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Structure–Activity Relationship, Biological, and Pharmacological Characterization of the Proline Sulfonamide ACT-462206: a Potent, Brain-Penetrant Dual Orexin 1/Orexin 2 Receptor Antagonist

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The orexin system consists of two G-protein-coupled receptors, the orexin 1 and orexin 2 receptors, widely expressed in diverse regions of the brain, and two peptide agonists, orexin A and orexin B, which are produced in a small assembly of neurons in the lateral hypothalamus. The orexin system plays an important role in the maintenance of wakefulness. Several compounds (almorexant, SB-649868, suvorexant) have been in advanced clinical trials for treating primary insomnia. ACT-462206 is a new, potent, and selective dual orexin receptor antagonist (DORA) that inhibits the stimulating effects of the orexin peptides at both the orexin 1 and 2 receptors. It decreases wakefulness and increases non-rapid eye movement (non-REM) and REM sleep while maintaining natural sleep architectures in rat and dog electroencephalography/electromyography (EEG/EMG) experiments. ACT-462206 shows anxiolytic-like properties in rats without affecting cognition and motor function. It is therefore a potential candidate for the treatment of insomnia.

Introduction

The orexin (hypocretin) system is an evolutionarily conserved neuropeptide–receptor system that acts as a central regulator of wakefulness and modulates emotional states related to stress or reward. The neuropeptides orexin A and orexin B are biosynthesized by a discrete number of neurons in lateral hypothalamic areas (LHA), regions historically implicated in arousal, emotional and metabolic regulation, and motivated behaviors such as feeding.[1–4] Orexins are released in a Ca^{2+}-sensitive manner at axonal terminals and can then bind to two closely related G-protein-coupled receptors (GPCRs): orexin receptor type 1 (OX_1) and orexin receptor type 2 (OX_2).[5–7] In neurons, activation of OX_1 and OX_2 leads to the activation of the G_q/phospholipase C/protein kinase C pathway which results in the modulation of ion channel activities, cellular depolarization, and increases in cytosolic Ca^{2+} concentrations.[8] Thus, orexin receptor signaling enhances synaptic transmission.

The orexin peptides and their receptors are highly conserved across mammalian species, and the neuroanatomical distribution of OX_1 and OX_2 supports their essential role in the regulation of vigilance states and circadian activity. Nerve fibers from orexin immunoreactive neurons of the LHA make wide and dense projections to the basal forebrain, corticolimbic structures, and brainstem, particularly to those regions related to waking/regulation of sleep (locus coeruleus, raphe nucleus, tuberomammillary nucleus), regions activated in anxiety/stress-related conditions (paraventricular nucleus, amygdala) as well as regions involved in reward processing and drug abuse (nucleus accumbens, ventrotegmental area).[3,4,9–18] Accordingly, infusing orexins intracerebrally in rats leads to enhanced behavioral activity, arousal, delayed onset of REM sleep, and maintenance of cortical activation. Furthermore, pharmacological inhibition of the orexin system in animal models of insomnia, stress/anxiety as well as drug abuse has demonstrated a direct role of an overactive orexin system in these pathologies and suggests orexin receptors as therapeutic targets in insomnia, stress/anxiety-related disorders and addiction.[19–30] Two dual orexin receptor antagonists have been studied in advanced clinical trials and demonstrated potential for the treatment of sleep disorders. In insomnia patients, both almorexant (**1**) and suvorexant (**3**) dose-dependently increased sleep efficiency and total sleep time by decreasing latency to persistent sleep and wake after sleep onset.[31–33] SB-649868 (**2**) has also been studied in humans with primary insomnia. In initial studies it exhibited a typical efficacy profile of dual orexin receptor antagonists.[34] Other orexin receptor antagonists have recently been tested by Merck as co-medication in depression, neuropathic pain, and migraine (http://www.clinicaltrials.gov).

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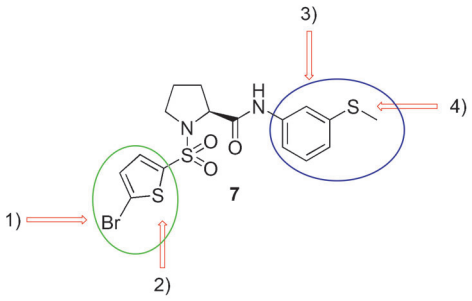
We carried out a high-throughput screening program using Ca^{2+} release assays in the search for dual orexin receptor antagonists. Herein we describe the structural optimization of our initial screening hit, leading to the preclinical candidate ACT-462206 (**24**), which is a new, selective, and competitive antagonist at OX_1 and OX_2. It shows excellent brain penetration following oral administration. It decreases wakefulness, decreases sleep latency, and increases sleep efficacy in rats and dogs. Compound **24** also shows some efficacy in paradigms of stress/anxiety and addiction.



$IC_{50}(OX_1)$: 21 nM
$IC_{50}(OX_2)$: 9.9 nM
IC_{50}(3A4T): 6.1 μM
HLM: 541 μL min^{-1} mg^{-1}
RLM: >1250 μL min^{-1} mg^{-1}

in vivo BBB experiment
@ 100 mg kg^{-1} in rat; sampling @ 3 h
[P]: 524 ng mL^{-1}
[B]: 403 ng g^{-1}
B/P ratio: 77%

MDR1-MDCK assay
E ratio: 1.3
P_{app} A —> B: 34.5 × 10^{-6} cm s^{-1}
P_{app} B —> A: 46 × 10^{-6} cm s^{-1}

Figure 2. Structure and data for the OX_1/OX_2 HTS hit.

All compounds shown in Figure 1 are or were in clinical trials for primary insomnia. Compounds **1**–**4** are described as potent dual OX_1/OX_2 antagonists, exhibiting an insurmountable inhibition profile at the orexin receptors.[34–38] Compounds **5** and **6** are reported to be selective OX_2 antagonists. Based on recent publications, selective OX_2 antagonists might show similar clinical efficacy as dual OX_1/OX_2 antagonists.[39,40]

Results and Discussion

Our high-throughput screen for orexin receptor antagonist activity on both OX_1 and OX_2 using calcium release assays resulted in the proline sulfonamide **7** as one of our preferred promising hit structures (Figure 2). Medicinal chemistry based optimization focused on the following aspects (see arrows in Figure 2): 1) effect of the bromine substituent exchange; 2) replacement of the electron-rich thiophene by a bioisostere; 3) determining if replacement of the phenyl ring with nitrogen-containing heterocycles can avoid the anilide subunit; and 4) identification of replacements of the oxidatively labile S-methyl substituent. After having scrutinized the available data, we decided to keep the sulfonamide as well as the amide functionality in the molecule.

The data collected for compound **7**, with a molecular weight of 461.42 Da and significant activity in the calcium release assay, could be considered optimal and illustrate the high quality and attractiveness of this starting point for a medicinal chemistry lead optimization program. Compound **7** exhibited good brain penetration properties and no liabilities toward being a P-glycoprotein (P-gp) substrate, reasonable in vitro human liver microsomal metabolic stability, and no obvious cytochrome inhibitory activity. From a synthetic chemistry point of view, the preparation of compound **7** and its derivatives was straightforward and is depicted in Scheme 1.

The preparation of screening hit **7** for confirmation purposes, and to obtain larger amounts for broader profiling, started from commercially available L-proline methyl ester hydrochloride **8**, which was treated with 5-bromothiophene-2-sulfonyl

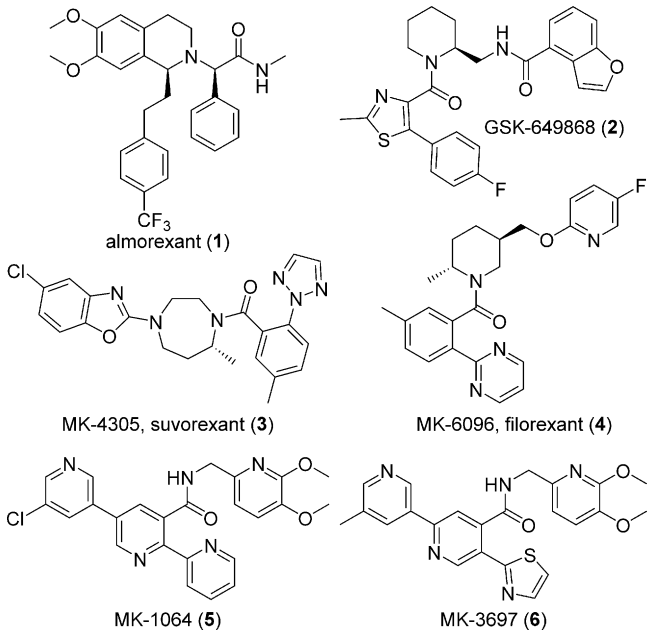
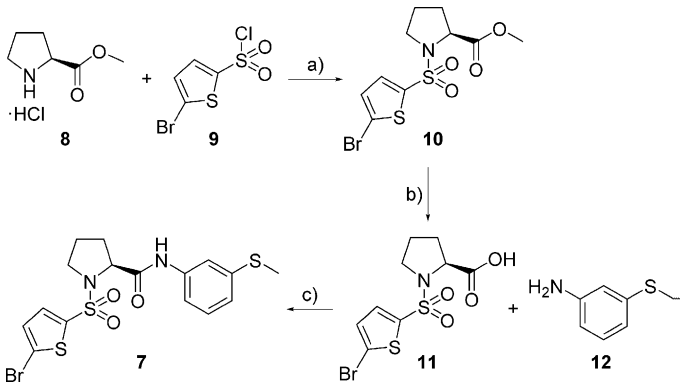


Figure 1. Compounds with clinical trial activity being reported.



Scheme 1. Synthesis of compound **7** and derivatives thereof (details given in the Supporting Information and in Ref. [44]). *Reagents and conditions*: a) DIPEA, CH_2Cl_2, RT, 12 h, 98%; b) MeOH/THF (1:1), 1 M NaOH (2 equiv), RT, 12 h, 93%; c) $POCl_3$, pyridine, 0 °C→RT, 30 min, 95%.

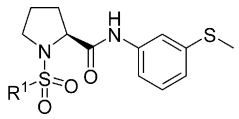
chloride (**9**) in dichloromethane in the presence of *N*,*N*-diisopropylethylamine at room temperature for 12 h to give the intermediate methyl ester **10** in 98% yield. Ester hydrolysis was achieved under standard conditions[41] by dissolving **10** in a 1:1 mixture of THF/methanol and adding two equivalents of aqueous sodium hydroxide. The resulting mixture was stirred for 12 h at room temperature to give the acid precursor **11** in 93% yield. The final step, reaction with 3-(methylthio)aniline (**12**), was achieved with phosphorous oxychloride in pyridine starting at 0 °C and slowly warming the reaction mixture to room temperature.[42,43] Screening hit **7** was obtained in 95% yield. Selection of the reaction conditions to form the anilide moiety was based on the potentially low nucleophilic reactivity of the anilines used in this step.

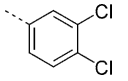
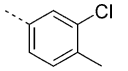
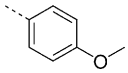
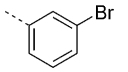
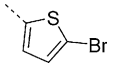
Scheme 2 represents the general possibilities to obtain final proline sulfonamide dual orexin receptor antagonists **17**. All synthetic steps necessary by either way can be performed with conditions mild enough to tolerate a diverse set of substituents at the aromatic or heteroaromatic rings contained in both substituents introduced to the proline template. The route chosen depended fully on the structure–activity relationship (SAR) question to be investigated. Experimental details and analytical data for the preparation of some of the final compounds can be found in the Supporting Information and in Ref. [44].

Our optimization efforts started by looking for replacements of the 5-bromothiophene unit present in the screening hit **7**. In an initial effort we kept the 3-methlythioanilide part fixed and combined it with variously substituted phenyl moieties at the sulfonamide end of the molecule, as depicted in Table 1. These changes allowed maintenance of the promising inhibitory activity at OX_2 and clearly improved the potency at OX_1. With respect to orexin antagonistic activity, compounds **20** and **21** were superior to the other examples. Unfortunately, the compounds containing a 3,4-disubstituted phenyl unit showed rather prohibitive inhibitory potency in a cytochrome P450 3A4 assay. Still very promising activities on both orexin receptors, combined with lower inhibition of cytochrome P450 3A4 activity were found for compound **22**, bearing a 4-methoxyphenyl sulfonamide unit. Comparing the 3-bromophenyl substituent present in **23** with the 2-bromothienyl substituent from parent compound **7** shows that the phenyl unit is a viable replacement for the thiophene moiety with respect to orexin antagonist activity as well as cytochrome inhibition. In the next step we investigated the SAR in the anilide area of the orexin antagonists by fixing the sulfonamide to the previously identified 4-methoxyphenyl moiety **22**. Table 2 summarizes the results obtained in this effort. Comparison of compounds **24**–**28** with compound **22** shows that with respect to orexin antagonist activity, exchanging the methylthio substituent generally results in decreased potency toward OX_1. Potency toward OX_2 suffered less from these changes or could be maintained as shown in **24**. In addition, inhibitory activity toward cytochrome P450 3A4 could be significantly decreased in **24** relative to **22**.

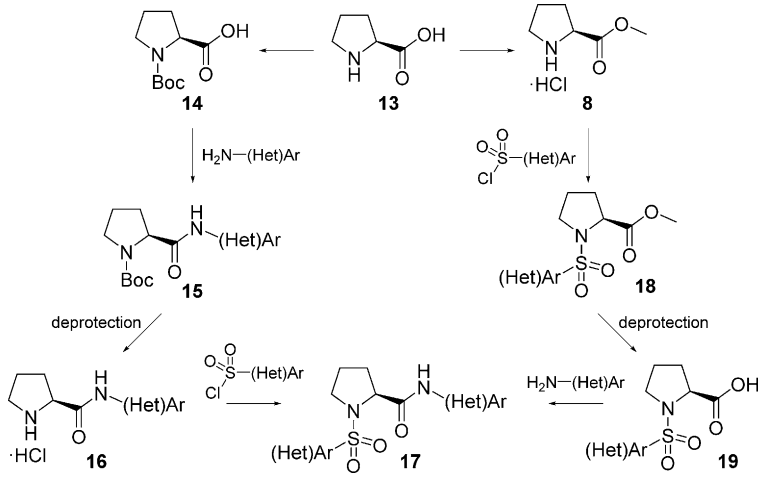
We followed up on these results by additional variations in the sulfonamide part of the antagonists by keeping the 3,5-dimethylanilide unit fixed. Results are summarized in Table 3. Comparison of compound **24** with the other mono-*para*-substituted residues, such as compound **30** containing a *para*-methyl substituent or compound **35** containing a *para*-bromo substituent, reveals that the *para*-methoxy substituent remains the most favorable unit with respect to orexin antagonistic activity. Moving the *para*-methyl substituent of **30** into the *ortho* position as shown in **33**, for example, results in a loss of activity at OX_2 and has no beneficial effect on cytochrome P450 3A4 inhibition. Attaching two substituents to the phenyl ring of the sulfonamide

Table 1. Structure–activity relationships: sulfonamide moiety part 1.



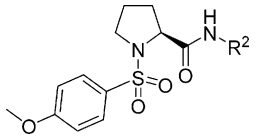
R^1	Compd	IC_{50} [nM][a] OX_1	OX_2	IC_{50} [μM][b] 3A4T
	**20**	2.2	1.9	3.2
	**21**	2.5	2.8	3.9
	**22**	31	6.9	7.8
	**23**	8.4	5.6	6.4
	**7**	21	9.9	6.1

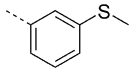
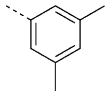
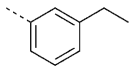
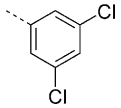
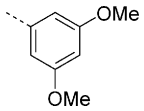
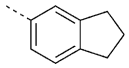
[a] Data are the geometric mean (±2-fold) of at least three independent experiments and were determined by FLIPR assay (see the Supporting Information for assay details). [b] Values are from single measurements with testosterone used as the marker substrate.



Scheme 2. Synthetic approaches toward proline sulfonamide dual orexin receptor antagonists: efficient strategies toward quick SAR development.

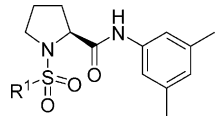
Table 2. Structure–activity relationships: amide moiety part 1.

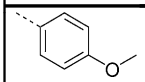
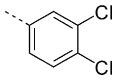
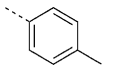
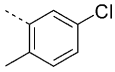
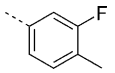
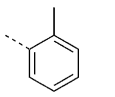
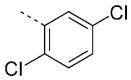
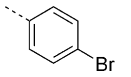


R^2	Compd	IC_{50} [nM][a] OX_1	IC_{50} [nM][a] OX_2	IC_{50} [μM][b] 3A4T
	**22**	31	6.9	7.8
	**24**	60	11	15
	**25**	61	23	17
	**26**	46	39	3.7
	**27**	99	24	ND[c]
	**28**	157	23	5.8

[a] Data are the geometric mean (±2-fold) of at least three independent experiments and were determined by FLIPR assay (see the Supporting Information for assay details). [b] Values are from single measurements with testosterone used as the marker substrate. [c] Not determined.

Table 3. Structure–activity relationships: sulfonamide moiety part 2.



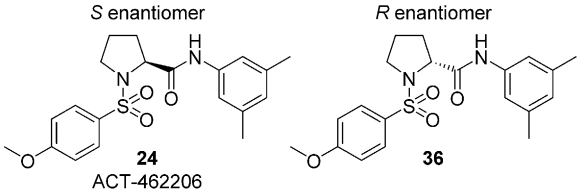
R^1	Compd	IC_{50} [nM][a] OX_1	IC_{50} [nM][a] OX_2	IC_{50} [μM][b] 3A4T
	**24**	60	11	15
	**29**	17	16	ND
	**30**	48	15	ND
	**31**	32	16	3.7
	**32**	41	18	ND
	**33**	50	22	3.2
	**34**	89	12	3.6
	**35**	53	24	ND

[a] Data are the geometric mean (±2-fold) of at least three independent experiments and were determined by FLIPR assay (see the Supporting Information for assay details). [b] Values are from single measurements with testosterone used as the marker substrate. [c] Not determined.

moiety, as in **29**, **31**, **32**, or **34**, results in derivatives with promising antagonistic potency toward OX_2. Activities at OX_1 are more variable, and for cases in which the inhibitory activity for cytochrome P450 3A4 was determined, it was less advantageous relative to compound **24**. This effort finally confirmed that derivative **24** was the best compound identified from this series.

Compound **24** was further profiled in an in vivo blood–brain barrier [BBB] penetration experiment in male Wistar rats (experimental details in the Supporting Information). We found that **24** showed excellent brain penetration with high absolute values for the brain concentration of [B] = 1219 ng g^{-1} and the plasma concentration of [P] = 2667 ng mL^{-1} when administered orally at a dose of 100 mg kg^{-1}, resulting in a [B]/[P] ratio of 46%. In parallel, we checked the influence of the proline core chirality and found that the non-natural *R* enantiomer **36** (Figure 3) exhibited almost no antagonistic activity for either of the orexin receptors, but behaved very similarly in terms of brain penetration properties, with [B] = 1041 ng g^{-1} and [P] = 1309 ng mL^{-1}, and thus a [B]/[P] ratio of 79%. This result was in agreement with theory and our expectations and excluded active uptake phenomena of the natural proline-amino acid based **24**.

We then investigated several other derivatives from the proline sulfonamide series for their brain penetration potential (Figure 4). We selected mainly 4-methoxyphenylsulfonamide



	24 ACT-462206	**36**
$IC_{50}(OX_1)$ [nM]:	60	6215
$IC_{50}(OX_2)$ [nM]:	11	6044
[P] (ng mL^{-1}):	2667	1309
[B] (ng g^{-1}):	1219	1041
[B]/[P] (%):	46	79

Figure 3. Influence of proline core chirality. BBB experiments were performed in Wistar rats at a p.o. drug dosing of 100 mg kg^{-1}; sampling at 3 h; drug formulation: PEG 400.

derivatives for these experiments. Comparison of **24** with compounds **22**, **26**, **27**, **28**, and **37** revealed that with respect to absolute brain concentrations [B], compound **24** was by far the best in this experimental setting. The same was true for absolute plasma concentrations [P]. Analyzing the [B]/[P] ratios revealed that the majority of the compounds exhibited good to excellent values (with the exception of **28** and **22**) pointing toward the fact that, at least in the rat, the proline sulfonamide derivatives did not seem to suffer from being efflux pump sub-

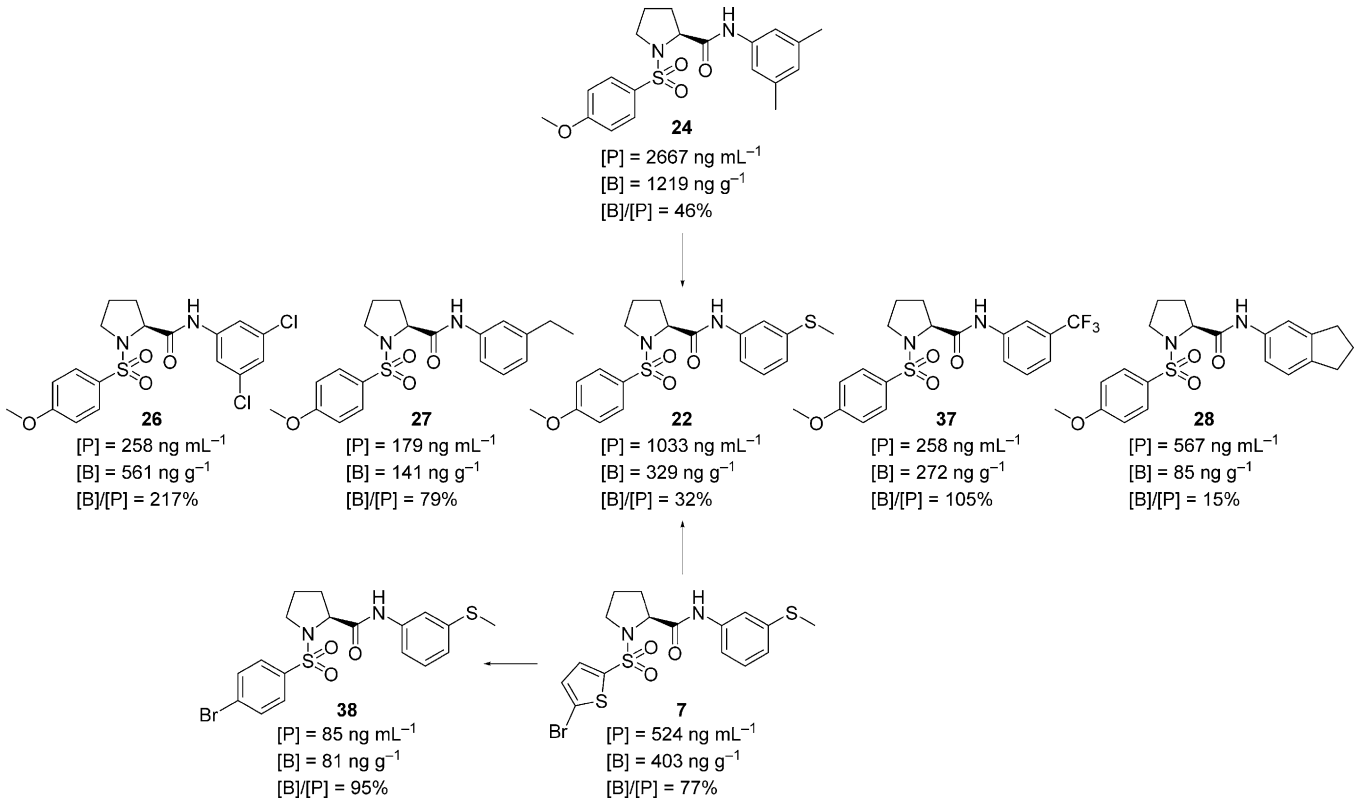


Figure 4. Structure–brain penetration analysis. BBB experiments were performed in Wistar rats at a p.o. drug dosing of 100 mg kg^{-1}; sampling at 3 h; drug formulation: PEG 400.

strates. Comparing **7** with **38** showed that replacement of the 2-bromothiophene moiety by the closest isostere, 4-bromophenyl, resulted in clearly inferior absolute brain concentrations without affecting the excellent [B]/[P] ratio. Finally, by moving from **7** to **22** it became clear that replacement of the 2-bromothiophene unit with a 4-methoxyphenyl group only resulted in increased plasma concentrations and only advancing one step, and replacing the 3-methylthiophenyl group by the 3,5-dimethylphenyl moiety adjusted all values in the desired range.

By going another step further in the analysis of the hit structure, further optimization possibilities consisted of scaffold hopping, as summarized in Figure 5. Compounds **7**, **22**, **42**, and **24** are depicted as the proline-based parent reference compounds for the respective substituents combined with new scaffolds. Comparison of **7** with **39** showed that the pyrrolidine template was strongly preferred over the piperidine template. Most of the activity lost in **39** was regained in **40**, which is based on a bridged bicyclic template containing a pyrrolidine and a piperidine moiety. Ring enlargement to a morpholine template was also not tolerated, as can be seen from comparison of **22** with **41**.

Compounds **43** and **44** seemed to represent the group of compounds with the most promising alternative scaffold. Further investigations resulted in many potent dual OX_1 and OX_2 antagonists with slight selectivity toward OX_2, but the series was not superior to the initial proline series with respect to cytochrome P450 inhibition profiles. In addition, in vivo rat BBB data (experiment done at 100 mg kg^{-1} p.o.) for compound **43** ([P] = 130 ng mL^{-1} and [B] = 228 ng g^{-1}) are exemplary for the whole series based on the 3-methylene proline (more than 10 compounds investigated in vivo) with good [B]/[P] ratios, but low to very low absolute concentrations. The bicyclic templates represented in **47** and **45** also did not exhibit advantageous activity and added synthetic complexity for the scaffold preparation. Therefore, it was decided not to continue investigations with these templates. Similar trends were observed for the compounds based on the tetrahydroisoquinoline template as represented by **46**, well in accordance with the results obtained for **39** and **41**. Based on these results it was decided to characterize compound **24** in detail. Figure 6 summarizes the data set.

The mode of antagonism of **24** at OX_1 and OX_2 was assessed in greater detail using Ca^{2+} release assays and stably transfected Chinese hamster ovary (CHO) cells recombinantly expressing human, dog, or rat orexin 1 or orexin 2 receptors. Orexin A concentration–response curves (CRC) were generated in the presence of increasing concentrations of **24**. The compound induced rightward shifts of the orexin A CRCs, demonstrating competitive antagonism, and Schild K_b values for 120 min antagonist pre-incubation were calculated to be $K_b = 17$ nM (human OX_1) and 2.4 nM (human OX_2). Schild K_b values were not different for 10 vs. 120 min antagonist pre-incubation time, indicating rapid association and dissociation kinetics of **24** from the orexin receptors. No species differences in potency, selectivity, or competitiveness were detected.

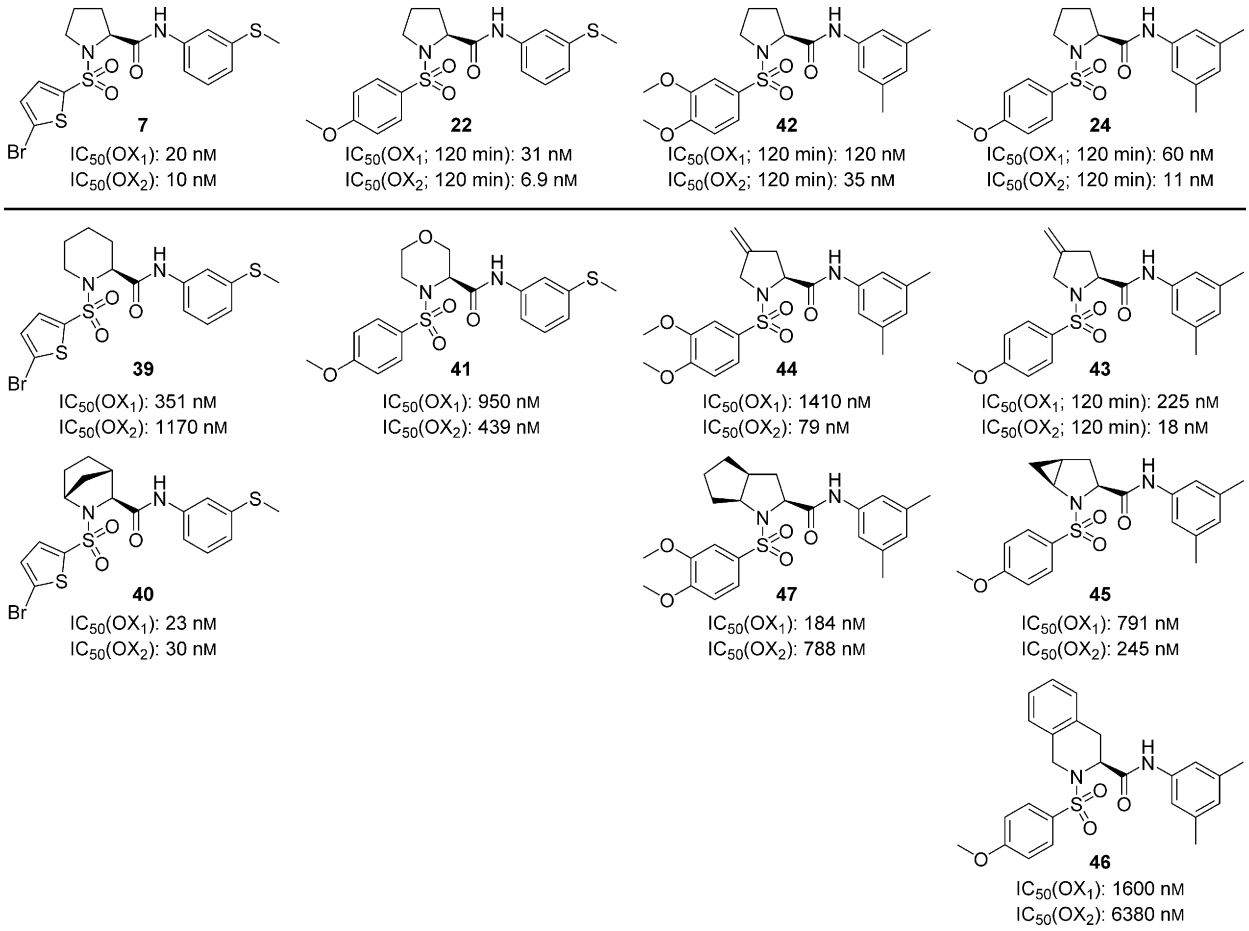


Figure 5. Scaffold hopping SAR. Orexin IC_{50} data represent the geometric mean of at least three independent experiments and were determined by using a fluorimetric imaging plate reader (FLIPR) assay. See the Supporting Information for assay details.

Compound **24** was tested on a panel of over 120 enzyme, radioligand binding, and tissue assays for established central and peripheral pharmacological targets. Compound **24** showed no significant activity against these targets at the tested concentration of 10 μM, thus demonstrating high selectivity for the orexin receptors. Detailed results are given in the Supporting Information.

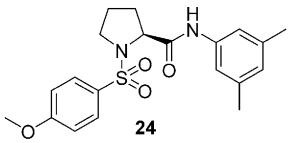
The pharmacokinetic characterization of compound **24** after single dose administration was performed in Wistar rats and Beagle dogs. The intravenous dose was 1 mg kg^{-1} in both species. Oral doses were 3 mg kg^{-1} in the dog ($n=4$) and 10 mg kg^{-1} in the rat ($n=5$). Table 4 gives an overview of the pharmacokinetic parameters in both species.

After intravenous dosing, compound **24** exhibited plasma clearances of 29 and 11 mL min^{-1} kg. Considering the blood-to-plasma partitioning of ~0.6, blood clearances in both species were between 50–70% of the respective liver blood flow. Notably, elevated clearance is required to achieve the appropriate

Table 4. Pharmacokinetic parameters of **24** after single intravenous and oral dosing to male Wistar rats and Beagle dogs.[a]

Intravenous:	$AUC_{0\rightarrow\infty}$ [ng h mL^{-1}]	CL [mL min^{-1} kg^{-1}]	V_{ss} [L kg^{-1}]	$t_{1/2}$ [h]
rat	586 (442–1020)	29 (16–38)	1.8 (1.3–2.8)	1.9 (1.6–3.1)
dog	1490 (1160–1780)	11 (9.4–11)	1.4 (1.0–2.0)	1.7 (1.1–3.3)
Oral:	$AUC_{0\rightarrow\infty}$ [ng h mL^{-1}]	c_{max} [ng mL^{-1}]	t_{max} [h]	F [%]
rat	2310 (1600–3080)	1600 (1290–1840)	0.5 (0.25–0.5)	39 (27–53)[b]
dog	2750 (1910–2960)	426 (351–611)	0.5 (0.25–0.75)	52 (43–57)[c]

[a] Data are the geometric mean (range in brackets); the median is given for t_{max}. [b] Bioavailability in rat was calculated with reference to the mean $AUC_{0\rightarrow\infty}$ after an i.v. dose of 1 mg kg^{-1}. [c] Bioavailability in dog was calculated from individual animals (cross-over design).



24

human OX receptors:	cytochromes:	in vivo pharmacokinetics:
$IC_{50}(hOX_1)$: 60 nM	IC_{50}(3A4T): 15 μM	
$IC_{50}(hOX_2)$: 11 nM	IC_{50}(3A4M): 29 μM	rat i.v. (1 mg kg^{-1})
$K_b(hOX_1)$: 17 nM	3A4 time shift: 2.7	AUC: 586 ng h mL^{-1}
$K_b(hOX_2)$: 2.4 nM	IC_{50}(2C9): 9.1 μM	CL: 29 mL min^{-1} kg^{-1}
	2C9 time shift: <1.0	V_{ss}: 1.8 L kg^{-1}
rat OX receptors:	IC_{50}(2D6): >50 μM	$t_{1/2}$: 1.9 h
$IC_{50}(rOX_1)$: 48 nM	2D6 time shift: none	rat p.o. (10 mg kg^{-1})
$IC_{50}(rOX_2)$: 9.6 nM		AUC: 2310 ng h mL^{-1}
$K_b(rOX_1)$: 28 nM	**in vitro metabolism:**	c_{max}: 1600 ng mL^{-1}
$K_b(rOX_2)$: 9.9 nM	HLM: 140 μL min^{-1} mg^{-1}	t_{max}: 0.5 h
	RLM: >1250 μL min^{-1} mg^{-1}	F: 39%
dog OX receptors:	DLM: 111 μL min^{-1} mg^{-1}	
$IC_{50}(dOX_1)$: 68 nM		dog i.v. (1 mg kg^{-1})
$IC_{50}(dOX_2)$: 26 nM	**protein binding:**	AUC: 1490 ng h mL^{-1}
$K_b(dOX_1)$: 27 nM	PPB (h): 99.3	CL: 11 mL min^{-1} kg^{-1}
$K_b(dOX_2)$: 4.2 nM	PPB (r): 99.3	V_{ss}: 1.4 L kg^{-1}
	PPB (d): 98.9	$t_{1/2}$: 1.7 h
		dog p.o. (3 mg kg^{-1})
	MDR1 assay:	AUC: 2750 ng h mL^{-1}
	P_{app} A—>B: 50.7	c_{max}: 426 ng mL^{-1}
	P_{app} B—>A: 56.4	t_{max}: 0.5 h
	E ratio: 1.1	F: 52%

Figure 6. Dataset generated with compound **24**. Orexin activity data represent the geometric mean of at least three independent experiments and were determined by using a fluorimetric imaging plate reader (FLIPR) assay. See the Supporting Information for assay details. CYP3A4T: testosterone as marker substrate; CYP3A4M: midazolam as marker substrate; HLM, RLM, and DLM values represent a normalized rate constant.

pharmacokinetic/pharmacodynamic profile of a sleep-promoting drug.

Mean volume of distribution at steady-state (V_{ss}) as a general measure of tissue distribution was 1.8 L kg^{-1} in rat and 1.4 L kg^{-1} in dog, i.e., exceeding total body water volume and indicating significant distribution of **24** into tissues. Compound **24** was rapidly absorbed after oral dosing as peak drug levels were reached within the first 30 min after dosing. Absorption from the gastrointestinal lumen was considered complete. Bioavailability was 39% in the rat and 52% in the dog, which is close to the maximally achievable bioavailability considering the respective blood clearances. Bioavailability of **24** therefore appears to be limited by clearance rather than by absorption under the conditions employed in this study.

The sleep-promoting effects of **24** were evaluated in male Wistar rats and in male Beagle dogs implanted with radiotelemetry probes recording continuously EEG/EMG and locomotor activity. Male Wistar rats were administered with single oral doses of 0, 10, 30, 100, or 300 mg kg^{-1} at the beginning of the nocturnal active phase, when endogenous orexin levels increase. Compound **24** significantly decreased the latency to the first persistent episode of non-REM sleep (60 s) and the first persistent episode of REM sleep (30 s) (one-way ANOVA; $p < 0.001$ and $p < 0.01$ respectively). The first episode of persistent non-REM sleep occurred within 10–15 min of treatment. Over the 6 h following administration, **24** was found to dose-dependently decrease total wake time and behavioral home cage activity (one-way ANOVA; $p < 0.001$), while increasing REM and non-REM sleep times (one-way ANOVA; $p = 0.001$ and $p < 0.001$) (Figure 7). The effect on sleep lasted between 2 and 12 h, depending on dose.

Non-REM and REM sleep increased in physiological proportion. Under treatment with **24**, sleep architecture was conserved, as the relative proportion of non-REM and REM sleep over the total sleep time were not significantly changed (one-way ANOVA, $p = 0.12$). Over the first 6 h night period post-administration, vehicle-treated rats slept a total of 36.8% of the time. Total sleep time was divided into 84.5% time spent in non-REM sleep and 15.5% time spent in REM sleep. At the highest tested dose, 300 mg kg^{-1} p.o., rats slept in total 52.9% of the first 6 h night period post administration. This total sleep time was divided into 80.0% non-REM sleep and 20.0% REM sleep.

Male Beagle dogs were treated with single oral doses of 0, 10, 30, 100, or 300 mg of **24** in a Cremophor RH40® formulation during their active phase, when endogenous orexin levels are naturally elevated. Compound **24** dose-dependently decreased behavioral signs of activity and electrophysiological signs of wakefulness (one-way ANOVA; $p < 0.001$ and $p < 0.001$). This decrease in wakefulness was accompanied by increases in

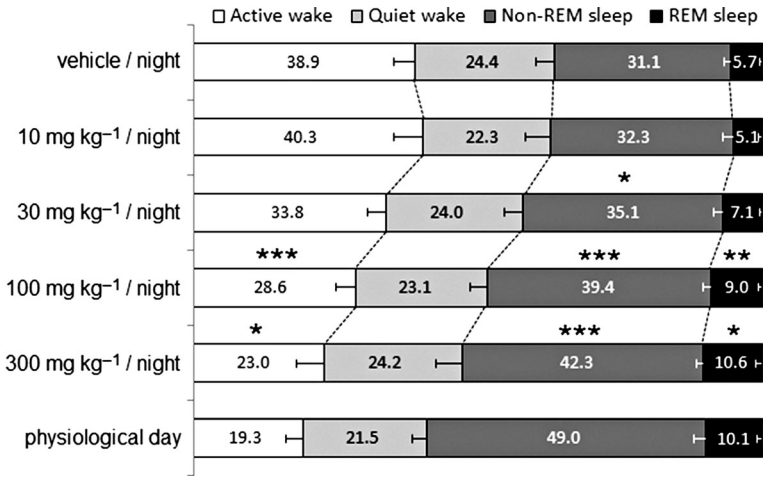


Figure 7. Effect of **24** on the relative time spent in sleep and wake stages (% of total time) during the first 6 h of the active period post-administration in male Wistar rats. Rats were administered single oral doses of vehicle (PEG 400) or compound **24** at 10, 30, 100 or 300 mg kg^{-1} at night, during their active phase. Data are the mean ± SEM. Paired *t*-test with Sidak correction for multiple comparisons: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to matched vehicle-treated rats; $n = 7$–15 per dose group. REM: rapid eye movement.

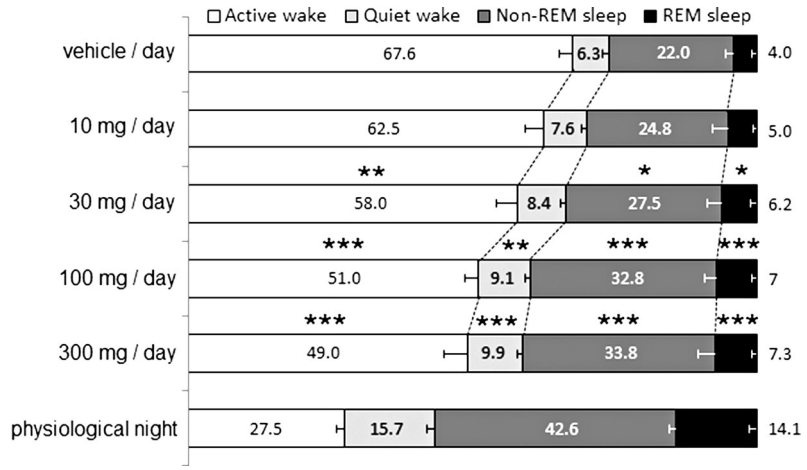


Figure 8. Effect of **24** on the relative time spent in sleep and wake stages (% total time) during 9 h of the active period post-administration in male Beagle dogs. Dogs were administered single oral doses of vehicle (Cremophor RH40®-based formulation) or compound **24** at 10, 30, 100, or 300 mg in the daytime, during their active phase. Data are the mean ± SEM. Bonferroni post-hoc analysis: $*p<0.05$, $**p<0.01$, $***p<0.001$ relative to matched vehicle-treated dogs; $n=13$/dose group. REM: rapid eye movement.

both REM and non-REM sleep times (one-way ANOVA; $p<0.001$ and $p<0.001$) (Figure 8). Onset of daytime somnolence occurred within 30 min of administration, and lasted between 2 and 9 h depending on the dose.

In dogs, similar to rats, non-REM and REM sleep increased in physiological proportions. Over the 9 h day period post-administration, vehicle-treated dogs slept in total 26.0% of the time. This total sleep time was divided into 84.6% time spent in non-REM sleep, and 15.4% time spent in REM sleep. At the highest tested dose, 300 mg, dogs slept in total 41.1% of the 9 h day period post-administration. This total sleep time was divided into 82.2% non-REM sleep and 17.8% REM sleep.

As mentioned in the introduction, the orexin system is implicated in regulating stress and anxiety-like reactions in rodents.[30] The dual orexin receptor antagonist **1** (almorexant) shows anxiolytic-like behavioral effects in a cued fear model[28] and decreases the autonomous nervous system response to certain types of stressors.[25] To probe the anxiolytic potential of **24**, rats were trained to associate an unpleasant electric foot shock with a cue light (conditioned stimulus) which was delivered through a metal grid within an animal enclosure. When these rats were later re-exposed to the same enclosure either under light (conditioned stimulus; cued fear) or in the dark (no conditioned stimulus; contextual fear), compound **24** decreased the fear-potentiated startle reflexes in response to a sudden loud noise with effective doses of 100 and 300 mg kg^{-1} (Figure 9a). Control experiments revealed that startle reflexes in naive rats remained unaffected by **24** (300 mg kg^{-1}; Figure 9b), as well as forepaw grip strength, as an indicator of potential muscle relaxation (Figure 9c).

Compound **24** (100 mg kg^{-1}) was also tested in a resident–intruder rat model where it significantly decreased the social-stress-induced increases of locomotion, body temperature, and heart rate (Figure 10a–c). Mean arterial blood pressure remained unaffected (Figure 10d).

In addition to exerting anxiolytic-like effects, dual orexin receptor antagonists are also known to decrease certain types of addiction-like behaviors in rodents.[27,29] Compound **24** (100 mg kg^{-1}) effectively decreased the expression of locomotor sensitization to morphine (Figure 11a) without affecting the conditioned place preference induced by conditioned morphine reward (Figure 11b), mimicking previous findings with **1** (almorexant).[29]

Finally, as part of our pharmacological characterization of **24**, we also assessed potential effects on cognitive and motor functions. In the Morris water maze, where rats are trained to use spatial navigation along external cues to locate a hidden platform in a large water tank, repeated treatment with **24** (300 mg kg^{-1}) during training was not different from vehicle treatment. Both groups, but not rats treated with the muscarinic antagonist scopolamine, used as positive control, established spatial memory. This was indicated by the larger amount of time spent searching for the platform in the target quadrant of the maze than in the other quadrants, or chance level (Figure 12a). In comparison with the positive $GABA_A$ receptor

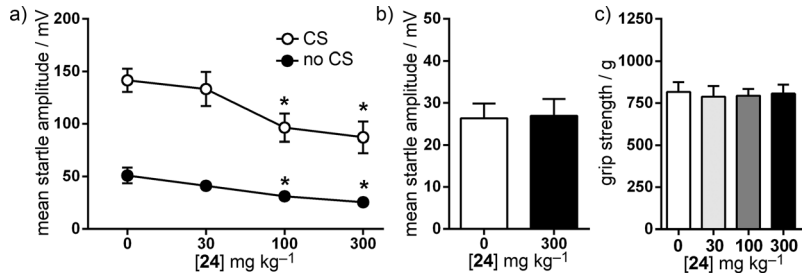


Figure 9. a) Rats were exposed to the fear-potentiated startle test 2 h after oral treatment with vehicle (0 mg kg^{-1}) or increasing doses (30, 100, 300 mg kg^{-1}) of **24**. The drug dose-dependently decreased the mean startle amplitude both upon presentation of the cued fear-eliciting conditioned light stimulus (CS) and under dark conditions (no CS) where only contextual fear traces remain. Data are the mean ± SEM; $*p<0.05$ vs. 0 mg kg^{-1} under CS or no CS conditions, respectively; within subjects cross-over design, $n=16$. b) Compound **24** at 300 mg kg^{-1} p.o. had no effect on basal startle reactions in the dark in naive rats; within subjects cross-over design $n=16$; data are the mean ± SEM. c) Compound **24** at 30, 100, 300 mg kg^{-1} p.o. had no influence on forepaw grip strength in rats; $n=6$–7; data are the mean ± SEM.

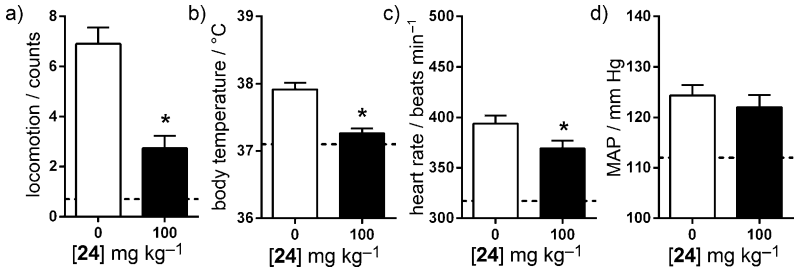


Figure 10. Rats equipped with telemetry transmitters were orally treated with vehicle (0 mg kg^{-1}) or **24** (100 mg kg^{-1}) 2 h before submission to social interaction stress with a male intruder for a duration of 1 h. During stress, a) locomotion, b) body temperature, and c) heart rate were significantly decreased by **24**, whereas d) mean arterial blood pressure (MAP) remained unaffected. Data are the mean ± SEM of transmitter recordings during the 1 h period of social interaction. Dotted lines represent the respective baseline values before vehicle/drug treatment and stress exposure. Within subjects cross-over design, $n = 12$; $^*p < 0.05$ vs. 0 mg kg^{-1}.

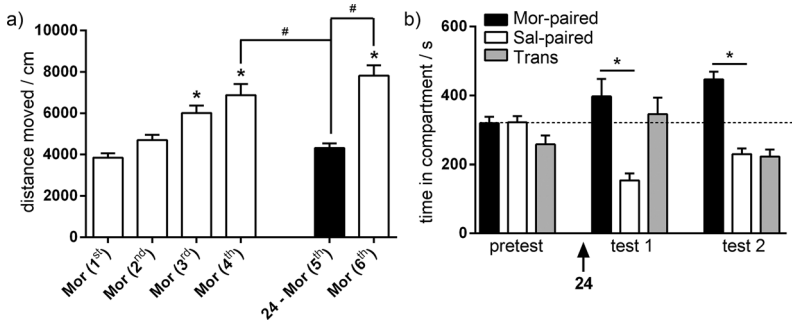


Figure 11. a) Locomotor sensitization was assessed as the increase in the distance moved in a box during a defined period (45 min) upon repeated injections (four times) with morphine (Mor; 10 mg kg^{-1} s.c.). Before the fifth morphine injection rats received **24** (100 mg kg^{-1} p.o., −2 h) which normalized the distance moved to those of non-sensitized levels. After compound **24** washout and an additional (sixth) morphine exposure, the previous level of locomotor sensitization was again observed; $^*p < 0.05$ vs. Mor (1st), $n = 16$. b) Rats were tested for the absence of initial place preference for either compartment in a pretest before conditioning with saline or morphine (10 mg kg^{-1} s.c.) for eight days. After conditioning, at the conditioned place preference (CPP) test 1, treatment with **24** (100 mg kg^{-1} p.o., −2 h) failed to inhibit the induced CPP for the morphine (Mor)-paired compartment over the saline (Sal)-paired compartment. Time spent in the neutral transition (Trans) compartment was, however, increased. This was a specific effect that can be ascribed to dual OXR blockade, which was previously observed with **1** (almorexant).[29] Indeed, after **24** washout, another control test (2) was conducted, which still revealed the place preference for the morphine-paired compartment, but showed the expected low time spent in the third neutral transition compartment. Data are the mean ± SEM; $n = 16$.

modulator zolpidem, which is frequently used as sleep medication in the clinic at present, **24** (300 mg kg^{-1}) did not impair contextual memory acquisition in the passive avoidance learning paradigm (Figure 12b), nor forced motor performance in the rotating rod test (Figure 12c). These data confirm the particular mode of action of DORAs, which induce sleep without negative impact on cognition or motor function in rats.[45–49]

Importantly, DORAs primarily promote sleep under little or non-stimulating environmental conditions (such as in the home cage).[22] For instance, dogs treated with DORAs are able to wake up just fine when presented with emotionally salient acoustic stimuli,[50] and rats treated with DORAs perform normally on the rotarod after being woken up during their daytime sleeping phase (Figure 12c and Ref. [48]). It is therefore unlikely that the anxiolytic-like effects of **24** observed in the present study are merely a consequence of increased sleepiness.

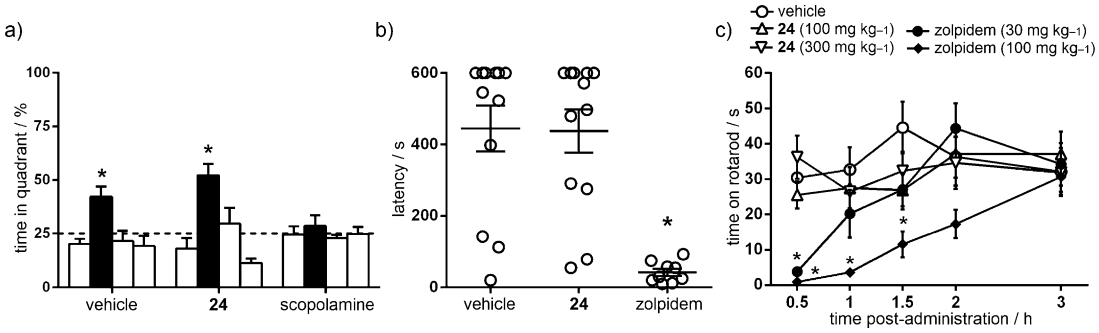


Figure 12. a) Rats were trained for two consecutive weeks under drug treatment in the Morris water maze to find a hidden platform in a water tank through orientation on external spatial cues. On the test day (probe trial) both vehicle- and **24** (300 mg kg^{-1} p.o.)-treated rats spent significantly more time than chance level (25%, indicated by the dotted line) in the target quadrant (black bars), where the platform was originally located, indicating successful spatial memory formation. Scopolamine (0.7 mg kg^{-1} s.c.)-treated rats failed to establish spatial memory; $n = 7$–8 per group; $^*p < 0.05$ vs. chance level. b) In the passive avoidance paradigm rats that were treated during the training trial with vehicle or **24** (300 mg kg^{-1} p.o.) showed significantly higher latency to step through to the aversive side of the shuttle box on the test day than rats that had been treated with zolpidem (30 mg kg^{-1} p.o.) during training, indicating intact aversion learning under OXR blockade but not under $GABA_A$ receptor activation; $n = 9$–12 per group; $^*p < 0.05$ vs. vehicle. c) Following oral treatment the forced motor activity of rats was tested repeatedly on the rotarod up to 3 h. Whereas **24** (30 and 100 mg kg^{-1})-treated rats behaved similarly to those treated with vehicle, zolpidem (30 and 100 mg kg^{-1})-treated rats showed impaired motor function in a dose-dependent manner; $n = 12$ per group; $^*p < 0.05$ vs. vehicle at the respective time point. Data are the mean ± SEM.

Conclusions

In summary, we have identified and broadly characterized compound **24** as a competitive small-molecule orexin receptor antagonist displaying activity toward both the orexin 1 and orexin 2 receptors. A broad receptor screen demonstrated that **24** was highly selective for the orexin receptors among more than 100 other potential neuronal targets. The in vivo rat blood–brain barrier penetration experiment confirmed the excellent brain penetration properties of **24**, and in vitro tests suggested that **24** is not a human P-gp efflux pump substrate. Pharmacokinetic studies in rats and dogs revealed that **24** exhibited a favorable pharmacokinetic profile for an insomnia drug, and this translated into beneficial pharmacological effects in sleep studies in both rats and dogs. Compound **24** was further characterized in pharmacological rat experiments assessing stress- and anxiety-related readouts exploring a broader therapeutic potential for dual orexin receptor antagonists, besides insomnia.[49,51] In the meantime, **24** has been investigated in a phase I human clinical trial.[52]

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Keywords: insomnia · neurotransmitters · orexin receptor antagonists · peptides · stress-related disorders

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