[¹⁸F]FEPPA: Improved Automated Radiosynthesis, Binding Affinity, and Preliminary in Vitro Evaluation in Colorectal Cancer

Neydher Berroterán-Infante,^{†,‡} Theresa Balber,^{†,§} Petra Furlinger,[†] Michael Bergmann,^{||} Rupert Lanzenberger,[⊥] Marcus Hacker,[†] Markus Mitterhauser,^{†,§,#} and Wolfgang Wadsak^{*,†,‡,▽}

[†]Division of Nuclear Medicine, Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna A-1090, Austria

[‡]Department of Inorganic Chemistry, Faculty of Chemistry, University of Vienna, Vienna A-1090, Austria

[§]Department of Pharmaceutical Technology, Faculty of Life Sciences, University of Vienna, Vienna A-1090, Austria

^{||}Surgical Research Laboratories, Department of Surgery, Medical University of Vienna, Vienna A-1090, Austria

[⊥]Department of Psychiatry and Psychotherapy, Medical University of Vienna, Vienna A-1090, Austria

[#]Ludwig Boltzmann Institute Applied Diagnostics, Vienna A-1090, Austria

[▽]Center for Biomarker Research in Medicine, CBmed GmbH, Graz A-8010, Austria

Supporting Information

ABSTRACT: The overexpression of the translocator protein (TSPO) has been amply reported for a variety of conditions, including neurodegenerative disorders, heart failure, and cancer. Thus, TSPO has been proposed as an excellent imaging biomarker, allowing, in this manner, to obtain an accurate diagnosis and to follow disease progression and therapy response. Accordingly, several radioligands have been developed to accomplish this purpose. In this work, we selected [¹⁸F]FEPPA, as one of the clinical established tracers, and assessed its in vitro performance in colorectal cancer. Moreover, we setup an improved radiosynthesis method and assessed the in vitro binding affinity of the nonradioactive ligand toward the human TSPO. Our results show an excellent to moderate affinity, in the subnanomolar and nanomolar range, as well as the suitability of [¹⁸F]FEPPA as an imaging agent for the TSPO in colorectal cancer.

KEYWORDS: TSPO, FEPPA, radiosynthesis, affinity, autoradiography, colorectal cancer

The translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor (PBR),¹ has emerged in the last years as an attractive target for diagnosis and treatment of several pathological conditions.^{2–5} Its up-regulation has been especially reported in activated microglia of neuroinflammatory disorders such as multiple sclerosis, Parkinson's, Alzheimer's, and Huntington's diseases, as well in some type of cancers, including breast, colon, and glioblastomas.^{6–8} Therefore, a variety of ligands have been developed for in vivo visualization, for instance, with positron emission tomography (PET), of the TSPO; including isoquinoline carboxamides, phenoxyphenyl acetamides, pyridazinoindoles, and others.^{9–11} The isoquinoline carboxamide [¹¹C](R)-PK11195 ((R)-1-(2-chlorophenyl)-N-[¹¹C]methyl-N-(1-methylpropyl)-isoquinoline) is one of the first and, until now, the most used ligand for TSPO imaging using PET.¹² However, a high nonspecific binding, due to its high lipophilicity, which ultimately leads to a low signal-to-noise ratio and a problematic radiosynthesis, have been a major drawback for the use of this tracer.¹³ To overcome these issues, a second generation of TSPO PET ligands with improved affinity and lipophilicity has arisen over the years. Nevertheless, a single nucleotide

polymorphism (rs6971) in the TSPO gene (Ala147Thr substitution) has hampered the development of such tracers because all these ligands exhibit different binding affinities toward human TSPO from subjects with different genotype. In this regard, three groups have been reported: high-affinity binders (HAB, homozygotes Ala147Ala), mixed-affinity binders (MAB, heterozygotes Ala147Thr), and low-affinity binders (LAB, homozygotes Thr147Thr).¹⁴ Some of those PET tracers are still widely used, although it is common to exclude LAB from clinical studies since low or no uptake of the tracers is observed. The second disadvantage is the interpretation of the data since the difference in the binding affinity must be considered, causing difficulties on the appropriate quantification of the tracer uptake.¹⁵ Among these tracers, the phenoxyphenyl acetamide [¹⁸F]FEPPA (N-acetyl-N-(2-[¹⁸F]-fluoroethoxybenzyl)-2-phenoxy-5-pyridinamine) shows out-

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standing properties regarding affinity, stability, lipophilicity, and radiosynthesis.¹⁶

[¹⁸F]FEPPA has been already used in several preclinical and clinical settings,^{17,18} however, only the in vivo effect of the polymorphism has been reported.¹⁹ To the best of our knowledge, no binding affinity profile, in terms of the inhibitory constant (K_i) toward the human TSPO, nor the evaluation in colorectal cancer have been performed. Hence, starting from the already reported automated radiosynthesis of [¹⁸F]FEPPA, we first established an improved and reliable method for the tracer production and subsequently assessed the in vitro binding affinity profile regarding the polymorphism rs6971. Furthermore, since the overexpression of TSPO in colorectal cancer is amply described,^{20–22} autoradiography experiments with [¹⁸F]FEPPA, using human colorectal cancer and healthy colon tissues, as well as real-time kinetic experiments utilizing the colorectal cancer cell line HT-29 were conducted.

The TSPO-PET tracer [¹⁸F]FEPPA was synthesized according to previously published procedures,^{16,17} with some minor modifications as outlined in Scheme 1.

Scheme 1. Radiosynthesis of [¹⁸F]FEPPA

^a[¹⁸F]KF/K222(dry), ACN, 90 °C, 10 min. Purification: preparative HPLC, SPE.

Briefly, aliphatic nucleophilic substitution was automated in a nuclear interface synthesizer between dried [¹⁸F]KF/K222 and the tosylate precursor (2-(2-((N-(4-phenoxy-3-pyridin-3-yl)-acetamido)methyl)phenoxy)ethyl-4-methylbenzenesulfonate). After the reaction time, the crude product was transferred to a semipreparative HPLC system, and the fraction containing the product ($t_R \approx 5$ min) was collected. The solvents were removed by SPE and the product reformulated in ethanol, sodium chloride, and phosphate-buffered saline. The (radio)chemical purity was assessed by means of analytical HPLC and TLC. All other quality control tests (visual inspection, pH, osmolality, radionuclide purity, K222, and residual solvents contents) were performed using regular procedures implemented in the PET Centre of the Vienna General Hospital. Complete details of the radiosynthesis and the quality control can be found in the Supporting Information.

The purity of [¹⁸F]FEPPA always exceeded 99%, and 4.2 ± 0.8 GBq of the product was afforded, representing a nondecay corrected radiochemical yield of $38 \pm 3\%$ (based on [¹⁸F]F[−] at EOB) with a molar radioactivity of 241 ± 13 GBq μmol^{-1} ($n = 15$) in a total synthesis time of 30 min. The quality control was always in accordance to the guidelines of the European Pharmacopeia.²³

With this new set of conditions, the main outcome was the time reduction of the purification step of the previously published method¹⁷ from 23 to 5 min, which drastically reduces the total synthesis time and affords a small increase in the radiochemical yield.

The affinity toward human TSPO of nonradioactive FEPPA (cold standard, *N*-acetyl-*N*-(2-fluoroethoxybenzyl)-2-phenoxy-

5-pyridinamine) was determined in a competitive binding assay, as reported elsewhere,²⁴ using [³H]PK11195 and a TSPO-expressing platelet membrane, from individuals previously genotyped regarding the polymorphism rs6971 and subsequently identified as HAB, MAB, and LAB (approved by the Ethics Committee of the Medical University of Vienna).

Inhibition constants in the subnanomolar and nanomolar range were obtained for FEPPA (Figure 1). K_i (HAB) = $(0.5 \pm$

Figure 1. Competition binding assays of nonradioactive FEPPA in HAB, MAB, and LAB using [³H]PK11195 and a TSPO-expressing platelet membrane.

0.1) nM, K_i (MAB) = (0.6 ± 0.1) , (37 ± 5) nM, and K_i (LAB) = (37 ± 5) nM. As expected, the in vitro binding affinity of FEPPA shows the typical behavior of the second generation of TSPO ligands regarding the TSPO polymorphism rs6971. Moreover, the inhibition constant for LAB shows a moderate binding affinity, which is 70 times lower than for HAB.

Although the up-regulation of TSPO in colorectal cancer is well-known, little research has been performed regarding TSPO imaging in this disease. TSPO-PET research has been more focused on neuroinflammation disorders and glioblastomas, as a consequence of the significant role of TSPO in glial activation.²⁵ Therefore, we deemed to evaluate the appropriateness of [¹⁸F]FEPPA as an imaging agent for colorectal cancer in vitro.

First, the expression of the TSPO in colon cancer and healthy mucosa (both obtained directly after tumorectomy with full informed consent from one patient bearing colon cancer and approved by the Ethics Committee of the Medical University of Vienna), as well as in HT-29 cells, was examined by Western blot. The presence of TSPO (above the 15 kDa mark, predicted molecular weight of 18 kDa) in HT-29 cells,²⁶ as well as in the tumor lysate, was confirmed (Figure 2). Interestingly, TSPO was not detected in healthy tissue lysate, although a moderate expression of TSPO in colon has been reported.²⁷

Moreover, in order to ensure the suitability of [¹⁸F]FEPPA for further evaluations and thus taking into account the polymorphism rs6971, we also conducted a PCR-based genotype assay on the cell line and the patient tissue, which distinguished the HT-29 cells and the patient bearing the tumor within the HAB group.

Accordingly, real-time binding experiments, to assess the tracer kinetics, were planned using HT-29 cells and LigandTracer Technology.²⁸ In a typical real-time binding experiment, the equilibrium was reached after 20 min, and in pursuance of displacing bonded [¹⁸F]FEPPA and thereby demonstrating specific binding to the TSPO, solutions of unlabeled FEPPA, PBR28, or PK11195 were used.

As shown in Figure 3, specific binding was observed for [¹⁸F]FEPPA since the signal decreased after the addition of displacement solutions, i.e., the radioactivity bound to the cells was reduced in all cases. Unlabeled FEPPA achieved a $(50.2 \pm$

Figure 2. Protein bands of ~15 kDa (theoretical molecular weight of TSPO, 18 kDa) were detected via Western blot with a specific anti-TSPO antibody in HT-29 cell and colorectal cancer tissue lysates, however not in healthy colon tissue lysate.

Figure 3. Normalized overlay real-time binding kinetics of $[^{18}\text{F}]$ FEPPA in HT-29 cells using LigandTracer Technology. Association of $[^{18}\text{F}]$ FEPPA was performed for 30 min, and subsequently, displacing agents (FEPPA, PK11195, or PBR28) were applied. Displacement was examined for additional 30 min.

11.2)% displacement, whereas PBR28 and PK11195 reached $(48.2 \pm 6.7)\%$ and $(32.2 \pm 10.6)\%$ displacement, respectively. In a similar set of experiments, only DMSO was used as vehicle control.

With these results in hand, we proceeded to perform autoradiography experiments with tumor and healthy colon tissues. Tissue slices were incubated with approximately 50 kBq per slice of freshly prepared $[^{18}\text{F}]$ FEPPA for 1 h. Analogously, blocking experiments were accomplished by coincubating the slices with radiotracer and unlabeled FEPPA or PBR28 (10 μM). After exposing the slices to a phosphor screen, radiotracer uptake was analyzed, and the amount of compound ($\text{fmol}\cdot\text{mm}^{-2}$) accumulated in each slice was determined.

Figure 4 shows a significantly higher (three to six times) uptake of the tracer in malignant tissue (baseline red) in comparison with healthy colon tissue (baseline blue), which is in accordance with the already mentioned upregulation of TSPO in colorectal cancer. Moreover, competitive blocking with unlabeled TSPO ligands was significantly observed in all cases.

Furthermore, an immunohistochemical investigation was realized in order to match the TSPO localization and the radiotracer uptake. For this purpose, an anti-TSPO antibody staining was performed using vicinal slices to those used for autoradiography. In Figure 5, a representative staining shows a stronger staining for tumor tissue in comparison to healthy colon. Accordingly, in the autoradiography counterpart, a higher uptake of the tracer in this tissue is observed as well,

Figure 4. $[^{18}\text{F}]$ FEPPA uptake ($\text{fmol}\cdot\text{mm}^{-2}$) in healthy colon compared to colon cancer tissue slices. Specific uptake was confirmed by blocking experiments with cold FEPPA and PBR28.

Figure 5. $[^{18}\text{F}]$ FEPPA accumulation observed in autoradiography corresponded to TSPO expression in the vicinal slices as revealed by immunohistochemistry. (a) TSPO IHC. (b) $[^{18}\text{F}]$ FEPPA autoradiography. Left/middle, colorectal tumor tissues. Right, healthy colon tissue. DLU, digital light units.

demonstrating once more the high sensitivity of $[^{18}\text{F}]$ FEPPA for TSPO recognition in colon cancer.

PET imaging of TSPO in colonic diseases seems to be challenging mainly due to the basal expression of this protein in healthy tissues and in less grade due to the availability of several probes with different pharmacological properties, which difficult the comparison of the data. Several reports^{29,30} suggest poor specificity of TSPO PET tracers for evaluation of such conditions; meanwhile, some investigations^{31–33} support the use of TSPO in inflammatory bowel diseases and colorectal cancer. In view of the high level of specificity demonstrated by our *in vitro* results and also the relatively low uptake of $[^{18}\text{F}]$ FEPPA *in vivo* in healthy colon and stomach as demonstrated elsewhere,¹⁷ we propose $[^{18}\text{F}]$ FEPPA as a potentially appropriate probe to be further *in vivo* evaluated as a TSPO PET tracer to evaluate this malignancy.

In summary, we have developed a faster and still reliable method for the radiosynthesis of $[^{18}\text{F}]$ FEPPA with higher radiochemical yields. Moreover, FEPPA showed similar behavior *in vitro* as other TSPO ligands regarding the polymorphism rs6971; although its affinity in human platelets is very potent in all identified genotypes. Additionally, we present here the first evaluation, to the best of our knowledge,

in colorectal cancer. Our results point toward the suitability of [^{18}F]FEPPA for imaging colorectal cancer with PET.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsmmedchemlett.7b00367](https://doi.org/10.1021/acsmmedchemlett.7b00367).

Detailed information about radiosynthetic procedures, binding affinity assays, including genotyping and membrane preparation, cell culture, real-time kinetics, autoradiography, immunohistochemistry, Western blot, and additional results (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +43-1-40400-55330. E-mail: wolfgang.wadsak@meduniwien.ac.at.

ORCID

Wolfgang Wadsak: [0000-0003-4479-8053](https://orcid.org/0000-0003-4479-8053)

Author Contributions

N.B.-I. performed all the radiosyntheses, the binding affinity experiments and genotyping assays, and writing of the paper and contributed to autoradiography and real-time binding assays. T.B. performed all immunohistochemistry, Western blot, autoradiography, and real-time binding experiments. P.F. contributed to autoradiography, immunohistochemistry, Western blot, and real-time binding experiments. M.B. performed the tumorectomy and contributed to the design of the study. M.H. and R.L. designed parts of the research and proofread the manuscript. M.M. conceived and supervised the in vitro experiments and proofread the manuscript. W.W. conceived and supervised the radiosyntheses and proofread the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TSPO, translocator protein; PBR, peripheral benzodiazepine receptor; HAB, high-affinity binders; MAB, mixed-affinity binders; LAB, low-affinity binders; t_R , retention time; ACN, acetonitrile; HPLC, high performance liquid chromatography; SPE, solid phase extraction; TLC, thin layer chromatography; EOB, end of bombardment; PCR, polymerase chain reaction; IHC, immunohistochemistry; DLU, digital light units

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