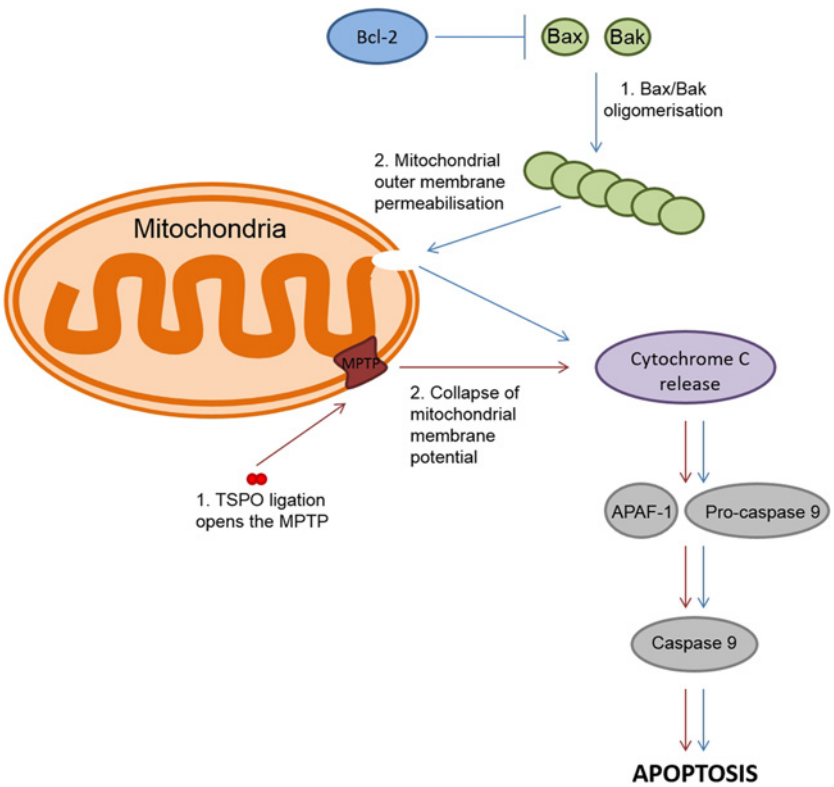
**Figure 1 | Ligation of TSPO may overcome GM resistance to apoptosis by bypassing the influence of anti-apoptotic Bcl-2**

The blue arrows show Bax and Bak oligomerization leads to mitochondrial outer membrane permeabilization, allowing cytochrome *c* release from the mitochondria. Cytochrome *c*, with apoptosis protease-activating factor-1 (APAF-1) and procaspase-9, activate caspase 9 which initiates a caspase cascade, resulting in apoptosis. Bcl-2 inhibits the first step of this process, preventing apoptosis. The red arrows show that ligation of TSPO, which may form part of the MPTP, brings about collapse of the mitochondrial membrane potential and subsequent cytochrome *c* release, which results in apoptosis via the same pathway as Bax/Bak oligomerization.




fact, we have identified compounds that appear to interact at the TSPO in an allosteric manner [40] (Eryn L. Werry, R. Narlawar and Michael Kassiou, unpublished data) and in consideration alongside the known influence of the rs6971 polymorphism on TSPO binding [41], more investigation into the influence of TSPO pharmacology on pro-apoptotic potential needs to occur.

## Invasiveness

The aggressive invasiveness of GM and other cancers may be partly due to the changes in cell adhesion to extracellular matrix proteins that promote cell migration [42–44]. Addition of PK 11195 or knockdown of TSPO in U118MG glioblastoma cells decreases adhesion to extracellular matrix proteins such as collagen, fibronectin and fibrinogen [44]. When these U118MG cells with decreased TSPO levels were implanted into a xenograft mouse model, they invaded larger areas of the brain than wild-type U118MG cells [44]. These results suggest endogenous TSPO may contribute to extracellular matrix adhesion and restrict invasiveness.

The mechanisms by which TSPO may influence extracellular matrix adhesion and migration are, as yet, unknown. Although TSPO is usually found primarily in the mitochondria in less-aggressive gliomas such as astrocytomas [45], there are reports of TSPO translocation to the nucleus in some glioma cell lines such as MGM-1 and in glioma biopsies [45]. Given that knockdown of TSPO can effect expression of genes involved in adhesion, migration and invasiveness, it may be that translocation of the TSPO to the nucleus facilitates invasiveness at a genetic level [44,45].

## Proliferation

TSPO density can influence the metastatic potential of GM. Overexpression of TSPO potentiated proliferation in C6 glioma cells [46], whereas knockdown of TSPO in ADF cells lowered proliferation rate [36]. Glioma cell lines with lower TSPO levels also show lower levels of proliferation [17]. Pharmacological data also provide evidence for an anti-proliferative role of TSPO, with a variety of TSPO ligands leading to decreased glioma cell line proliferation [32,35,38].

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The mechanism by which TSPO ligands affect proliferation is unclear. The extent to which the anti-proliferative effect is independent of the pro-apoptotic actions is not known. If the anti-proliferative effect is independent, the translocation of TSPO to the nucleus in some glioblastoma cell lines may indicate a direct regulation of proliferation at the nuclear level [45]. Furthermore, TSPO ligands can affect steroid biosynthesis [45], which may also influence GM proliferation [47].

### Pre-clinical trials

To the best of our knowledge, there have not been any pre-clinical trials examining the effectiveness of TSPO ligands to restrict *in vivo* GM growth. Promisingly, the use of a TSPO ligand restricted the growth of ingrafted colorectal tumour cells in mouse thighs [48] and of xenografted human prostate cancer cells without inducing toxicity [49]. Similar work exposing GM xenografts to TSPO ligands will be useful in further exploring the anti-GM therapeutic potential of TSPO ligands.

## Using TSPO as a Trojan horse

Apart from direct ligation of TSPO to circumvent the apoptotic-resistant, invasive and proliferative nature of GM, TSPO may have use as a 'Trojan horse' through which to administer other anti-GM treatments. As there are greatly up-regulated levels of TSPO in GM compared with normal brain tissue, combining other treatments with TSPO ligands allows a way of directing treatments to regions of need, sparing healthy tissue from treatment-induced damage.

One example of this is boron neutron capture therapy (BNCT), which is an experimental GM treatment that uses non-radioactive $^{10}B$ nuclei taken up by GM. On capture of thermal neutrons, these nuclei release high linear energy transfer particles ($^{4}He^{2+}$ and $^{7}Li^{3+}$), which travel approximately 10 $\mu$m. This results in ionizing damage in cells containing $^{10}B$ but not surrounding cells [50], suggesting an effective BNCT agent must deliver an adequate quantity of 10B atoms selectively to GM sites. Current BNCT agents, such as BPA [BPA-fr (L-boronophenylalanine–D-fructose complex)] and borocaptate ion $[B_{12}H_{11}SH]^{2-}$ (BSH) only have limited success against GM. High-affinity TSPO ligands engineered to contain $^{10}B$ atoms however deliver 10 times as many $^{10}B$ atoms to GM cell cultures than BPA and BSH, suggesting TSPO ligands may be effective BNCT agents [50]. Further *in vivo* work will reveal the potential use of TSPO ligands for BNCT agent delivery to GM.

## Challenges and future work

Although numerous ligands have shown an ability to circumvent the challenging nature of GM, some challenges must be overcome before clinical trials of these ligands can proceed. One major challenge is that TSPO ligands frequently induce apoptosis in GM cells with a potency that is often more than 1000-fold higher than their affinity for the TSPO [28,32,35]. This discrepancy has raised questions about whether the anti-GM effects of these ligands may be through non-specific pathways that do not require the TSPO. As PK 11195 was able to sensitize the MPTP to $Ca^{2+}$ in conditional TSPO knockout mice, the authors suggested PK 11195 may target other important mitochondrial proteins such as ATP synthase [51]. To address these concerns, Chelli et al. [36] showed that impairments in GM cell line viability and mitochondrial membrane potential induced by PK 11195 were prevented when TSPO was silenced with siRNA. Costa et al. [52] also showed GM cell line viability impairment induced by a high-affinity TSPO ligand was decreased by knocking down TSPO levels. The authors showed this compound did not affect ATP synthase activity in GM cells. Furthermore, Castellano et al. [34] showed a 4-phenylquinazoline-2- carboxamide derivative did not activate common non-specific pathways such as G-protein coupled receptors and 13 GM-related kinases, despite impairing viability and depolarizing mitochondrial membrane potential in a GM cell line. These studies suggest, at least *in vitro*, the discrepancy between affinity and potency of anti-GM effects may be attributable to a mechanism other than non-specificity. Recently, insight into what this mechanism may be was provided by Costa et al. [52], who identified potency could be increased by increasing ligand residence time. A reversibly binding TSPO ligand that showed micromolar potency for impairing GM cell line viability could be transformed into a ligand that produced effects at low nanomolar doses by engineering it to covalently bind to TSPO. This suggests further development of covalent TSPO binders may produce high potency ligands that could be brought forward to clinical trial.

## Conclusions

*In vitro*, *in situ* and clinical evidence suggests levels of TSPO correlate well with tumour grade and outcome and are greatly up-regulated in GM compared with normal brain tissue, making it an attractive GM diagnostic marker. GM represents a challenge to treatment as it is highly invasive, resistant to apoptosis and potential chemotherapeutics must overcome the blood–brain barrier. Ligands that bind TSPO have shown an ability to disrupt extracellular matrix interactions, thereby reducing invasiveness. They have also shown an ability to circumvent the resistance to apoptosis that is a hallmark of GM, inducing apoptosis in a range of GM cell lines and *in vivo*. TSPO ligands with good stability and bioavailability in humans have been described, hence pre-clinical and clinical trials with these agents may present an avenue for the development of novel chemotherapeutics for GM.

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