The Translocator Protein (TSPO, 18kDa) as a Potential Molecular Target for Improved Treatment Efficacy in Photodynamic Therapy

Luke Rogers,^a Mathias O. Senge^{a*,b}

* Author for correspondence.

^a School of Chemistry, SFI Tetrapyrrole Laboratory, Trinity Biomedical Sciences Institute, 152-160 Pearse Street, Trinity College Dublin, Dublin 2, Ireland

^b Medicinal Chemistry, Institute of Molecular Medicine, Trinity Centre for Health Sciences, Trinity College Dublin, St James's Hospital, Dublin 8, Ireland

Tel.: +353 1 896 8537

Fax: +353 1 896 8536

E-mail: sengem@tcd.ie

Abstract: Since its serendipitous discovery by researchers over thirty years ago, the translocator protein (18 kDa) (TSPO) has been demonstrated to play an important role in a multitude of critical biological processes. Although implemented as a novel therapeutic and diagnostic tool for a variety of disease states, its most promising role is as a molecular target for anti-cancer treatments such as photodynamic therapy (PDT). This review gives an overview of the attempts made by researchers to design porphyrin based photosensitizers for use as anti-cancer therapeutics in PDT as well as improved imaging agents for diagnostic purposes. With a better understanding of the structure and function of the TSPO, the synthesis of porphyrins for use in PDT with optimum binding affinities will become ever more possible.

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PHOTOSENSITIZER

A photosensitizer is a molecule that typically sensitizes living cells or tissue to light. The photosensitizer achieves this through promotion to an excited state upon absorption of light. This excited species then may undergo inter-system crossing to produce singlet oxygen.

REACTIVE OXYGEN SPECIES

Oxygen species such as singlet oxygen, hydrogen peroxide or superoxide anions can be formed by several different biological mechanisms. These molecules can damage cell structures or other molecules within close proximity.

PET

Positron emission tomography (PET) is a clinical three dimensional imaging method which relies on the incorporation of radiolabelled molecules into tissue. The emitted γ -rays are detected as a tool for constructing tomographic images by computer analysis.

PHOTOTHERAPY

The use of light of a specific wavelength to treat a number of biological disorders such as eczema, acne vulgaris and cancer.

PORPHYRIA

A group of inherited or acquired disorders caused by deficiencies in the enzymes involved in the biosynthetic pathway of heme. These disorders vary in severity and may manifest with skin problems, muscle weakness vomiting, abdominal pain, seizures or neurological issues or occasionally a combination of the above.

TSPO

■ Background

The translocator protein (18 kDa) is a five transmembrane domain protein that is localized primarily in the outer mitochondrial membrane [1] and is expressed predominantly in steroid-synthesizing tissues and the brain [2]. The TSPO was identified in 1977 whilst researchers were searching for binding sites for benzodiazepines such as diazepam in peripheral tissue [3]. Thus, the receptor was initially named the peripheral-type benzodiazepine receptor. This aimed at distinguishing these 'peripheral type' benzodiazepine receptors from the central benzodiazepine receptor, which is part of the GABA_A receptor complex, typically found in the brain [4]. A number of contentious issues arose with the term 'peripheral-type benzodiazepine receptor' as numerous ligands other than benzodiazepines bind to it. Additionally, this name did not give an accurate representation of the tissue distribution of the molecule as well as the fact that the protein is not in strict terms a receptor itself. To resolve these issues Papadopoulos *et al.* suggested the term translocator protein (18 kDa) (TSPO), as it has since been discovered to be a subunit of the mitochondrial permeability transition pore (MPTP). This pore consists of the TSPO, voltage-dependent anion channel (VDAC) and the mitochondrial inner membrane adenine nucleotide translocator (ANT) (Figure 1) [5,6].

[<Figure 1>](#)

While an important regulatory complex in its own right, the evidence is mounting that the TSPO could be used as a target of choice in cancer therapy. A major cause of treatment failure in human cancers is the evasion of cell death [7,8]. Thus, drugs designed to activate the cell death machinery may represent a more effective therapeutic option. A potent way of unleashing the machinery composed of activators such as catabolic hydrolases, proteases and nucleases is through the permeabilization of the mitochondrial outer membrane. Multiple apoptosis-inducing and necrosis-inducing biochemical cascades converge on mitochondria to cause their deregulation and permeabilization [9]. Mitochondrial dysfunctions have been linked to multiple hallmarks of cancer including insensitivity to anti-growth signals, limitless proliferative potential, impaired apoptosis, enhanced anabolism and decreased autophagy, making mitochondrially-targeted compounds a promising approach to eradicate chemotherapy-refractory cancer cells [10,11]. The anti-apoptotic effect of Bcl-2 proteins and myeloid cell leukemia sequence 1 (Mcl1) were shown to be blocked by the TSPO [12,13], which suggests that Bcl-2 imposed chemoresistance may be avoided by exploiting the TSPO [13,14].

■ Function

As a major component of the outer mitochondrial membrane, TSPO mediates various mitochondrial functions, including cholesterol transport and steroid hormone synthesis, mitochondrial respiration, mitochondrial permeability transition (MPT) pore opening, apoptosis and cell proliferation [15]. It is involved in numerous other functions including, heme biosynthesis, porphyrin transport and calcium channel modulation [16]. In addition, TSPO plays an important role in the regulation of stress response, anxiety disorders and neurodegenerative disorders (Parkinson's disease and Alzheimer's disease) [2]. Elevated TSPO expression is also related to certain cancers, such as those of the breast, colon-rectum, and prostate tissue, and has

been proposed as a novel prognostic indicator of an aggressive phenotype for these indications [17]. The same situation was revealed for glial cells, where different types of cancers show different TSPO expression, characteristic for specific lines [18].

As a result, the possible use of the TSPO as a target for chemotherapy and as a marker of neoplastic growth has begun to attract considerable attention due to its up-regulation within tumors. The unrestricted growth of tumorigenic tissues is partially due to the altered up-regulation of cellular processes, such as those connected with cholesterol transport and the mitochondria [19].

TSPO ligands induce cell-cycle arrest and, for example, the apoptosis of human colorectal carcinoma cell lines *in vitro* compared with in normal human colon tissues [20,21]. In addition, a correlation between the progression of prostate, breast and colorectal cancer and the overexpression of TSPO has been shown [22,23,24,25,26]. There may, however, be other contributing factors such as the tissue of origin that may be instrumental in determining the role of the TSPO in cell proliferation [27]. Researchers were able to demonstrate that deregulation of TSPO expression or function contributes to cellular changes such as apoptosis during tumor progression [28]. These observations indicate that the TSPO could be an interesting target for the development of future anti-cancer therapeutics.

As mentioned previously, the TSPO plays a crucial role in a number of biological functions such as normal cellular homeostasis (Figure 2). This pivotal role is demonstrated by a TSPO $-/-$ mutant, which results in an embryonic lethal phenotype in mice [29]. As a member of the mitochondrial permeability transition pore it is responsible for binding and transporting cholesterol into the mitochondria, where it is converted to pregnenolone. The translocator is also involved in the transport of preproteins into mitochondria. These preproteins are synthesized on cytosolic polysomes and are located within mitochondrial subcompartments. Significant mitochondrial resources and bioenergetics activity are required for cellular proliferation and thus the import of these proteins for mitochondrial biogenesis and metabolism is an important factor in the cell cycle.

[<Figure 2>](#)

The MPTP maintains transmembrane potential through active pore opening and closing during normal homeostatic conditions. Apoptotic factors, such as cytochrome *c* or apoptosis-inducing factor (AIF) may be released from the mitochondria into the cytosol during periods of prolonged opening of the MPTP [16,30]. Cell death may arise from this subsequent release as these apoptotic factors can induce osmotic swelling of the mitochondrial matrix which initiates a necrotic signaling cascade, ultimately ending in cell death [31].

■ Structure

Thus far, detailed X-ray crystallographic structural investigations of the TSPO have been hampered by difficulties expressing, purifying and stabilizing this membrane-bound protein. Other approaches, such as nuclear magnetic resonance and thermodynamic simulations have been employed and resulted in its description as a 18 kDa protein with five transmembrane spanning domains [32]. Recently, single-particle helical reconstruction and electron cryo-microscopy were used to obtain a three dimensional structure of the *Rhodobacter sphaeroides* TSPO at 1 nm resolution [33]. This study details that two TSPO monomers form a tightly associated symmetric dimer in the membrane plane, each with five transmembrane α -helices, although the exact functional role of the dimerization is not clear yet.

■ Ligands

The two classes of ligands which have been pivotal to the elucidation of the structure and function of the TSPO have been the benzodiazepines and isoquinoline carboxamides (Figure 3). Both classes of compounds are selective for the TSPO and display nanomolar affinities [34]. [^{11}C]PK-11195 **4** was the first radiolabelled example of an isoquinoline carboxamide ligand for the TSPO. Although the benzodiazepine [^{11}C]Ro 5-4864 **5** is a competitive binder for TSPO derived from rat kidney, it fails to maintain its affinity across species. Cloning studies have shown a high affinity of **5** for rodent-derived TSPO ($K_D = 1\text{--}9\text{ nM}$) but a markedly reduced affinity for human TSPO ($K_D = 54\text{ nM}$) [35].

<Figure 3>

However research has shown that TSPO ligands have no general structure, and porphyrins such as protoporphyrin IX (PP IX, the biosynthetic precursor of heme) **11** have shown to be the only endogenous ligands that bind with nanomolar affinity to TSPO [36]. This high affinity is selective for the TSPO by a factor of 1000 compared to the affinity for central benzodiazepine receptors. In this context it was postulated that the TSPO facilitates the transport of porphyrins into the mitochondria, as the first and final stages of heme biosynthesis take place in the mitochondria. This hypothesis would then explain the strong affinity of protoporphyrin for the receptor and would create a possible link between a deficiency in this receptor and development of porphyrias (*vide infra*).

Despite being extensively studied for over 30 years, the TSPO's role in pathophysiology has still not completely been elucidated. Partly this is the result of the absence of high resolution structures and of difficulties in interpreting imaging and pharmacological data [37]. Growing evidence from recent studies has confirmed that multiple binding sites exist and it is now logical to assume that not all newly discovered ligands for the TSPO will only be competitive binders for the isoquinoline site [38,39].

As a consequence of the marked up-regulation of TSPO in active disease states it has proven an attractive target for *in vivo* imaging of disease progression using functional imaging modalities such as positron emission tomography (PET) [34,40]. Although radiolabelled derivatives of PK11195 **4** [41] and Ro 5-4864 **5** [42] have been the gold standard of imaging agents for the TSPO (Figure 3), these molecules still suffer from some limitations, such as the poor pharmacokinetic profile of the isoquinolines and poor performance of the benzodiazepines as imaging agents. Thus, a need exists to develop improved TSPO labeling agents for the *in vivo* study of the TSPO [42].

As progression of diseases and diminished survival rates can archetypally be linked with TSPO expression it has been an obvious target for imaging studies. Notably, Manning's group has explored the use of TSPO ligands as imaging agents for colon [43] and breast cancer [44], and glioma [45,46] with agents which potentially could be used as cancer imaging biomarkers. They focused on the pyrazolopyrimidine scaffold, specifically modified at the 5-, 6-, and 7-positions in an attempt to synthesize higher affinity ligands for the TSPO, which in turn may serve as more robust PET agents *in vivo*. They identified 2-(5,7-diethyl-2-(4-(2-fluoroethoxy)phenyl)pyrazolo[1,5-a]-pyrimidin-3-yl)-*N,N*-diethylacetamide **7** to be a selective ligand for the TSPO (Figure 4). It exhibited an excellent 36-fold enhancement in affinity compared to the known pyrazolopyrimidine (DPA-714) [47]. Next they synthesized a ^{18}F radiolabeled analogue of **7**, which displayed negligible accumulation in normal brain tissue, but gave first-rate imaging contrast due to

strong accumulation in tumor tissue in *in vivo* studies of healthy rats and a preclinical model of glioma. Clearly, there is therapeutic potential for **8** as a novel PET ligand for assessing TSPO expression in tumors [48].

<Figure 4>

Photodynamic Therapy

■ PDT: Principle

Photodynamic therapy (PDT) is a selective treatment, which can be used as an alternative or in addition to classical therapies, e.g., chemotherapy [31,49]. PDT involves the administration of a tumor localizing photosensitizing agent followed by activation of the agent by light of a specific wavelength (Figure 5). This approach generates a sequence of photochemical and photobiological processes that cause irreversible photodamage to tumor tissue. PDT as a treatment holds many advantages over traditional cancer treatments such as radiation therapy, chemotherapy and surgery as PDT is a relatively non-invasive treatment which has the potential to selectively destroy tumor cells whilst sparing healthy tissue. It can be applied to places deemed non-viable by surgery and it is more controllable and less destructive on the body meaning older patients and others vulnerable to other treatment modalities can be treated with PDT.

<Figure 5>

Selectivity is achieved partly by the photosensitizer (PS), such as the compounds described below, which have a natural proclivity to accumulate in malignant cells/tissue [50], and by localized application of the incident light to the desired area. Interest currently lies in the advancement of efficient and specific carrier delivery platforms for systemic PDT, be it as bioconjugates [51], by encapsulating them in liposomes [52] or even connecting them to nanoparticles [53,54]. These modifications focus on designing systems to impart greater selectivity and specificity on the photosensitizer in order to enhance cellular uptake.

An alternative method to improve the efficacy and selectivity of these PS's may come through the selection of 'new' molecular targets, e.g., the TSPO. Verma *et al.* observed that TSPO density in tumors parallels their susceptibility to porphyrin-catalyzed phototoxicity [55]. Such data suggests that mitochondrial damage is the proximal event that leads to observed cell death. Porphyrins used in photodynamic therapy may thus enter cells and accumulate on mitochondrial porphyrin transport sites [56,57,58,59,60]. Since the lifetime of the porphyrin triplet state is extremely short and their diffusion in space is limited, the photo-induced reactions primarily affect the cell organelles labelled by the PS.

■ PDT: Photosensitizers

The principal characteristic of any photodynamic therapy sensitizer is its capacity to preferentially accumulate in malignant tissue and *via* the production of cytotoxic species, induce a desired biological effect [61]. By now a wide range of dyes has been screened for their efficacy in PDT. For a molecule to be considered a viable candidate as a photosensitizer it must meet a number of requirements. Generally, they should exhibit a strong absorption in the near infrared region of the electromagnetic spectrum (600-850 nm), which allows for a deeper penetration of light and show no dark toxicity. Typically they are pure compounds with a stable

shelf life, constant composition and produce a high quantum yield of singlet oxygen production.

The high quantum yield of singlet oxygen is seen as the most important characteristic when evaluating a dye for its PDT efficacy, as it is this reactive oxygen species which is a key mediator of cell death. A number of different chromophores can generate sufficient quantities of this reactive oxygen species and thus be considered potential photosensitizers for PDT. Examples are methylene blue, porphyrins and hypericin [62,63]. Of these different molecules, a number have seen clinical success as photosensitizers. These range from simple molecules such as δ -aminolaevulinic acid (ALA) [64] to tetrapyrrole formulations such as Foscan[®] **9** [65] or Photofrin[®] **10** (Figure 6) [66].

<Figure 6>

As porphyrins meet most of the traits desired for a photosensitizer and due to the relative commercial success of some of the aforementioned compounds, they have become the most extensively studied class of photosensitizers. Current research is centered on developing successful 3rd generation sensitizers to overcome some of the drawbacks present with Foscan[®] and Photofrin[®] and in particular, post-treatment photosensitivity and tissue selectivity [65,66].

As it stands, many, but not all of the “better” photosensitizers for photodynamic therapy are tetrapyrroles which tend to localize in the mitochondria. The induction of apoptosis resulting from the opening of the MPTP by ligands implicates the TSPO in the regulation of apoptotic and necrotic cell death [16]. Ligands of the TSPO also inhibit cell proliferation in cancer cell lines, resulting in an accumulation of cells in the G₁/G₀ phase of the cell cycle. These actions ultimately inhibit the progression of cells to the S and G_{2/M} phase, in which cell proliferation occurs [67]. By targeting the TSPO with endogenous or exogenous porphyrins, one may be able to selectively induce cell death *via* pro-apoptotic and anti-proliferative pathways.

TSPO as a Porphyrin Target

■ Porphyrins as Endogenous Ligands

But the focus on porphyrins in connection with the TSPO story begins with Snyder and coworkers discovering in 1987 that porphyrins are endogenous, potent competitive inhibitors of the TSPO [36,68,69]. They found that pure hemin and PP IX **11** competitively inhibit TSPO binding of [³H]PK-11195 and [³H]Ro 5-4864 with *K_i* values of 41 and 15 nM, respectively. While their binding activity at central-type benzodiazepine receptors saw a 1000-fold lower affinity. Tetra- and octacarboxylic acid porphyrins were to a small degree able to displace isoquinoline and benzodiazepine derivatives from binding to the TSPO and inactive in comparison to their dicarboxylic acid porphyrin counterparts, which displayed nanomolar potencies. This finding of porphyrins as endogenous TSPO ligands opened a new field of studies into porphyrins as therapeutic or diagnostic agents pertaining to TSPO related diseases.

Clearly, if porphyrins can bind to TSPO, this must effect the action of porphyrin-based photosensitizers in PDT. One of the most insightful strategies in PDT involves the administration of δ -aminolaevulinic acid (ALA), a biosynthetic precursor of heme, with the aim to boost protoporphyrin IX production in the target cells [70]. Indeed, Furre *et al.* showed that PP IX produced endogenously by

hexaaminolaevulinic acid (HAL) localized primarily in the mitochondria of the human cell line Reh [71]. Upon PDT treatment, post irradiation analysis showed that >80% of cells died by apoptosis, as indicated electron microscopy. HAL-PDT turned out to be both light dose and time course dependent.

In the biosynthetic pathway of heme, coproporphyrinogen III traverses the channel from the matrix to the cytosol [72]. It is theorized by Furre *et al.* [71] that HAL-induced endogenous PP IX may also advantageously use this channel for its own transportation into the cytosol. However, the study also showed that exogenous PP IX localizes differently intracellularly than endogenous PP IX and may not target the TSPO sufficiently to produce the same degree of apoptosis. Analysis of competitive binding studies with ligands for ANT suggests that, as a result of its close spatial proximity to the TSPO, the mitochondrial inner membrane adenine nucleotide translocator may be an additional target for HAL-PDT [73]. Their propinquity to HAL-induced endogenous PP IX synthesis at the mitochondrial inner membrane and the possibility that the porphyrin may use ANT for its transportation validates the TSPO as a possible therapeutic target for HAL-based PDT treatment of cancer.

With the exception of ALA and Photofrin® most PS in clinical use or development have chemical substituent pattern quite distinct from PP IX and this raises the question of porphyrin ligand specificity when targeting the TSPO. Kessel *et al.* conducted a study into the comparative binding of protoporphyrin IX **11** and two structural analogs, PP III **12** and PP XIII **13**, using murine leukemia L1210 cell cultures (Figure 7) [74]. The amphiphilicity of porphyrin based PS plays a significant role in the localization and membrane passage [75]. As such, it is important to consider the distribution of polar and hydrophobic substituents around the macrocycle as well as the charge of the side chains present on the molecule [63,76]. Kessel *et al.* suggest that porphyrin accumulation and affinity of the TSPO must be governed by hydrophobic considerations and that a major determinant of PDT efficacy within the PP IX series may be the relative hydrophobicity of the different analogues. All three porphyrins displayed equal hydrophobicity and all three agents induced 30% to 40% cell death *via* apoptosis after light exposure, although only PP IX had a strong affinity for the TSPO. These results suggest that only sensitizers with a configuration similar to protoporphyrin IX may display PDT efficacy as a result of TSPO affinity.

<Figure 7>

The next question is to what extent can the binding of porphyrins to the TSPO be affected by other factors? Next to hypoxia one other important factor is the intracellular pH. Healthy cells grow in pH 7.4 while cancerous ones, depending on their kind and age, can live in pH 5.5-6.5 [77]. Bombalska and Graczyk observed a decline in binding constants of a series of protoporphyrin and haematoporphyrin derivatives with increasing acidity of the environment [78]. With decreasing pH value porphyrin-type photosensitizers occur in different ionic forms and it is possible that the receptor changes its structural conformation in varying environment conditions. PP IX demonstrated the greatest binding affinity across the pH range, followed by haematoporphyrin (Hp), Hp(Arg)₂ and PP(Arg)₂ [78]. There is also the possibility that a lower pH value results in porphyrin core protonation [79] with attendant conformational distortion [80] which could impair binding with the receptor [81,82].

This work was followed by studies on the interaction of diamino acid derivatives of protoporphyrin IX, where the vinyl groups have been substituted with amino acids **36-49**. The amino acids were installed to facilitate the entry of the

photosensitizer into the cell by binding to amino acid receptors present in the tumor cell membrane. Preliminary studies indicate selectivity of accumulation of the dyes and low cytotoxicity to normal cells [83]. The strongest binding was observed at pH 5.5 for all four amino acid derivatives, which was supported by an observed increase in association constants as compared to pH 7.4. With these results, one can hypothesize that with the progression of cancer, the strength of the binding efficacy will increase as the pH decreases and therefore an increase in photosensitizer concentration is not necessary so as to achieve the required photodynamic effect.

■ Exogenous PDT Drugs

If endogenous porphyrins bind to TSPO or their level and interaction can be modulated through additional protoporphyrin IX availability what effects can be observed upon the exogenous administration of porphyrin-type PDT drugs? Several studies have addressed questions such as this. For example, Pandey's group used a synthetic library of long-wavelength photosensitizers derived from bacteriopurpurinimide and bacteriochlorin p_6 for comparative *in vitro* and *in vivo* PDT studies in RIF tumors [84]. The capacity of the rhodochlorins **14-18** (Figure 6) to displace the labeled high-affinity TSPO ligand PK11195 **4** was measured and it was found that photosensitizers **15**, **16** and **17** displaced more than 70% of ^3H -PK11195 at 10^{-4} M, whilst **18** displaced about 51% of ^3H -PK11195. However, no direct correlation between TSPO binding and PDT efficacy was found even though some molecules showed encouraging results in respect to PDT use.

Next, they synthesized a phytochlorin derived from chlorophyll *a* and evaluated its PDT and tumor imaging capacity *in vitro* and *in vivo* [85]. They found methyl 3-(1'-*m*-iodobenzyloxyethyl)-3-devinylpyropheophorbide *a* (HPPH) **19** to be a prospective photosensitizer and imaging agent for photodynamic therapy (Figure 8). Five C3H mice bearing RIF tumors were treated with the non-radiolabelled photosensitizer analogue of **20** (**21**) at a drug dose of $1.5 \mu\text{mol.kg}^{-1}$ and a light dose of 128 J.cm^{-2} , 14 mW.cm^{-2} for 2.5 h ($\lambda_{\text{max}} = 665 \text{ nm}$) at 24 h post injection. A 100% cure of tumors was achieved, *i.e.* all mice were tumor free within 60 days. The photosensitizer exhibited promising tumor fluorescence and PET imaging abilities and clearly supports the Pandey's concept of this ^{124}I -labeled photosensitizer finding use as a "multimodality agent". In the future, the design and efficacy may be improved upon by incorporating more tumor-avid and/or target specific photosensitizers.

<Figure 8>

Another example involved the use of bacteriochlorophyll *a* derivatives. These relatively unstable bacteriochlorins from *Rhodobacter shaeroides* were converted *in situ* into a series of stable *N*-hexylimide analogues **22-24** and tested *in vitro* and *in vivo* for their efficacy as PDT agents (Figure 9) [86]. The bacteriopurpurinimide **22** gave limited *in vivo* PDT efficacy in C3H mice bearing RIF tumors (drug dose $0.2 \mu\text{mol.kg}^{-1}$, $\lambda_{\text{max}} = 780 \text{ nm}$, 135 J.cm^{-2} at 24 h post injection). Two of the more efficacious analogues were **23** and **24** however, despite their improved efficacy, displacement studies suggest that they do not have a significant affinity for the TSPO. Comparative intracellular localization studies with Rhodamine 123 found the less effective photosensitizer to be localized in the lysosomes, whereas the most effective one was localized in the mitochondria indicating the possibility for future improvements.

<Figure 9>

Competitive binding studies of a ^{14}C phthalocyanine photosensitizer against ^3H -PK11195 (**4**), for binding to rat kidney mitochondria or intact Chinese hamster ovary cells were conducted by Morris *et al.* [87]. Their results suggest the presence of various binding sites for Pc4 (**25**) in mitochondria and cells, other than those attributed to PK11195 binding. Chemically this makes sense as phthalocyanines have chemical properties distinct from those of protoporphyrins. Although a low affinity binding site for **25** was identified, it appears that other mitochondrial events, such as photodamage to Bcl-2 are more pertinent to the phototoxicity of Pc4 (**25**) PDT than the binding of the phthalocyanine to the TSPO. This suggests that the observed inhibition of Pc4 (**25**) PDT induced apoptosis by PK11195 likely occurs through a mechanism separate to the TSPO.

■ Porphyrins for PET and PDT

^{11}C and ^{18}F are the most common PET isotopes used to label drugs [88,89]. Unfortunately, both suffer from relatively short half-lives which limits their use in experiments involving monoclonal antibodies or photosensitizers which take a long time (hours compared to minutes) for accumulation in a tumor. Due to a half-life of 4.2 days, ^{124}I is a more appropriate candidate and this longer half-life makes it compatible for sequential biological imaging using microPET [90,91,92].

With these facts in mind Pandey and coworkers aimed to develop a TSPO targeting photosensitizer for both imaging (PET) and PDT. As mentioned previously, numerous reports in the literature have shown overexpression of TSPO for both colon and breast cancers [22,24]; hence Balb/c mice bearing Colon-26 (colon adenocarcinoma) and EMT6 (well characterized, undifferentiated mouse breast cancer) tumors were selected. The synthesis of ^{124}I labeled **26** was achieved with high (>95%) radioactive specificity [93].

Use of the radiolabelled derivative of PK11195 (^{124}I -PK11195, **26**) resulted in a significant improvement of the PDT efficacy of the well-known photosensitizer HPPH (**19**), once conjugated. Compounds **27** and **28** possess similar binding affinity to the TSPO, however, **28** exhibited stronger tumor affinity as it possessed a higher standardized uptake value (% ID/g) at every time point (Figure 10). Both compounds **27** and **28** are good imaging agents and, interestingly, compound **28** produced significantly less skin phototoxicity than HPPH (**19**) in Scid mice bearing MDA-231 tumors; one of the main drawbacks of contemporary clinical PDT. Optimal tumor imaging for these compounds were obtained at 48, 72 and 96 h post injection, which further rationalizes the necessity to use ^{124}I instead of the shorter lived ^{18}F (Figure 11 95). These results clearly indicate that a conjugation of PK11195 to photosensitizers increases the PDT efficacy and target specificity compared to the photosensitizer alone.

<Figure 10>

<Figure 11>

Related studies used pyropheophorbides and their respective metal complexes to ascertain their viability as nonradioactive TSPO binding probes [94]. The TSPO binding and photosensitizing efficacy was influenced substantially by the presence of a core metal and as these compounds display strong fluorescence, they have significant potential to replace radioactive TSPO probes. For example, porphyrin **29**, an indium(III) complex proved to be the most efficacious with respect to photosensitizing efficacy. *In vitro* experiments with mice bearing RIF tumors (light dose $135\text{ J}\cdot\text{cm}^{-2}$, $75\text{ mW}\cdot\text{cm}^{-2}$, $\lambda_{\text{max}} = 665\text{ nm}$) revealed an order of efficacy for the central metal of $\text{In(III)} > 2\text{H} > \text{Zn(II)} > \text{Ni(II)}$. This might be related to their singlet

oxygen production capabilities. Although the Nickel complex **30** produced no PDT effect as expected, it did have the strongest binding affinity to TSPO, comparable to that of PK11195. Again *in vitro* efficacy and TSPO binding efficacy showed no direct correlation and no relationship was seen between TSPO binding and *in vitro* PDT efficacy.

As synthetic bacteriochlorins are good prospective PDT agents [63,95] some of them were also investigated in the context of the TSPO [96]. Benzobacteriochlorins derived from *vic*-dihydroxybacteriochlorins have long wavelength absorption in the range of 737 to 805 nm and were used in *in vitro* (RIF tumor cells) and *in vivo* (C3H/HeJ mice bearing RIF tumors) tests (Figure 12). The benzobacteriochlorin **31** was found to be the most efficacious and displayed reduced skin phototoxicity compared to Photofrin® at their respective therapeutic doses. Displacement studies were undertaken against ³H-PK11195 (**4**) and indicated that increasing concentrations of mitochondrial located benzobacteriochlorins **31-35** did not result in a higher displacement of ³H-PK11195. This suggests that, for these benzobacteriochlorins, the TSPO is not the target site.

<Figure 12>

Likewise, octaethylporphyrin-derived benzochlorins with varying degrees of lipophilicity were evaluated in an *in vitro* and *in vivo* SAR study [97]. A number of different structural features, such as the length of alkyl or fluoroalkyl groups attached to the exocyclic ring either by a carbon-carbon or ether linkage, were assessed. All of the (benzochlorinato)zinc(II) compounds were found to be effective with analogues **40**, **41** and **42** giving the best *in vivo* (C3H/HeJ mice) results, with three out of six mice tumor free after 90 days (1.0 $\mu\text{mol.kg}^{-1}$ dose). Of the two compounds **40** and **43** the Zn(II) complex produced 100% cell kill (RIF tumor cells) at 4.0 and 6.0 J.cm⁻². Comparative intracellular localization and binding studies with Rhodamine-123 and ³H-PK11195 (**4**) indicate that the PSs localize in the mitochondria. However, no specific displacement of ³H-PK11195 (**4**) was observed, *i.e.* no direct correlation exists between binding affinities and photosensitizing efficacy.

■ Modelling and Structural Investigations

Recent studies on the role of the interaction of PP IX and the TSPO in porphyrin-based photodynamic therapy focused on structural and modelling studies. By using *Escherichia coli* Papadopoulos *et al.* were able to express recombinant mouse TSPO in order to investigate the validity of their hypothesis that the interaction of PP IX with the TSPO is involved in the regulation of heme biosynthesis [98]. The *E. coli* protoplasts produced a recombinant gene product that showed specific affinity for PP IX binding.

The interaction of PP IX and TSPO in various cell models [99,100,101] has been suggested to assist the *modus operandi* of porphyrin based photosensitization in photodynamic therapy (PDT) of tumors [102]. These experiments found that neither PK 11195 nor Ro5-4864 could increase the PP IX binding or uptake, which was in accordance with previous studies [103]. Protoporphyrin IX could displace a radiolabeled analogue of PK 11195 from the TSPO but not that of Ro 5-4864, which indicated the presence of separate but overlapping binding sites [103,104]. Interpretation of these results suggest that the TSPO protein is solely responsible for PP IX binding and transport [74,98].

Bacterial TspO have been used to develop a functional model of the receptor. A number of TSPO mutants were synthesized by Yeliseev and Kaplan to investigate

the significance of a number of amino acid residues on the functional activities of TspO [105]. Many of the amino acid residues involved in TSP function are highly conserved among *R. capsulatus* CrtK, *R. sphaeroides* TspO and their mammalian analogues. The first putative periplasmic loop of TSPO, located between the first and second membrane spanning domain, contains a clustering of highly conserved amino acid residues. These residues are Leu³⁴, Lys³⁶, Trp³⁰, Trp³⁹, Trp⁴⁴, Trp⁵⁰, Trp¹³⁵, Gln⁸⁰, and Arg⁹⁶ [106]. This might suggest the direct interaction of the first putative loop with a specific ligand or cofactor, e.g., a tetrapyrrole. This loop contains a number of tryptophan residues, which could support its involvement in tetrapyrrole ligand binding. This is based on the heme binding motif (WWD), which is found in proteins involved in transmembrane heme delivery [107].

This proposed model, where the N-terminus of TSPO is exposed to the cell exterior may contradict the theorized structural model for the mammalian TSPO, which proposes that the N-terminal region of the protein is located on the matrix side of the outer mitochondrial membrane and that the first putative loop is exposed to the cytoplasm [38,108]. However, it appears permissible to anticipate that the tetrapyrrole binding site on the TSPO be exposed to the periplasmic space due to its part in the biosynthetic pathway for heme. The synthesis of TSPO in the cytoplasm and subsequent importation into the mitochondrial matrix before re-entering the mitochondrial outer membrane supports this new inside-out topology relative to bacterial topology [108]. Previous studies indicated the significance of Lys³⁹ (corresponding to Lys³⁶ of the TspO sequence) in binding of Ro5-4864, and of Trp⁴² and Trp⁴⁷ (corresponding to Trp³⁹ and Trp⁴⁴ of the TspO sequence) in binding of PK11195 and Ro5-4864 [38]. This supports the participation of the conserved amino acids discussed above in binding TSPO ligands in the mammalian TSPO [38]. Parallels between the structural and functional properties of the bacterial TspO and the mammalian homologue are mounting and this study provides further support for the theory [109].

Some information on the binding sites for endogenous ligands of the TSPO dimer model, could be derived from competitive binding studies using PK11195, PP IX and cholesterol. Some level of interaction among all three binding sites of the TSPO appears to be present [110]. These results are representative of a model in which PK11195 and PP IX interacting at somewhat different positions on extra-membrane loop 1 (Figure 13). The model suggests that the porphyrin may initially bind at loop 1, en route to transport *via* the dimer interface, as demonstrated in Figure 12. This proposed binding model matches previous *in vivo* studies in *RsTspO* [106], which also suggested that loop 1 plays a critical role in the binding and export of porphyrins [111,105,112]. Despite these correlations, it is unlikely that the first putative loop 1 is the only contributor to the binding of PP IX. Using a tryptophan fluorescence quenching assay for TSPO investigations it was observed that the tryptophan fluorescence was quenched evenly and completely by PP IX while no such effect was found with PK11195 and other ligands. Thus, PP IX may interact with a rather large area its binding induces a global conformational change [33].

<Figure 13>

One suggestion is that protoporphyrin IX may bind to a large area or induce a global conformational change, both of which are conceivable if PP IX is being bound and transported through the dimer interface [110]. This was based on the observed quenching of the tryptophan fluorescence by PP IX but not by PK11195 and other ligands. Decreased porphyrin export activity in phenylalanine mutants of W44 and W50 (single amino acid substitution with elevated levels of accessory pigments),

indicates changes in TSPO function [106]. This indicates that aromatic residues on transmembrane helix II play an important role in porphyrin binding and transport. A W38C mutant showed a ~6-fold reduction in affinity of PP IX for *R.s.* TSPO which is the location in loop 1 that initial binding occurs [98]. Although PK11195 was reported to compete with PP IX binding and bind to loop 1 of the TSPO, results from tryptophan quenching experiments indicate that they do not bind in precisely the same location.

All modelling studies suggest that the last two-thirds of loop 1 are highly conserved across all species. The observation that the K_d values of both PK11195 and PP IX were distinctly lower in the W38C mutant suggests some overlap between PK11195 and PP IX [110]. These independent lines of evidence allowed Miller *et al.* [110] to propose that within the monomer, PK11195 interacts mainly on the first half of loop 1, extending to residue W38 (S41 in human).

■ The Situation in Plants

Although unrelated to clinical applications it may be illustrative to take a quick look at the situation in plants. After the initial impetus from studies in mammals the search began for analogous mitochondrial import mechanisms in plants [113] and such a system was first identified by Lindemann *et al* in 2004 [114]. Substantial similarities to the *Rhodobacter* TspO protein and the mammalian TSPO were found in protein sequences from *Arabidopsis thaliana* [114,115]. The recombinant gene product of a TSPO-homologues *Arabidopsis* sequence was cloned and expressed in *E. coli* and displayed high binding affinity for benzodiazepines [114]. A comparative benzodiazepine-stimulated uptake of PP IX and cholesterol was observed when the protein was applied to *E. coli* protoplasts. This TSPO-like protein appears from these results to be involved in the directing of protoporphyrinogen IX to the mitochondrial site of protoheme formation and steroid import.

The gene sequencing of *Arabidopsis thaliana* identified a single TSPO related gene with a 40 amino acid N-terminal extension compared to the bacterial or mammalian species. This discovery suggest that site of localization may be in the mitochondria or chloroplast [113]. It has also been suggested that the At-TSPO plays a part in the response of *Arabidopsis* to high salt stress as re-localization of the At-TSPO from the ER to chloroplasts through its N-terminal extension occurs under high salt stress.

Further studies on different plant species investigated the central and peripheral-type benzodiazepine receptors in pods and leaves of *Ceratonia Siliqua* [116]. Using [3 H]PK 11195 and [3 H]RO 15-1788 (8-fluoro-3-carbethoxy-5,6-dihydro-5-methyl-6-oxo-4-imidazo-[1,5-a]-1,4-benzodiazepine) as specific ligands in a radioreceptor binding assay, the authors observed the presence of compounds which could readily displace the radiolabelled ligands from their respective binding sites. TSPO acting compounds were found to be extremely concentrated in young leaves suggesting that these compounds may play a significant role in cell growth and proliferation.

■ TSPO and Porphyrrias?

The TSPO seems to play a critical role in the production of neurosteroids, which are able to alter the electrical properties of neuronal membranes and thus the firing patterns of neurons. Porphyrrias, inherited or acquired disorders of certain enzymes that normally participate in the production of porphyrins and heme, are known to give

rise to certain aspects of neuropsychiatric disorders, such as schizophrenic-like symptoms [16].

Intriguingly, the binding capacity of TSPO for heme precursors is significantly reduced in schizophrenia patients with predominantly negative symptoms (residual type) compared to schizophrenia patients with predominantly positive symptoms. Additionally, no correlation was observed between TSPO density and age, gender and anti-psychotic medication, nor between variations of the TSPO gene and schizophrenia [117]. However, there was a decrease in TSPO density in post-mortem brains of chronic schizophrenia patients [117]. Additionally, Ritsner *et al.* found a connection between decreased TSPO density in blood platelets of schizophrenic patients exhibiting aggressive behavior [118]. The decrease in TSPO density was around 30% in patients and the $B_{\max}$ value was inversely correlated with aggressive behavior scores ($r=0.36$, $p=0.023$).

Porphyrias typically arise due to an accumulation of heme precursors, *i.e.* atypical porphyrins, in tissue due to a deficiency in one or more of the enzymes associated with the heme biosynthetic pathway. Medical conditions associated with porphyrias vary depending on which enzyme in the biosynthetic pathway is deficient. Porphyrias associated with coproporphyrinogen III oxidase and protoporphyrinogen oxidase are the most likely to display neurological symptoms [119]. Interestingly, no research has been conducted into the possible correlation between this decreased TSPO density in aggressive schizophrenic patients and the manifestation of a porphyria. As the TSPO is a transporter for heme precursors into the mitochondria, a 30% decrease in TSPO density may cause an accumulation of porphyrins in the cytosol and therefore potentially a porphyria.

FUTURE PERSPECTIVE

It has now been demonstrated that there are additional binding sites on the TSPO that are not related to isoquinoline compounds and due to this the current screening methods fall short of addressing all potential binding sites. Furthermore, noted species differences suggest that *in vitro* results using animal-derived TSPO, as well as *in vivo* imaging of animals, cannot necessarily be translated to human studies. Also, while establishing the K_i of a compound allows for relative comparison of affinity it does not address the capacity to measure increased binding site density ($B_{\max}$).

Despite all the major advances in the understanding of the structure and function of the TSPO, it is still unclear precisely how the TSPO acts in the binding of tetrapyrrole substrates [120,121,122,76]. Several groups have trialed numerous synthetic analogues of the endogenous ligand PP IX but still have no definitive understanding or predictive model of whether a compound will bind to the TSPO. Due to the flexible nature of loop 1, further modeling studies need to be conducted and ligand-protein interactions need to be deciphered to give a more rational approach to their drug design. Ultimately, this will require a high-resolution structure with bound ligand.

Although PDT efficacy and TSPO binding can still not conclusively be correlated the TSPO still represents an intriguing target for PDT research. One possibility may be the use of a larger library of carboxylic acid based porphyrins with varying degrees of hydrophobicity in order to obtain a better understanding of the structural tolerance, if any, of the TSPO for macrocycle variation.

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The authors have no financial interest or conflict of interest related to the subject matter of this review.

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Figures, Tables and Boxes

Figure 1. Schematic representation of the mitochondrial permeability transition pore and TSPO complex. The translocator protein (TSPO) is labelled yellow and the voltage-dependent anion channel (VDAC) is colored red. The B-cell lymphoma 2 protein (Bcl-2) is shown in green with the adenine nucleotide translocator (ANT) depicted blue.

Figure 2. Flowchart of the different biological functions of the TSPO.

Figure 3. Classes of TSPO Radioligands. The phenoxyphenyl acetamides [123,124,125], e.g., $[^{18}\text{F}]d_2$ FMDAA1106 have seen great success as radioligands for TSPO. Other classes of suitable compounds include the pyrazolopyrimidines, e.g., $[^{18}\text{F}]$ DPA-714, vinca alkyls [126] such as $[^{11}\text{C}]$ vinpocetine. Aryloxodihydropurines [127,128], for example $[^{11}\text{C}]$ AC-5216 have been used extensively and successfully in animal models.

Figure 4. PET imaging of a preclinical glioma using the pyrazolopyrimidine 8. "Reprinted (adapted) with permission from [48]. Copyright (2013) American Chemical Society."

Figure 5. Graphical depiction of PDT treatment. (A) (i) PS is injected into the body. (ii) The PS is allowed accumulate at the tumor site before irradiation with light. (iii) Selective destruction of tumor. (B) Chemically this method is based on the formation of singlet oxygen and other ROS.

Figure 6. Porphyrins used in PDT.

Figure 7. Protoporphyrins and rhodochlorin derivatives.

Figure 8. Pyropheophorbide derivatives and their *in vitro* photosensitizing activity. Variable drug concentrations and light doses of **20** and HPPH **19** in RIF tumor cells at 24 h post-incubation. "Reprinted (adapted) with permission from [85]. Copyright (2013) American Chemical Society."

Figure 9. Stable bacteriopurpurinimide and phthalocyanine based photosensitizers.

Figure 10. Library of phytochlorin derivatives.

Figure 11. Conjugation of the photosensitizer HPPH to a radiolabeled derivative of PK11195. For MDA-231 tumors bearing Scid mice, **26** possesses strong tumor imaging capability. MicroPET emission imaging (coronal view) at 24 h (A), 48 h (B), 72 h (C), and 96 h (D) post-injection of **28** (i.e., ${}^{124}\text{I}$ -**26**) (dose: 50 μCi (~40 ng)/mouse). (E) Kaplan-Meier plot for the *in vivo* PDT efficacy of compounds HPPH (**19**) and **26** at 0.4 $\mu\text{mol/kg}$ dose. Light dose: 135 J/cm^2 , 75 mW/cm^2 , ten mice for each group. **26** produces significantly better *in vivo* PDT efficacy than HPPH (**19**) ($P < 0.0001$). "Reprinted (adapted) with permission from [93]. Copyright (2011) American Chemical Society."

Figure 12. A range of tetrapyrrole-based photosensitizers.

Figure 13. Model of PK11195 and PpIX binding in the proposed dimeric *RsTSPO* state. A suggested model of the binding sites of PP IX, PK11195, and cholesterol on *RsTSPO*. The ligands have been manually inserted into the model of *RsTSPO* on one monomer. Roman numerals have been used to illustrate the helices: W38 (green sticks), PK11195 (orange sticks), PP IX (dark purple sticks), and cholesterol (yellow sticks). Proposed PP IX binding sites on loop 1 were colored light purple and PK11195 binding site was colored light orange. The potential role of the dimer in the loading and transport of PP IX has been illustrated by a magenta dotted line indicating the potential route. "Reprinted (adapted) with permission from [111]. Copyright (2013) American Chemical Society."