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Brief Article

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Identification of a Novel Benzoxazolone Derivative as a Selective, Orally Active 18 kDa Translocator Protein (TSPO) Ligand

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Supporting Information

ABSTRACT: Optimization of the pharmacokinetic properties for a series of benzoxazolone derivatives led to the identification of **9b**, which showed anxiolytic effect in a rat model. However, **9b**, like known benzodiazepines, induced motor impairment. Investigation into the cause of this unexpected side effect and management of **9b** off-target binding affinity led to the identification of **10d**, which showed oral anxiolytic effect in the rat model with improved safety profile.

INTRODUCTION

Benzodiazepines such as diazepam are widely used for the treatment of anxiety owing to their fast onset and potent efficacy. However most benzodiazepines produce adverse events, such as drug dependence, development of tolerance, and withdrawal symptoms, which limit their clinical use.[1−3] Antidepressants, including selective serotonin reuptake inhibitors, are also prescribed in the treatment of anxiety disorders, although their anxiolytic effect is often delayed by several weeks.[3] Considering these drawbacks, there is great need for new antianxiety agents with fast onset and potent effect but minimum unfavorable side effects.

The 18 kDa translocator protein (TSPO),[4] which was first identified as a discrete receptor for diazepam,[5] is known to be functionally and structurally distinct from the central benzodiazepine receptor (CBR).[6,7] TSPO functions as a heterotrimeric complex with the 32 kDa voltage-dependent anion channel (VDAC) and the 30 kDa adenine nucleotide transporter (ANT)[8] and is mainly distributed in the mitochondrial membrane of peripheral organs, including the kidney, heart, and endocrine glands, and in glial cells in the central nervous system (CNS).[9] Although the physiological role of TSPO in the CNS remains to be investigated, accumulated evidence suggests that TSPO in the CNS most likely promotes the synthesis of neurosteroids.[10−12] Indeed, it has been suggested that stimulation of TSPO increases neurosteroid concentration in the CNS by activating cholesterol transport from the outer to the inner mitochondrial membrane,[10−12] which is known as the rate-limiting step of neurosteroids biosynthesis.[13] As neurosteroids have been reported to have beneficial effects in psychiatric disorders,[14,15] TSPO ligands are expected to be useful in the treatment for such disorders.

A wide variety of TSPO ligands have already been reported (Figure 1). In addition to the classical TSPO ligands Ro5-4854 (**1**)[16] and PK11195 (**2**),[17] several other TSPO ligands have been identified. Among them are the indole derivative FGIN-1-27 (**3**),[18] the 8-oxopurine derivative AC-5216 (**4**),[19,20] the 2-phenylindolglyoxylamide (**5**),[21] and the pyrazolo[3,4-*b*]-quinoline derivative (**6**).[22]

We have recently reported a series of benzoxazolone derivatives designed by opening of the diazepine ring of Ro5-4864 (**1**), including **7a** and **10a** as potent and selective TSPO ligands.[23] Benzoxazolone derivatives' high affinity for TSPO was rationalized in light of a pharmacophore model comprising three lipophilic pockets and a hydrogen-donor group.[24,25] Additionally, we have found in a preliminary study on benzoxazolone derivatives that substitution at the amide part and the C-5 position in the benzoxazolone ring plays an important role as hydrophobic group in TSPO binding. Owing to the necessity of this hydrophobic group in TSPO binding, a number of benzoxazolone derivatives have poor druglike properties. Although the introduction of hydrophilic group into the amide part or the C-5 position in the benzoxazolone ring seems to be difficult, our structure−activity relationship (SAR) survey led to the discovery of compounds bearing a hydrophilic substituent with acceptable TSPO affinity. Among these compounds, **10a** selectively bound to TSPO and showed anxiolytic effect in the rat Vogel conflict model.[26] Although **10a** showed acceptable anxiolytic effect in rats, there was room for improvement of its PK profile. On the basis of the results of a preliminary SAR study of benzoxazolone derivatives, optimization of **10a** PK profile led to the identification of **9b** as a potential anxiolytic worthy of further development. However,

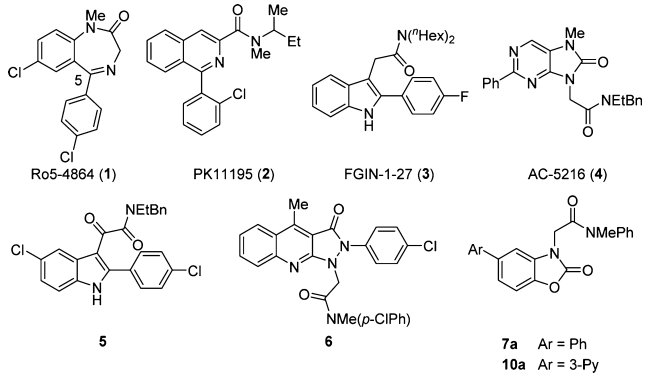

Figure 1. Chemical structures of selected TSPO ligands.

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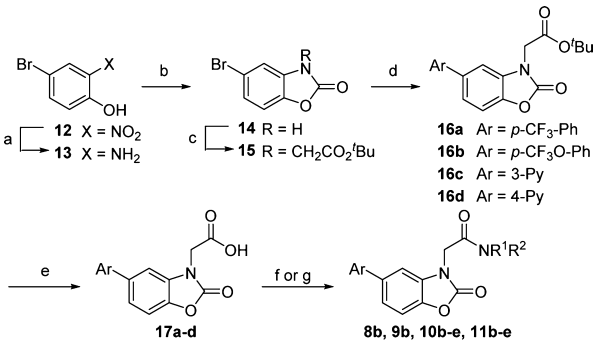
regardless of its high selectivity for TSPO over CBR, **9b**, like conventional benzodiazepines, showed motor impairment.

In this paper, we describe our investigation into the cause of **9b** unexpected side effects on motor coordination, and management of **9b**'s off-target binding affinity. Our efforts led to the identification of **10d**, which showed high binding affinity and good selectivity for TSPO and exhibited anxiolytic effect in the rat Vogel conflict model, producing few side effects associated with benzodiazepines.

CHEMISTRY

The target compounds in this study were synthesized as illustrated in Scheme 1. Compounds **16a–d** were prepared via

Scheme 1. Synthesis of Benzoxazolone Derivatives[a]



[a]Reagents and conditions: (a) H_2, 5% Rh-C, THF, rt; (b) CDI, THF, rt; (c) *tert*-butyl bromoacetate, K_2CO_3, DMF, rt; (d) $ArB(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, 1,4-dioxane, H_2O, reflux; (e) HCl/dioxane, AcOH, 50 °C; (f) amine, BOPCl, Et_3N, 1-hydroxybenzotriazole, DMF, rt; (g) amine, EDCI, DMF, 50 °C.

Suzuki–Miyaura coupling reaction of **15**[23] with a selected boronic acid. Deprotection of **16a–d** with hydrogen chloride (HCl) provided the key intermediate acid derivatives **17a–d**. Condensation of **17a–d** with a selected amine was carried out in the presence of phosphoric acid bis(2-oxooxazolidide) chloride (BOPCl) or by combination of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole to afford the corresponding amide derivatives **8b**, **9b**, **10b–e**, and **11b–e** (Table 1). Compounds **7a–d**, **8a**, **9a**, **10a**, and **11a** were obtained as indicated in our previous report.[23]

RESULTS AND DISCUSSION

The affinity of the prepared compounds for TSPO and for CBR was evaluated by measuring each compound's ability to displace [^{3}H]PK11195 and [^{3}H]flumazenil binding to membranes prepared from rat kidney and rat cerebral cortex. All tested compounds had negligible affinity for CBR (<50% inhibition at 10 μM).

In a previous paper,[23] we have shown that incorporation of a para electron-withdrawing substituent onto the phenyl ring at the C-5 position of the benzoxazolone (**8a** and **9a**) led to an increase in TSPO binding affinity and metabolic stability (Table 1). However, the obtained compounds **8a** and **9a** suffered from poor solubility (<1.0 μg/mL at pH 7.4 and pH 2.5). To improve the aqueous solubility of these compounds, we decided to modify the amide part of selected compounds. Our preliminary research on the amide part of benzoxazolone derivatives revealed that **7c** with a 3-pyridyl substituent has moderate affinity for TSPO with improved solubility (45 μg/mL at pH 7.4). On the basis of this finding, we selected the 3-pyridyl unit as the amide part and prepared **8b** and **9b**. As **9b** exhibited improved TSPO affinity and metabolic stability, it was selected for further PK examination. Both **9b** and its HCl salt (**9b**·HCl) showed acceptable solubility (20 and 23 μg/mL) in a pH 6.8 buffer with bile acids, suggesting good bioavailability after oral administration. Accordingly, we evaluated the PK profile of **9b**·HCl in rats after oral administration at 10 mg/kg. As shown in Table S1 in the Supporting Information, **9b**·HCl displayed good oral absorption with low clearance and good CNS penetration (brain/blood ratio of 1.3). Encouraged by these results, we evaluated the anxiolytic effect of **9b**·HCl in the rat Vogel conflict model. As shown in Figure S1 in the Supporting Information, oral administration of **9b**·HCl (3.0 mg/kg) significantly produced an anxiolytic effect in the rat model.

We examined the safety profile of **9b**. As mentioned in the Introduction, benzodiazepines are known to cause adverse effects by allosterically modulating the action of GABA via CBR. On the other hand, recent studies have shown that selective TSPO ligands can have anxiolytic effect without causing the side effects associated with benzodiazepines.[19,20] However, contrary to our expectation, **9b**·HCl, like conventional benzodiazepines, induced motor impairment in the horizontal rotarod test at a dose of 300 mg/kg (Table S3 in the Supporting Information). Investigation of the **9b**·HCl pharmacokinetics profile revealed that **9b**·HCl's concentration in the rats does not correlate with the dose administered, i.e., 2-fold increase in concentration for a 30-fold increase in dose (10–300/kg) (Table S2 in the Supporting Information). As similar concentration was observed (at 10 mg/kg, po) in mice, **9b**·HCl would have the potential to induce motor impairment at concentrations relatively close to the concentration needed for anxiolytic effect.

To identify the mechanism by which **9b**·HCl induces motor impairment, a profiling of **9b** using a broad package of in vitro radioligand binding assays was conducted. In all assays, **9b** was tested in duplicate at 10 μM. The results of these assays showed that **9b** has potent activity at the rat brain site 2 sodium channel (108% inhibition at 10 μM, IC_{50} = 0.70 μM) without significant inhibitory effect on other receptors (Table S7 in the Supporting Information). As compounds that have affinity at the site 2 sodium channel are reported to induce failure of motor coordination,[27–29] we decided to evaluate the affinity of previously reported benzoxazolone derivatives (**7a,c**, **9a**, **10a**, and **11a**) at the rat site 2 sodium channel. As shown in Table 1, a substitution at the C-5 position mainly affects site 2 sodium channel binding. Among the tested compounds, **10a** and **11a** showed negligible effect on the rat site 2 sodium channel and were therefore selected for further modifications at the amide part.

As shown in Table 1, **10b** with R^1 converted to an ethyl showed 2-fold increase in TSPO affinity compared to **10a**, albeit reduced metabolic stability. As introduction of a substituent into the phenyl of the amide part was expected to increase affinity for TSPO by preliminary study results, we prepared **10c–e** with substitutions at the phenyl ring of the amide moiety. Contrary to our expectation, introduction of a methoxy group (**10c**) or a trifluoromethoxy group (**10e**) decreased the affinity for TSPO. On the other hand, a better overall in vitro profile was identified with **10d** having a *p*-trifluoromethylphenyl as the amide moiety. Compound **10d**

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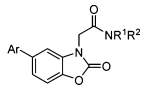
Table 1. In Vitro Profile of Benzoxazolone Derivatives



compd	Ar	R^1	R^2	TSPO K_i (nM)[a]	CBR inhibition (%)[b]	metabolic stability[c]	solubility (μg/mL)[d] pH 7.4	solubility (μg/mL)[d] pH 2.5	Na^+ channel (site 2) IC_{50} (μM)[e]
7a	Ph	Me	Ph	1.6	5	1	<1.0	1.0	>1.0
7b	Ph	Me	2-Py	23	9	0	1.0	3.0	NT[f]
7c	Ph	Me	3-Py	28	29	0	45	180	>1.0
7d	Ph	Me	4-Py	270	2	NT[f]	NT[f]	NT[f]	NT[f]
8a	*p*-CF_3-Ph	Me	Ph	0.68	0	26	<1.0	<1.0	NT[f]
8b	*p*-CF_3-Ph	Me	3-Py	9.8	6	24	<1.0	<1.0	NT[f]
9a	*p*-CF_3O-Ph	Me	Ph	0.65	0	67	<1.0	<1.0	0.19
9b	*p*-CF_3O-Ph	Me	3-Py	4.9	0	32	<1.0	4.0	0.70
10a	3-Py	Me	Ph	11	14	24	5.0	440	>1.0
10b	3-Py	Et	Ph	4.4	21	0	NT[f]	NT[f]	NT[f]
10c	3-Py	Me	*p*-MeO-Ph	19	0	9	NT[f]	NT[f]	NT[f]
10d	3-Py	Me	*p*-CF_3-Ph	8.6	0	38	1.0	420	>10
10e	3-Py	Me	*p*-CF_3O-Ph	26	0	NT[f]	NT[f]	NT[f]	>1.0
11a	4-Py	Me	Ph	5.3	8	27	1.0	730	>10
11b	4-Py	Et	Ph	3.0	5	24	2.0	860	NT[f]
11c	4-Py	Me	*p*-MeO-Ph	17	2	47	<1.0	130	NT[f]
11d	4-Py	Me	*p*-CF_3-Ph	6.6	0	35	3.0	>1000	NT[f]
11e	4-Py	Me	*p*-CF_3O-Ph	23	NT[f]	NT[f]	NT[f]	NT[f]	NT[f]
PK11195				1.7					

[a] K_i values represent the mean of one to three separate experiments run in duplicate using four concentrations of each compound. [b] Percent inhibition of [^{3}H]flumazenil specific binding at 10 μM compound. [c] Metabolic stability data refer to percent of compound remaining after incubation with rat liver S-9 fraction (~2.0 mg·protein/mL) and NADPH (~3.0 mM) for 30 min at 37 °C. The initial concentration of each compound was 1.0 μM. [d] The solubility was determined by HPLC using the supernatant obtained after shaking of the buffered solution (pH 7.4 and pH 2.5) (0.4 mL) containing 1 mg of tested compound, followed by centrifugation. [e] IC_{50} represents displacement of [^{3}H]batrachotoxin (5.0 nM) binding to rat brain by each compound. [f] Not tested.

showed slightly increased TSPO affinity (K_i = 8.6 nM) and metabolic stability (38%) without affinity for the rat site 2 sodium channel. The same modification was carried out in the 4-pyridyl derivative **11a**, and a trend similar to that of **10a** was observed for TSPO affinity. Among the 4-pyridyl derivatives prepared, **11b** and **11d** showed high affinity for TSPO (**11b** K_i = 3.0 nM, **11d** K_i = 6.6 nM) with acceptable metabolic stability (**11b**, 24%; **11d**, 35%). However, further profiling of the 4-pyridyl derivatives **11b** and **11d** revealed that these compounds at 1.0 μM significantly inhibit cytochrome P450 in isozyme 2C19 (**11b**, 71%; **11d**, 73%), which could translate into potential drug−drug interaction. Although not reported here, introduction of a substituent in the adjacent position of the 4-pyridine nitrogen resulted in a decrease of metabolic stability. On the basis of these findings, **10d** was selected as the most promising compound for further evaluation.

Next we evaluated the pharmacokinetic properties of the HCl salt of **10d** (**10d**·HCl) in rats after intravenous and oral administration at doses of 1 mg/kg iv and 10 mg/kg po, respectively (Table S1 in Supporting Information). Compound **10d**·HCl showed moderate bioavailability and good CNS penetration (brain/blood = 1.2). Furthermore, **10d**·HCl showed a dose-dependent increase in serum concentration in rats, presumably due to improved aqueous solubility. Additionally, we confirmed serum concentration of **10d**·HCl after oral administration (10 mg/kg) in mice which was similar to that in rats (Table S2 in Supporting Information). Then we assessed the anxiolytic effect of **10d**·HCl in the rat Vogel-type conflict model and evaluated its potential for benzodiazepines side effects. Oral administration of **10d**·HCl (1.0 mg/kg) significantly increased the number of shocks in this model (Figure S2 in Supporting Information). As shown in Tables S3−S6 in the Supporting Information, diazepam produced motor impairment in the horizontal rotarod test, memory impairment in the passive avoidance test, decrease in locomotor activity, and elongation of hexobarbital-induced sleep at doses close to those that produced anxiolytic effect. On the other hand, **10d**·HCl did not induce motor impairment in the horizontal rotarod test, memory impairment, and decrease of locomotor even at the high dose (Tables S3, S4, and S6 in Supporting Information). Although **10d**·HCl slightly prolonged hexobarbital-induced sleep at 300 mg/kg, the extent of this effect was much less than that of diazepam (Table S5 in Supporting Information). These findings are consistent with those from previous reports on TSPO ligand.[19]

Finally, **10d** profiling in various receptor and transporter assays using proteins involved in the commonly observed adverse events in the central nervous, cardiovascular, and pulmonary systems was conducted. In all assays, **10d** was tested in duplicate at 10 μM. No significant affinity for any of the tested receptors or transporters was observed (Table S7 in Supporting Information). Profiling of **10d** across species revealed that **10d** has high affinity for human TSPO with a K_i of 8.0 nM and good metabolic stability in human liver S-9 (84% remaining after 30 min). Further pharmacological investigation of **10d**, including its effects on the synthesis of neurosteroids, is underway.

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CONCLUSION

We report here the optimization of a series of benzoxazolone derivatives as a novel selective TSPO ligand. Our efforts led to the identification of **10d** as a potent and highly selective ligand for TSPO. Compound **10d**·HCl exhibited oral anxiolytic effect in the rat model with few side effects associated with conventional benzodiazepines. Compound **10d**·HCl also showed satisfactory pharmacokinetics profile, brain exposure, and preliminary safety profile and has now progressed to toxicity studies. Further detailed pharmacological evaluation of **10d** is presently in progress.

EXPERIMENTAL SECTION

Chemistry. General procedures are in the Supporting Information. The purities of tested compounds were determined by analytical reverse phase HPLC using the area percentage method on the UV trace recorded at a wavelength of 254 nm. Compounds were found to have >95% purity unless otherwise specified.

General Procedure A for the Synthesis of Amide Derivatives 8b, 10d,e, and 11d. To a solution of **17** in DMF were added a selected amine (1.2 equiv), phosphoric acid bis(2-oxooxazolidide) chloride (BOPCl) (1.2 equiv), and triethylamine (2.5 equiv) at room temperature. The mixture was stirred at room temperature for 3 h. Following the general workup procedure, silica gel column chromatography afforded amide derivatives.

General Procedure B for the Synthesis of Amide Derivatives 9b, 10b,c, and 11b,c,e. To a solution of **17** in DMF were added a selected amine (2.5 equiv), 1-hydroxybenzotriazole (1 equiv), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) (3 equiv) at room temperature. The mixture was stirred at 50 °C for 1 h and cooled to room temperature. Following the general workup procedure, silica gel column chromatography afforded amide derivatives.

ASSOCIATED CONTENT

Supporting Information

Pharmacokinetic and pharmacological data of compounds **9b**·HCl and **10d**·HCl, procedures for pharmacological experiments, general chemistry directions, synthetic procedure, spectroscopic and analytical data for compounds, and spectra of tested compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

TSPO, 18 kDa translocator protein; CBR, central benzodiazepine receptor; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide transporter; CNS, central nervous system; CDI, 1,1′-carbonyldiimidazole; BOPCl, phosphoric acid bis(2-oxooxazolidide) chloride; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; SEM, standard error of the mean

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