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Graphical Abstract

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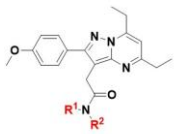

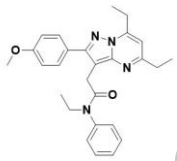

21 K_i = 0.28 nM

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New structure-activity relationships of *N*-acetamide substituted pyrazolopyrimidines as pharmacological ligands of TSPO

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ABSTRACT

Translocator protein (TSPO) represents an attractive target for molecular imaging and therapy due to its prevalence and critical roles played in oncology and other pathologies. Based upon our previously optimized pyrazolopyrimidine scaffold, we elucidated new structure activity relationships related to *N,N*-disubstitutions of the terminal acetamide on pyrazolopyrimidines and further explored the impacts of these substituents on lipophilicity and plasma protein binding. Several novel chemical probes reported here exhibited significantly increased binding affinity, suitable lipophilicity and protein binding compared with contemporary TSPO ligands. We illustrate that *N,N*-acetamide disubstitution affords opportunities to introduce diverse chemical moieties distal to the central pyrazolopyrimidine core, without sacrificing TSPO affinity. We anticipate that further exploration of *N*-acetamide substitutions may yield additional TSPO ligands capable of furthering the field of precision medicine.

Imaging now plays a critical role in cancer diagnosis and the molecular characterization of actionable lesions. Thus, imaging is central in the successful deployment of precision cancer medicine. Among clinical modalities available for cancer imaging, the sensitivity and quantitative nature of positron emission tomography (PET), coupled with the ability to produce biologically active tracers bearing positron-emitting isotopes, renders PET imaging uniquely capable of detecting tumors and profiling their molecular features. Despite this potential, a lack of specific and biologically validated tracers that are capable of providing detailed molecular information about individual tumors limits the breadth of biological questions addressable with PET. By far, the most widely used PET tracer in oncology is 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG), which accumulates in tissues as a function of glucose uptake. Many tumors utilize glycolysis to partially fuel their growth, which has made FDG PET a mainstream tool for cancer detection and assessment. Importantly, however, FDG has several limitations. FDG uptake in tumors can be confounded by accumulation in tissues possessing increased glycolytic metabolism such as the brain. Conversely, not all tumors exhibit high metabolic rates such as prostate cancer.[1] These issues highlight a currently unmet need to explore and validate additional molecular targets for cancer imaging.

Previously termed as peripheral benzodiazepine receptor (PBR), translocator protein (TSPO) is an 18 kDa protein typically localized to the outer mitochondrial membrane whose primary role is to facilitate cholesterol metabolism. TSPO expression and function is shown to be linked with steroid biosynthesis, cellular proliferation, and apoptosis.[2] In oncology, TSPO overexpression has been reported in many human cancers, including glioma, colon cancer, and breast cancer.[3] As a promising target for cancer imaging, TSPO ligands have been synthesized based upon a diversity of core scaffolds.[4-6] Many TSPO ligands were previously developed to image CNS disorders and thus, have sub-optimal pharmacokinetic properties for peripheral organ

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sites. Our lab has focused on the development and characterization of high-affinity TSPO ligands applicable to a wide variety of imaging modalities, especially in tumors that arise outside of the central nervous system.[1, 7-13] We previously

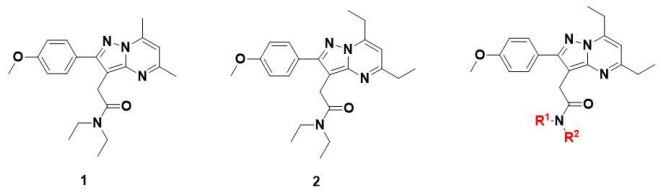
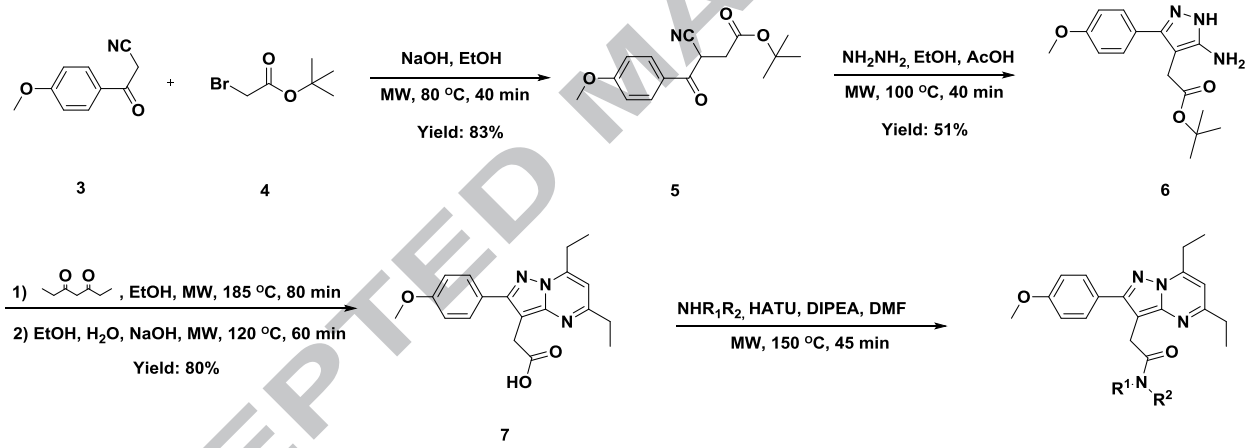


Figure 1. Structure of DPA-713 (1), 2-(5,7-diethyl-2-(4-methoxyphenyl)pyrazolo[1,5-*a*]pyrimidin-3-yl)-*N*,*N*-diethylacetamide, previously reported by our group (2) and indicated substituents of new pyrazolopyrimidine-based ligands.

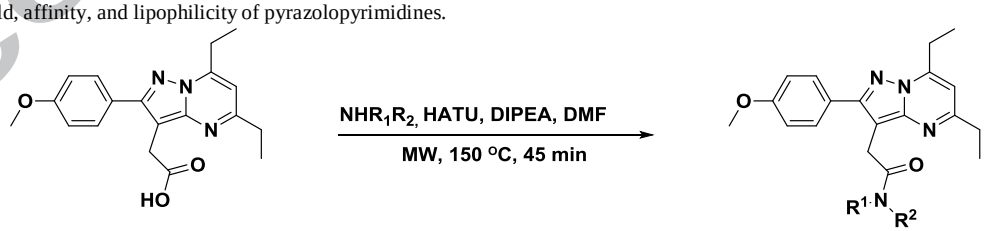
optimized the 5, 6, and 7 positions of a pyrazolopyrimidine, DPA-713[6,14] and reported a novel fluorine-18-labeled PET tracer (VUIIS 1008) with improved affinity and pharmacokinetic properties for cancer imaging.[1,11] Based upon our prior work and motivated by the work of Selleri et al.,[15] we further elaborated on the pyrazolopyrimidine structure activity relationships related to *N*,*N*-disubstitutions of the terminal acetamide (**Figure 1**). Herein, we report a variety of novel pyrazolopyrimidine TSPO candidate ligands (n = 16), bearing alkyl, alicyclic, aryl, and heterocyclic pendant acetamides. In doing so, we also report the development of a novel synthetic route to these compounds that allows for a rapid library expansion approach.

Our prior synthetic route to pyrazolopyrimidine-based libraries required a divergent synthesis at the first step,[1] which reduced the efficiency of producing products with various substituents. To achieve a more efficient pathway, we developed a new route which allows diversification in the final step, providing a facile approach to a diverse library. To further accelerate the library generation, we utilized microwave-assisted organic synthesis (MAOS) as shown previously.[7,16] As shown in **Scheme 1**, starting from commercially available compounds **3** and **4**, compound **5** was synthesized in a single step. Subsequently, the pyrazolo ring of compound **6** was formed by the reaction of compound **5** with hydrazine in ethanol, in the presence of acetic acid. Preparation of compound **7** was accomplished in a one-pot reaction procedure, combining compound **6** with 3,5-heptanedione to close pyrimidine ring, followed by deprotection to reveal the free carboxylic acid. Finally, a variety of secondary amines were subsequently reacted with carboxylic acid **7** to generate the final chemical probes evaluated here. In total, 16 probes were synthesized with a diversity of *N*-acetamide substituents.



Scheme 1. Synthetic route to *N*-acetamide substituted pyrazolopyrimidines.

Table 1. Structure, yield, affinity, and lipophilicity of pyrazolopyrimidines.



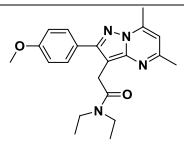
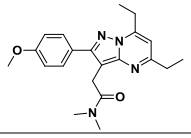
Compound	Structure	Yield	K_i (nM)[c,d]	log $P_{7.5}$[e]
8[a] **(DPA-713)**		---	4.7	2.40
9		97%	37.59±5.96	2.47

Table 1 (*continued*)

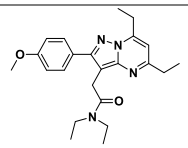
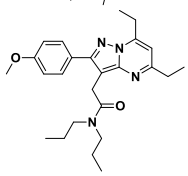
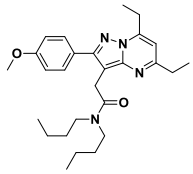
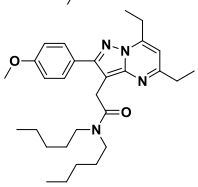
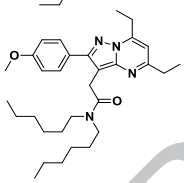
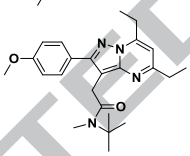
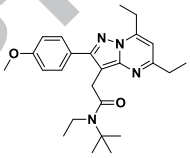
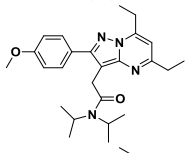
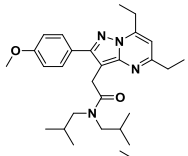
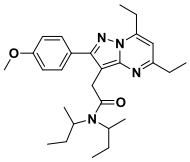
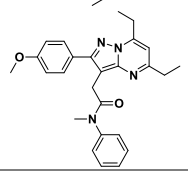
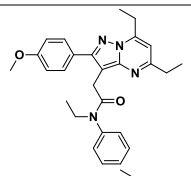
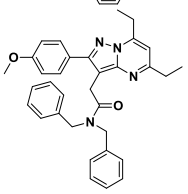
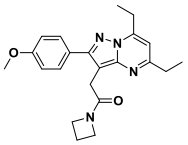
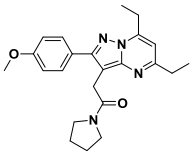
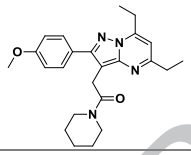
Compound	Structure	Yield	K_i (nM)[c,d]	log $P_{7.5}$[e]
10[b]		86%	0.18	2.84
11		82%	5.57±2.98	3.35
12		82%	7.34±0.89	3.81
13		80%	2.17±0.74	4.29
14		79%	18.57±10.27	4.78
15		64%	94.79±43.05	3.13
16		70%	59.12±16.12	3.32
17		67%	20.03±1.56	3.26
18		85%	13.25±1.76	3.77
19		76%	14.40±8.07	3.75
20		51%	6.44±1.44	3.11

Table 1 (*continued*)

Compound	Structure	Yield	K_i (nM)[c,d]	log $P_{7.5}$[e]
21		49%	0.28±0.14	3.27
22		76%	397.29±69.39	3.73
23		96%	87.75±37.96	2.58
24		83%	127.20±13.13	2.72
25		84%	49.22±9.24	2.96

[a]DPA-713, see ref 6. [b]Probe 5b, see ref 1. [c]K_i versus ^{3}H-flunitrazepam > 10000 nM. [d]All K_i values were averaged from triple runs with corresponding standard derivation. [e]log $P_{7.5}$ were averaged from triple runs with 1% or less standard deviation for each sample.

Upon synthesis and analytical characterization, TSPO affinities of the chemical probes were determined using a C6 glioma cell lysate binding assay previously described.[1,9,17] Lipophilicity was determined using analytical reverse phase HPLC as previously reported.[1] Plasma protein binding was performed using the equilibrium dialysis method. After extraction, the buffer and plasma fractions were analyzed using standard LC-MS methods.[18]

Previous investigation of 5/7 substitutions illuminated that TSPO affinity was highly sensitive to alkyl substitutions and intolerant of steric bulk.[1] However, we found the acetamide position particularly tolerant to *N*-alkyl substitution, with entries of differentially substituted alkyl groups exhibiting affinities in the nano molar to sub-nano molar range with the chain length ranging from one to six carbons. Among them, *N*-alkyl substitutions of up to 5 carbon length exhibited affinities analogous or better than DPA-713.

Chemical probes possessing branched alkane substituents typically maintained reasonably high affinity, yet branching generally diminished affinities relative to straight chain alkyl groups of similar carbon number. It was noted that the bulky t-butyl group significantly decreased binding affinity. When substituting ethyl groups with t-butyls, the affinity decreased by over 300-fold, from 0.18 nM (compound **10**) to 59.12 nM (compound **16**).

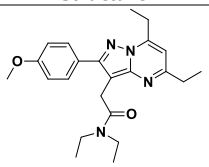
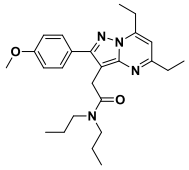
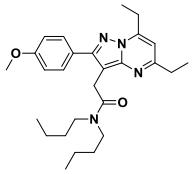
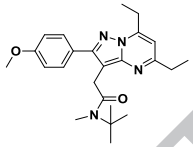
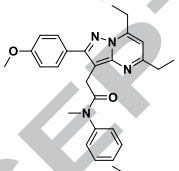
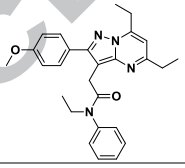
Interestingly, the introduction of a single phenyl ring rescued the binding affinity. We observed the heterozygous phenyl-ethyl substitution pattern to be particularity favorable, resulting in a novel ligand with pico-molar activity (K_i = 0.28nM), which is among the highest binding affinity for TSPO ligands reported to date. In contrast, when both *N*-acetamide substitutions were homologated to benzyl groups, affinity was decreased significantly (K_i = 397.29nM). It was also found that acetamide substitution with ethyl group was generally well-tolerated in combination with many other *N*-substitutions on the acetamide. For example, mixed ethyl acetamides fared better than other substituents of similar structure, *e.g.* **15** vs **16**, **20** vs **21**. In contrast to acyclic derivates, alicyclic constrained derivatives (**23**, **24** and **25**) exhibited very poor binding affinity.

Recognizing acetamide substitution as an opportunity to diversify the binding affinity of novel TSPO ligands, we further evaluated the lipophilicity and plasma protein binding. Lipophilicity is an important factor that dictates biodistribution and clearance. Chain length was a critical determinant of lipophilicity within the *N*-alkyl series. Long carbon chain substitutions, such as **12** (n=4), **13** (n=5) and **14** (n=6), were extremely lipophilic as expected. Compared to straight chain

alkyl groups of similar carbon number, branched alkyl substituents tended to exhibit more moderate lower lipophilicity. The lipophilicity of phenyl substitution compounds (**20** and **21**, 3.11 and 3.27 respectively) appeared to represent a compromise between longer alkyl chains and bulky branched substituents.

The majority of the compounds reported here exhibited unbound fractions of approximately 1%, including phenyl substituents (**Table 2**). It was noted that chemical probes exhibited higher lipophilicity tended to also exhibit lower unbound fraction. For example, the unbound fraction of compound **12** (n=4, $logP_{7.5}$=3.81) was 0.3%, the lowest among the examined library members.

Table 2. Protein binding of selected compounds.

Compound	Structure	Fraction Unbound
10		1.1%
11		0.7%
12		0.3%
15		1.3%
20		1.1%
21		0.6%

In summary, we illustrate here that the pendant acetamide represents an opportunity to diversify the chemical and physical properties of pyrazolopyrimidine-based TSPO ligands. Several new chemical probes exhibited suitable binding properties, lipophilicity and protein binding to potentially serve as TSPO imaging tracers when functionalized with an appropriate positron emitter such as Carbon-11. We anticipate that these results may expand opportunities to deploy TSPO ligands to image and treat diseases especially relevant to peripheral tissues.

Acknowledgments

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