Neuropeptide S receptor ligands: a patent review (2005-2016)

Abstract

Introduction: Neuropeptide S (NPS) is a 20-residue peptide and endogenous ligand of the NPS receptor (NPSR). This receptor was a formerly orphan GPCR whose activation increases calcium and cAMP levels. The NPS/NPSR system is expressed in several brain regions where it controls important biological functions including locomotor activity, arousal and sleep, anxiety, food intake, memory, pain, and drug addiction.

Areas covered: This review furnishes an updated overview of the patent literature covering NPSR ligands since 2005, when the first example of an NPSR antagonist was disclosed.

Expert opinion: Several potent NPSR antagonists are available as valuable pharmacological tools despite showing suboptimal pharmacokinetic properties *in vivo*. The optimization of these ligands is needed to speed up their potential clinical advancement as pharmaceuticals to treat drug addiction. In order to support the design of novel NPSR antagonists, we performed a ligand-based conformational analysis recognizing some structural requirements for NPSR antagonism. The identification of small-molecule NPSR agonists now represents an unmet challenge to be addressed. These molecules will allow investigation of the beneficial effects of selective NPSR activation in a large panel of psychiatric disorders and to foresee their therapeutic potential as anxiolytics, nootropics, and analgesics.

Keywords: addiction, anxiety, neuropeptide S, neuropeptide S receptor antagonists, psychiatric diseases, sleep disorders.

1. Introduction

Neuropeptide S (NPS, primary sequence in humans: SFRNGVGTGMKKTSFQRAKS) was identified in 2002 as the endogenous ligand of the previously orphaned G protein coupled receptor (GPCR) GPR154, now referred to as neuropeptide S receptor (NPSR), using a reverse pharmacological approach.[1] In 2004, an elegant study by Xu and colleagues [2] described for the first time some functional features of the NPS/NPSR system. As with other neuropeptides, NPS is cleaved from a larger precursor protein. The NPS sequence is highly conserved among vertebrates with few variations located in the centre and at the C-terminus of the peptide while the N-terminal sequence SFRNGVG is identical in all species, thus suggesting that this may represent the bioactive core of the peptide.[3] Interestingly, NPS is absent in fish genomes and is also not found in amphibian or reptile DNA sequences, indicating that this neuropeptide arose relatively late during vertebrate evolution.[3] The NPSR is a typical GPCR and it shows moderate homology to other members of the GPCR family, the closest relatives being the vasopressin receptors. The *in vitro* pharmacology of the human and mouse NPSR has been mainly studied in heterologous expression systems. These studies showed that NPS increases both intracellular calcium levels and cAMP accumulation with EC_{50} values in the low nanomolar range. This indicates that the NPSR can signal via both G_q and G_s pathways to increase cellular excitability.[2, 4] Moreover, it has recently been reported that the activation of the NPSR can also lead to an increase of the phosphorylation of the extracellular signal-regulated kinases (ERK) 1 and 2.[5, 6] Multiple single-nucleotide polymorphisms (SNP) and several splice variants have been identified in the human NPSR gene. The most interesting SNP described for the human NPSR results in an Asn-Ile exchange at position 107. This receptor polymorphism has functional implications since the hNPSRIle^{107} receptor displayed similar binding affinity but higher NPS potency (by approx. 10-fold) than the hNPSRAsn^{107}.[4]

The distribution of NPS and NPSR in the brain has been studied in rodents using quantitative RT-PCR and in situ hybridization experiments. These studies reveal that the NPS precursor mRNA is

strongly expressed in the brainstem in only three distinct regions: the locus coeruleus, the principal sensory trigeminal nucleus, and the lateral parabrachial nucleus. [2, 7, 8] In these areas, the peptide co-localizes with glutamate and corticotropin-releasing factor.[7] In contrast, NPSR mRNA is widely distributed in rat and mouse brain with the strongest expression in the olfactory nuclei, amygdala, subiculum, and some cortical structures, as well as various thalamic and hypothalamic regions.[2, 7, 8] This pattern of NPSR distribution has been confirmed with an NPSR antibody.[9] A recent study investigated the distribution of NPS and NPSR mRNA in the human pons. NPS positive neurons were detected in the parabrachial area, in the Kölliker-Fuse nucleus and around the adjacent lateral lemniscus. NPSR mRNA expressing neurons were found in the rostral laterodorsal tegmental nucleus, in the cuneiform nucleus, in the microcellular tegmental nucleus region and in the periaqueductal gray.[10]

The *in vivo* biological actions of NPS have been studied in rodents. A large amount of data, performed in different laboratories and using different validated assays (i.e. elevated plus, zero and T maze, light-dark box, open field, marble burying, stress induced hyperthermia, and defensive burying), demonstrated that NPS injected intracerebroventricularly (i.c.v.) in rodents elicits anxiolytic and panicolytic-like effects [2, 11-15] and facilitates the extinction of fear.[16-19] The amygdala is the brain area crucial for NPS anxiolytic action.[16, 20, 21] Importantly, in the same range of doses, NPS evokes stimulant and wake promoting effects.[2, 12, 22-25] The stimulant-anxiolytic profile represents the peculiarity of the NPS/NPSR system. Indeed the main side effect of classical anxiolytic drugs is sedation while stimulant drugs are in general anxiogenic. Regarding the effects of NPS on emotional states, in addition to its anxiolytic activity, the peptide modulates social behaviours. NPS was shown to reduce intermale aggression in rats [26] and mice,[27] to reverse fear of unknown con-specifics induced by social fear conditioning and to reduce avoidance of known aggressive con-specifics.[28] Another biological function modulated by NPS is memory. NPS injected i.c.v. facilitates spatial [29-31] and object recognition,[32-34] memory and counteracts the memory deficit

caused by scopolamine and MK801 [29, 32] in rodents. Finally, several recent studies demonstrated analgesic activity of NPS. Supraspinal injection of NPS reduced nociception in different models of acute and chronic pain.[35-40] Moreover, Yang et al.[35] reported that the intra-amygdala injection of a selective NPSR antagonist increases microglia and astrocyte reactivity and TNF-α, IL-1β, and IL-6 production in the spinal cord of a rat model of neuropathic pain. However, no phenotype was detected in NPSR(-/-) mice in the formalin test,[41] thus the role of the NPS system in pain and inflammation is still unclear. Considering these NPS actions, NPSR selective agonists are promising candidates as anxiolytics without sedative side effects, nootropics, and analgesics. Recent preclinical findings suggest an important role for the NPSergic system in drug abuse (for review see [42] and [43]). In particular NPS promotes relapse to cocaine [23] and alcohol [44] seeking in rats, while the administration of NPSR antagonists strongly reduced reinstatement of cocaine seeking behaviour (see Table 1).[45-47] Thus, NPSR antagonists may be useful in the treatment of craving and relapse in drug dependent subjects. Furthermore, selective NPSR antagonists, together with NPSR(-/-) mice, have been largely employed as valuable research tools to investigate the biological functions controlled by the NPS/NPSR system (*in vivo* activities of NPSR antagonists and behaviour of NPSR(-/-) mice are summarized in Tables 1 and 2, respectively).

The major purpose of this article is to review the chemical and pharmacological features of NPSR ligands so far described in patent literature. Starting from 2005, both peptide and small molecule NPSR antagonists have been identified. While small molecule NPSR antagonists will be discussed in detail in the present review, peptide antagonists are not included in this work, since no patents have been disclosed on this topic. However, structure-activity studies that led to identification of the three most used peptide NPSR antagonists [D-Cys(^{t}Bu)^5]NPS, [D-Val^5]NPS, and [^{t}Bu-D-Gly^5]NPS and their pharmacological features have been extensively discussed by Guerrini and colleagues.[48] Of note, the same research group recently published a new NPSR antagonist [49] and a tetrameric derivative of NPS named PWT-NPS.[50, 51]

2. NPSR ligands in the patent literature: small-molecule NPSR antagonists

The medicinal chemistry and pharmacology of both peptide and non-peptide modulators of the NPSR as a potential drug discovery target have been reviewed in the past with particular attention given to the main academic contributions to this research area.[48, 52] In the following section, we provide a comprehensive analysis and an updated overview of the patent literature that to date is almost exclusively focused on small-molecule NPSR antagonists. We will describe several heterocyclic templates that have been developed since 2005 when the first example of an NPSR antagonist was disclosed.

2.1. Oxazolo[3,4-*a*]pyrazine derivatives

The first patent application describing non-peptide NPSR antagonists was filed by Takeda Pharmaceuticals in 2005 when the company discovered the oxazolo[3,4-*a*]pyrazine template with general structure **1** (Figure 1) following an HTS program.[53] More than two hundred compounds were obtained from the combination of different substitutions at the 1- and 7-positions of the byciclic core. The reported structures were bis-functionalized at the 1- position with methyl, cyclopropyl, cyclohexyl, benzyl moieties or (substituted)phenyl rings (see R_2 in Figure 1) even though a 1,1-diphenyl is present in almost 90% of the investigated ligands. On the other hand, the chemical space around the piperazine nitrogen N^7 was more deeply scrutinized, with the introduction of a wide range of urea, carbamate, carboxamide or sulphonamide functions (see R_1 in Figure 1). The lack of an English translation of this patent application hampers the definition of clear structure-activity relationships (SAR); nevertheless, some compounds were quoted to have NPSR inhibitory potency below 100 nM and from these were excluded all derivatives bearing di-(cyclo)alkyl/benzyl groups at the 1-position. At the 7-position, a *N'*-(substituted)benzyl urea function was particularly beneficial for potency. SHA-68 (**1a**, Figure 1) and SHA-66 (**1b**), are largely recognized as the most

representative members of this class. In particular, the *in vitro* activity of SHA-68 was studied in different laboratories using different experimental conditions, assays (Ca^{2+} and cAMP), protocols (inhibition and Shild plot analysis) and NPSR isoforms. In all these studies the *in vitro* potency of this ligand was quite comparable, with pA_2/pK_B values ranging from 7.28 to 8.16 at the hNPSRAsn^{107}, from 7.55 to 8.03 at the hNPSRIle^{107}, and from 7.74 to 8.16 at the mNPSR.[6, 54-59] In addition, SHA-68 exhibited high affinity for the hNPSR in radioligand binding experiments (pK_i = 7.32) and high selectivity over fourteen unrelated GPCRs. Thus, the potencies of the different NPSR antagonists described below were normalized to that of SHA-68 in Table 3.

Marked enantioselective interaction of SHA-68 with the mouse and human NPSR has been recently demonstrated.[55] Specifically, the *R*-isomer was able to antagonize the stimulatory effect of NPS with slightly higher potency than the racemic mixture (see Table 3). By contrast, the *S*-enantiomer showed a substantial loss of potency (> 100-fold). In addition, with the synthesis of a series of 7-substituted analogues, Zhang and co-workers contributed to the elucidation of the structural determinants for NPSR antagonist activity in this class of compounds.[60] Briefly, alkylation of the free urea nitrogen or its replacement with carbon (to give amide) or oxygen (to give carbamate derivatives) significantly reduced potency at both human NPSR isoforms. This would indicate the particular importance of the urea moiety for antagonist activity. In the same way, the mono methyl substitution of the methylene spacer between the urea function and the 4-F-phenyl ring or its elongation led to a critical reduction of potency. Some molecular modelling studies were performed as a first attempt to rationalize the binding mode of these ligands [61] and docking analysis seems to corroborate the importance of the NH residue of the urea moiety as a potential hydrogen bond donor in an interaction with Asp 297 of the NPS receptor. In addition, the 1,1-diphenyl portion would face a cluster of aromatic residues (Phe 177, Tyr 290, Phe 293) taking π-stacking interactions.

The *in vivo* pharmacological profile of SHA-68 has been thoroughly investigated in a large panel of assays including locomotor activity, recovery of righting reflex, open field behavior, elevated

plus maze, defensive burying and palatable food intake.[16, 56, 62] Upon i.p. administration, the molecule reaches pharmacologically relevant (at or above its K_b value) plasma and brain levels [54], nevertheless, its effectiveness *in vivo* suffers from high variability according to the different assays and administration routes, thus suggesting suboptimal pharmacokinetic properties.[56] With the aim to overcome these limits, the 4-F-benzyl group was replaced with a salifiable piperidine function such as in **1c** (RTI-118, Figure 1). RTI-118 still shows acceptable potency (pA_2 of 6.31 and 6.96 at the hNPSRAsn^{107} and hNPSRIle^{107}, respectively) with the advantage of a higher water solubility (3600-fold improved over that of SHA-68 at pH 7),[57] that may be the reason for a slightly better *in vivo* efficacy in reducing cocaine self-administration.[45, 47, 63] A further effort to enhance the druglikeness of SHA-68 and RTI-118 was later reported by C. Hassler et *al*.[57] who tried to replace the benzyl moiety of **1a**/**1b** in an attempt to provide compounds that could be protonated in a suitable *in vivo* formulation and at a physiological pH. The 2-pyridyl analogue **1d** (Figure 1) resulted in a 5-fold decrease of potency compared to SHA-68 (see Table 3). On the other hand, the aniline derivative **1e** displayed a similar potency to SHA-68. Interestingly, this molecule was also equipotent with the *n*-butyl urea **1f**, somewhat contradicting the assumption that a benzyl moiety would be needed for promoting hNPSR antagonist potency. While any attempt to modify RTI-118 led to a substantial loss of activity, the separation of its individual enantiomers allowed for confirmation that the bioactive isomer displays an *R* configuration (see Table 3). *R*-**1c** was about 4-fold more active than the racemic mixture, while *S*-**1c** was inactive up to high micromolar concentrations. These data are totally in agreement with previous findings regarding SHA-68.[55] *In vivo* analysis in mice indicated a significant blockade of NPS induced locomotor activity upon i.p. administration of *R*-**1c** at 50 mg/kg. In addition, the compound showed rapid systemic absorption with an apparent half-life of 34 min and satisfactory brain penetration. Together, these data demonstrate that *R*-**1c** behaves as an NPSR antagonist *in vivo* with central action after peripheral administration.

2.2. Furo[3,4-c]pyridine derivatives

A series of 4,5,6,7-tetrahydrofuro[3,4-*c*]pyridine-1(3*H*)-one derivatives showing antagonistic activity at the NPSR have been recently developed by the Research Triangle Institute, North Carolina.[64] The therapeutic potential claimed for such molecules embraces the possible treatment of drug addiction, including substance abuse and relapse. Representative compounds from this series are reported in Figure 2 (general structure **2**). Essentially, the chemotype has been explored in relation to the effect of substitutions at the C^3- and N^5-positions. However, a 3-isobutyl-3-phenyl pattern was widely maintained along the series, while the chemical space around N^5 was probed with the synthesis of urea, thiourea and carboxamide analogues (see R_1 Figure 2). Unfortunately, the results from the screening assays were provided for a very limited number of compounds, thus making it difficult to understand the rationale behind the choice of such substitutions and of the related SAR profile. The urea derivative R06039-447 (**2a**, pA_2 = 5.83, Figure 2) and the carboxamides R06039-478 (**2b**, pA_2 = 7.51) and R06039-286 (**2c**, pA_2 = 6.18) are in fact the only members of this class for which the antagonist potency has been disclosed. Nevertheless, it is worth mentioning that the tetrahydrofuro[3,4-*c*]pyridine scaffold clearly mimics the oxazolo[3,4-*a*]pyrazine nucleus of **1c** (Figure 1), developed by the same inventors, since unambiguous key superimposition elements can be distinguished (specifically, 1-, 3- and 5- positions of structure **2** would correspond to 3-, 1- and 7-positions of **1c**, respectively). Interestingly, the notable potency of **2b** would suggest the possibility for replacement of one of the two phenyl rings in the SHA-68 series with branched alkyl chains. It should be also noted that the tetrahydrofuro[3,4-*c*]pyridinone template is deprived of the asymmetric piperazine carbon of particular importance for SHA-68 analogues (see above). However, chirality was restored around the C^3 atom of the furanone ring even though the currently available biological results are limited to racemic mixtures. To the best of our knowledge, to date only *in vitro* data are available for this chemotype although furo-pyridinone derivatives were apparently profiled for their *in vivo* activity in mouse models of locomotor activity and anxiety (elevated plus maze).[64]

2.3. Indanone, indandione derivatives

In 2013 Actelion Pharmaceuticals reported a large series of indanone and indandione derivatives claiming their possible effectiveness in the prevention or treatment of several NPSR-related diseases.[65] In Figure 3 we depicted some structural modifications of the bicyclic core detailed in the patent claims. Roughly 900 compounds have been evaluated for their antagonist activities against the endogenous ligand through fluorescence-based Ca^{2+} measurement (FLIPR assay) carried out in HEK cells. Structures and potencies (pIC_{50} at the hNPSRAsn^{107}) of selected members of this family (**3a-w**) are reported in Figure 3.

2-(4-Benzyl-piperazin-1-yl)-2-phenyl-indan-1,3-dione (**3a**, pIC_{50} = 7.57) can be considered as the reference compound for SAR comparisons within both the series. As for the substitution of the *N*-benzyl ring, NPSR inhibitory potency seems to be enhanced in compounds featuring a small *ortho*-alkyl (**3b**, pIC_{50} = 8.52) or *ortho*-alkoxy (**3c**, pIC_{50} = 8.22) group. Bulkier and/or electron withdrawing radicals (such as a CF_3) were particularly detrimental for potency especially when introduced at the p-position.[65] In addition, lengthening the methylene spacer of the benzyl group with an *N*-phenyl-ethyl or an *N*-phenyl-propyl one, progressively compromised potency.[65] In compounds **3d-l**, the effect of the functionalization of the 2-Ph ring or its replacement by heteroaryl moieties, have been scrutinized. The results indicated the *meta* substitution of the 2-Ph ring as preferred to promote potency (i.e. **3d**, pIC_{50} = 8.70; **3e**, pIC_{50} = 8.40; **3f**, pIC_{50} = 8.70). Conversely, the replacement of the 2-Ph ring with unsubstituted 5- or 6- membered heterocycles (see compounds **3g-j**, Figure 3), was poorly tolerated. Nevertheless, decoration of the 2-heterocycle with proper alkyl chains fully restored the activity reaching, in some cases, low nanomolar potencies (i.e. **3k**, pIC_{50} = 9.00; **3l**, pIC_{50} = 8.40). The pIC_{50} values of compounds **3m-p** (Figure 3), suggest that an unsubstituted indandione core would be recommended for ligand-receptor interaction. This is also confirmed in derivatives **3q-s** in which

the intact indandione bicycle was combined with key 2,2-disubstitutions, resulting in very high potency.

The removal of the 3-carbonyl function led to indanone derivatives that have been investigated with analogous substitution patterns (see **3t-w**, Figure 3). With few exceptions, this structural modulation induced a maintenance of activity suggesting that the 3-ketone moiety of the previous series would not be involved in determinant interactions with NPSR (compare for example **3t-v** with **3a-c**, respectively, or **3w** with **3q**). For the same reason the SAR profile around 2-Ph substitution of this series was practically unchanged. More interestingly, the enantioselectivity of the interaction between a number of indanones with the NPSR has been investigated in view of the chiral nature of such a chemotype. Similar to SHA-68-related antagonists, a clear and complete preference was detected for one of the two enantiomers even though its exact configuration was not declared. For example for compound **3t**, the pIC_{50} value of the racemate and the two optically pure isomers were of 7.77, 8.22 and 5.63, respectively. Unfortunately, although very potent compounds were identified among this series *in vitro*, no data are available to date about their *in vivo* potency.

2.4. Imidazo[1,2-*a*]pyridines

NIH laboratories recently developed a high-throughput homogeneous time-resolved fluorescence (HTRF) assay to measure NPS-induced formation of cAMP in CHO cells.[58] Using this assay more than 200,000 molecules were screened, among which imidazopyridinium analogues with general structure **4** (Figure 4) were identified.[66] MLS001018695 (**4a**) was initially recognized as valuable hit compound (cAMP pIC_{50} = 5.77-6.13) with even higher potency in calcium mobilization (pIC_{50} = 7.11-7.29) and ERK (pIC_{50} = 7.52) assays, along with good affinity in radioligand binding experiments ($[^{125}I]Tyr^{10}$-hNPS pIC_{50} = 6.97-7.07).[58] SAR analysis of these derivatives highlighted the importance of both the 2-methyl group and the thiophosphoryl function that were variously modulated (see Figure 4). Hit to lead optimization was elegantly guided by a

molecular model in which the diphenylphospho group was overlapped with the geminal diphenyl substituents of **1a** (Figure 1).[58] Accordingly, the N^1 imidazole nitrogen would reasonably match the N^7 piperazine nitrogen of **1a**. This suggested the need to restore a hydrophobic N^1-phenyl-alkyl moiety on the imidazo[1,2-*a*]pyridine core in order to mimic the 4-F-benzyl urea of **1a** (see R1 substituent in Figure 4). The best result was obtained with the N^1-cinnamyl derivative NCGC84 (**4b**, also labeled as NCGC00185684) featuring weak biased antagonist properties, due to the preferential blockade of ERK-phosphorylation over intracellular cAMP or Ca^{2+} responses to NPS. Further studies, with more selective compounds are needed to elucidate the biological significance of these kind of ligands. Compound **4b** was 15-fold more potent than **1a** in the calcium assay (Table 3) and roughly 10-fold more potent in the cAMP assay when evaluated under the same experimental conditions.[6] Interestingly, when the binding selectivity of this ligand was assessed over 55 different biological targets, significant affinities in displacing a μ opioid agonist (pIC_{50} = 7.22), a k opioid agonist (pIC_{50} = 6.68) and a Na^+ channel antagonist (pIC_{50} = 6.70) were observed.[58] The molecule revealed better *in vitro* metabolic properties if compared with previously reported NPSR antagonists. Therefore it was selected for further *in vivo* preclinical evaluation. Systemically administered, **4b** (i.p., 10 mg/kg in mice) crosses the blood-brain barrier, keeping brain concentrations above its *in vitro* IC_{50} for over 48 h.[58] In addition, **4b** (10 μg) completely reversed NPS (10 μg)-induced suppression of palatable food intake in rats following i.c.v. administration. This compound (1 mg/kg, i.p.) also inhibits alcohol-induced central ERK phosphorylation in the rat central amygdala[6] and decreased alcohol self-administration and motivation for alcohol reward at a dose that does not influence locomotor activity.

2.5. Quinoline derivatives

In an effort to identify new NPSR modulators, Merck Research laboratories provided a new chemotype with the development of a series of 1,3-disubstituted 2-oxoquinolines (see the structural manipulation of the bicyclic scaffold in Figure 5, general structure **5**).[67] The parent compound of

this family is the piperazine derivative **5a** that displays high antagonist potency (hNPSR pIC_{50} = 7.74) but was shown to be a substrate for the P-glycoprotein transporter (P-gp) *in vitro*, that generally leads to low brain concentration.[68] Any attempts to modify the cyclohexylamide portion of the molecule resulted in a marked loss of affinity. In addition, the piperazine side chain was recognized as responsible for P-gp-mediated efflux at the blood-brain barrier, thus it was chosen for an extensive computationally assisted optimization. This led, for example, to some piperidine analogues such as **5b** (Figure 5) with enhanced NPSR activity but still poor CNS exposure. The substitution with morpholine induced the desired balance between antagonist potency and P-gp properties as in compound QA1 (**5c**). Also in this case, one of the two enantiomers of **5c** (configuration not disclosed) seems to have greater activity than the other with a relevant eudysmic ratio (0.013, see Table 3). Of note, these quinoline derivatives are the most potent inhibitors of the NPSR according to several *in vitro* assays. For this reason, **5c** (as racemic mixture) has been profiled as potential pharmacological tool *in vivo*, apparently showing no off-target activities along with high affinity for the rat isoform of the NPSR (pIC_{50} from displacement studies of 8.89). In addition, i.p. administration of **5c** in rats (30 mg/kg) was linked to good plasma, brain and CSF exposure. In consideration of this data, V. Camarda et al. recently contributed to the in vivo characterization of **5c** by evaluating its effect in mouse models (i.p., 30 mg/kg) of righting reflex and locomotor activity.[59] Despite the high in vitro potency of this compound, only poor or partial in vivo activities were observed in both assays. The lack of effectiveness of **5c** in mice was interpreted as a possible lower CNS exposure than that observed in rat and therefore species-specific pharmacokinetic properties may be the reason of this discrepancy.[59]

2.6. 5*H*-imidazo[2,1-*a*]isoindoles

An unusual tricyclic template exhibiting NPSR antagonist properties has been claimed in 2010 by Merck Sharp & Dohme.[69] The general structure **6** of these molecules is depicted in Figure 6

where the most representative structural modulations of the central 5*H*-imidazo[2,1-*a*]isoindole core have been summarized. Of this class, the 5-benzyl-5-(4-bromophenyl) derivative **6a** was selected by Trotter et *al.* as the starting point for subsequent SAR studies.[70] The racemate of this compound inhibits the NPSR activation with a pIC_{50} value of 6.42, while one of its enantiomers is 2-fold more potent (pIC_{50} = 6.79, configuration not disclosed). The replacement of the 4-bromophenyl moiety with variously substituted benzene or pyridine rings generally resulted in lower potency. Nevertheless, when a 4-Cl-Ph was combined with other structural changes the antagonist activity was enhanced as in **6b** and **6c** (Figure 6). This also improved drug-likeness/pharmacokinetic properties (e.g. plasma free fraction, membrane permeability, P-glycoprotein 1 interaction, clearance). Specifically, the introduction of a tetrahydropyranyl-methyl (like in **6b**, pIC_{50} = 7.09) or an amide framework (like in **6c**, PI1, pK_B = 8.45, enantiopure but configuration not disclosed) through an alkyl spacer of the proper length were particularly effective especially if combined with a methoxy group at the 6- or 7-positions.

Derivatives **6b** and **6c** were chosen for further preclinical characterization. Against a panel of 163 enzymes, receptors and ion channels, **6b** shows an IC_{50} value lower than 5 μM in two cases: melatonin (1.3 μM) and sodium channel site 2 (4 μM) receptors. Compound **6c** displays even higher selectivity with some affinity for the CB1 receptor (6 μM) only. However, the plasma exposure of each compound is relatively low when dosed orally in rats, while, upon i.p. administration their peripheral and CNS concentrations are at or above the respective IC_{50} for *in vitro* NPSR antagonism. Of note, compound **6c** was subjected to the same *in vivo* assays (righting reflex and locomotor activity) described above for **5c** (Figure 5).[59] Intriguingly, despite the different chemotype, a limited *in vivo* efficacy was observed also in the case of **6c** administration, thus restricting the possibility to exploit the tricyclic imidazole scaffold for *in vivo* investigations in mice.

3. Conclusion

We have provided a comprehensive overview of the patent literature describing NPSR antagonists since the first small-molecule modulator was claimed in 2005.[53] In addition to the patent contributions discussed, the tetracyclic naphto-pyrano-pyrimidine **7** [71] and the pyrrolo-imidazole **8** (Figure 7) [72] are representative of further classes of NPSR antagonists solely described in scientific literature and previously reviewed.[52] Thus, we can conclude that the initial medicinal chemistry efforts to date led to the identification of few potent *in vitro* chemotypes whose subsequent optimization generated a limited number of pharmacological tools currently used in preclinical *in vivo* studies. In table 1 we summarized the different animal models in which these molecules have been used contributing to the understanding of the translational potential of NPSR as a therapeutic target. To the best of our knowledge, no non-peptide NPSR agonist has been identified to date.

4. Expert Opinion

The therapeutic potential of selective NPSR ligands, particularly in psychiatric disorders, can be suggested on the basis of the robust evidence coming from preclinical studies performed in the last decade. This has aroused the attention of both academia and pharma companies in terms of patents filed claiming selective NPSR antagonists.

Eight different chemotypes with NPSR antagonist activity have been identified as a result of the first medicinal chemistry efforts in this field. Available studies suggest that NPSR antagonists might be useful for treating drug addiction, as they have been demonstrated to reduce cocaine/alcohol seeking and relapse in rats. Noteworthy, the complex neuropsychiatric nature of drug addiction contributes to the typical resistance of drug dependent subjects to the very few treatments currently marketed. The efficacy of these is however limited to the management of opioid (methadone, buprenorphine, naltrexone) or alcohol (naltrexone, acamprosate, disulfiram) dependence. Although some promising treatments with novel mechanisms of action are currently being developed,[73] there are no medications approved by FDA or EMA to treat cocaine or amphetamine addiction. Therefore, the development of innovative drugs for these indications is a research priority [74] and NPSR

antagonists may contribute in this area. Moreover, NPSR antagonists may be useful to treat conditions in which the endogenous NPSergic system is activated and ongoing studies could extend their putative indications. However, controversial results have been occasionally observed regarding the *in vivo* efficacy of compounds with potent *in vitro* activities (i.e. **5c** and **6c** [59], Figures 5 and 6, respectively). Species-specific pharmacokinetic concerns have been suggested as one of the main reason of such inconsistencies. This is likely due to the fact that all of the known chemotypes derive from HTS programs that often lead to suboptimal physicochemical properties. For example, **4b** (Figure 4), apparently the most potent *in vivo* tool (see Table 1), has limited solubility that compromises its versatility.[6] Intriguingly, **4b** has also shown low selectivity over the μ opioid receptor [58] largely recognized for the capability to trigger brain reward systems causing addictive behaviours.[75] In addition, unfavourable cLogP values and a number of violations of Lipinski's guideline of five have been previously discussed for some derivatives.[52] However, the first efforts aimed at enriching druglikeness properties led to a small increase of *in vivo* potency (compare **1a** to its more soluble congener **1c** in Table 1) thus suggesting that other issues may affect the variable predictive potential of *in vitro* assays. For example an important limitation of this system is that NPSR ligands have been screened only in recombinant systems that may differ considerably from native tissues. There is a clear need for the identification of cell lines naturally expressing NPSR. Actelion Pharmaceuticals have described [65] two native cell lines expressing NPSR (colon carcinoma cells Colo205 and LS174T) but results of experiments performed with these cells are not reported.[65]

This overview indicates that, **1a** still remains the reference pharmacological tool in this research area, despite the fact that more potent compounds *in vitro* have been discovered (see for examples series **4** and **5** in Table 3). Thus, future optimization of existing chemotypes along with the identification of new NPSR antagonists is needed to speed up potential clinical advancements. Unfortunately, the structural determinants of NPSR antagonism are largely unknown. To gain insight into hypothetical structure-activity relationships, we envisioned the possibility of building a three-

dimensional (3D) topological model that could provide for the first time an estimation of structural similarities among different NPSR antagonist scaffolds based on their alignment in terms of shared pharmacophoric and pharmacosteric features (Figure 8). The lowest energy conformation of compound **1a** (Figure 8, top-left panel) has been chosen as the reference template to which the most potent and flexible compounds of each of the other chemical classes (**2b**, **3r**, **4b**, **5c**, **6c** and **8** in Figure 8) have been individually superimposed. All the selected compounds displayed potency values similar to or higher than **1a**. Based on their low potency ($pIC_{50}/pA_2 < 6.50$), naphto-pyrano-pyrimidine derivatives were excluded from this analysis. Results from this computational investigation have been reported in the right bottom panel of Figure 8. Specifically, three distinct regions occupied by lipophilic fragments (L_{1-3} = aryl, branched alkyl, or cycloalkyl moieties) have been recognized in all compounds with the only exception of **6c** not entirely matching the L_3 region. Moreover, our model would point to the presence of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA). Interestingly, HBD, corresponding to the NH residue of the urea moiety of **1a**, is actually the less conserved feature of our model. Compounds **4b**, **6c** and **8** are indeed devoid of any hydrogen bond donor in the putative HBD area. This would suggest that the previously highlighted interaction between the NH residue of **1a** and the side chain of NPSR Asp 297 [61] could be important but not mandatory for NPSR antagonism. In contrast, the HBA moiety, which is present in all of the molecules investigated, seems to be a strict chemical feature for NPSR antagonism. This previously unreported structural requirement, along with the general information provided by our model, might be instrumental in the rational design of new NPSR antagonists.

Finally, it should be underscored that the therapeutic potential is much wider for NPSR agonists than antagonists and that this might produce greater pharmaceutical interest. In particular, several studies performed by injecting NPS supraspinally in rodents consistently suggest a therapeutic indication for selective NPSR agonists as anxiety, including panic and post-traumatic stress disorders. Of note, because of their lack of sedative action, NPSR agonists may represent an entirely new class

of anxiolytics. On the other hand, this raises the problem that NPSR antagonists may have, as side effects, anxiogenic activity; this can limit the development of these compounds as therapeutics. Nevertheless, the majority of the studies performed with NPSR antagonists (small molecules and peptides) demonstrated the inactivity of these ligands in rodent models of anxiety. Similar findings were obtained with NPSR(-/-) mice, that did not show an anxiogenic-like phenotype. Thus, conscious that the anxiogenic potential must be taken into account in developing NPSR antagonist drugs, we are convinced that this will likely not be a limiting factor. Importantly, the potential of the NPS/NPSR system in the control of anxiety related behaviours in humans has been underlined by several epidemiological studies demonstrating that the single nucleotide polymorphism Asn107Ile of the receptor is associated with panic disorders, anxiety and fear sensitivity.[76-81] Moreover, NPSR selective agonists may be useful for the treatment of pain, memory and food intake disorders. Unfortunately, despite their promising translational potential, to the best of our knowledge non-peptide NPSR agonists have not yet been reported in the scientific or patent literature. Clearly, the peptide nature of NPS strongly limits its development as a drug. The only inherent patent application described the possible employment of peptide NPSR agonists in intranasal formulations as non-sedative anxiolytic drugs.[82] This has also been described in several research papers reporting that NPS produces anxiolytic [33, 83, 84] and analgesic [83] effects and facilitates memory [33] after intranasal administration. Together with the route of administration, the short half-life is one of the major limitations to the use of peptides as drugs. This issue has been recently addressed in a patent [51] and in a research paper [50] describing the pharmacological features of a tetrabranched derivative of NPS named PWT-NPS. This compound displayed an *in vitro* pharmacological profile similar to NPS with higher potency and longer lasting effects *in vivo*. Despite these approaches to overcome typical pharmacokinetic concerns of peptide molecules, the identification of small-molecules acting as selective agonists is clearly mandatory. Such molecules, whose discovery has been probably prevented to date by the known difficulty of designing non-peptide drug-like agonists for peptide

GPCRs, will facilitate investigation of the beneficial (and eventually unwanted) effects of selective NPSR activation and to foresee the best therapeutic indications of these innovative drugs.

LIST OF ABBREVIATIONS

CSF: cerebrospinal fluid

ERK: extracellular signal-regulated kinases

HBA: hydrogen bond acceptor

HBD: hydrogen bond donor

HTS: high-throughput screening

NPS: Neuropeptide S

NPSR: Neuropeptide S Receptor

SAR: structure-activity relationships

SNP: single-nucleotide polymorphisms

Article highlights box

- Via selective activation of the NPSR receptor, NPS modulates several biological functions including locomotor activity, arousal and sleep, anxiety, food intake, memory, pain, and drug addiction.
- Several small-molecule chemotypes featuring NPSR antagonist activity have been identified as a result of the first medicinal chemistry efforts in this field of research. Herein we provided a three-dimensional (3D) topological model indicating structural similarities among different NPSR antagonists.
- Available preclinical evidence suggests therapeutic utility of NPSR antagonists as innovative drugs to treat drug addiction, as they demonstrated to reduce cocaine/alcohol seeking and relapse in rats.
- The scientific and patent literature pointed out how NPS produces non-sedative anxiolytic, analgesic and pro-mnemonic effects after intranasal administration in rodents.
- Important epidemiological studies demonstrated the role of the NPS/NPSR system in the control of anxiety related behaviours in humans since the single nucleotide polymorphism Asn107Ile of the receptor is associated with panic disorders, anxiety and fear sensitivity.
- The identification of small-molecule agonists of the NPSR now represents a challenge to be addressed in order to pave the way to a new non-sedative approach for the treatment of a panel of psychiatric disorders, including anxiety.

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Figure legends

Figure 1. Oxazolo[3,4-*a*]pyrazines as NPSR antagonists.

Figure 2. Furo[3,4-c]pyridines as NPSR antagonists.

Figure 3. Indandione and indanone derivatives as NPSR antagonists.

Figure 4. Imidazo[1,2-*a*]pyridines as NPSR antagonists.

Figure 5. Quinoline derivatives as NPSR antagonists.

Figure 6. Imidazo[2,1-*a*]isoindoles as NPSR antagonists.

Figure 7. Naphto-pyrano-pyrimidine **7** and pyrrolo-imidazole **8** as NPSR antagonists.

Figure 8. Energy-minimized 3D structures of NPSR antagonists; compound **1a** (SHA-68) has been compared with different chemotypes and the right bottom panel depicts the topological model developed from the superimposition of all the structures. **1a**: *N*-(4-fluorobenzyl)-3-oxo-1,1-diphenyltetrahydro-1*H*-oxazolo[3,4-*a*]pyrazine-7(3*H*)-carboxamide; **2b**: 5-(3-(azepan-1-yl)propanoyl)-3-isobutyl-3-phenyl-4,5,6,7-tetrahydrofuro[3,4-*c*]pyridin-1(3*H*)-one; **3r**: 2-(3-methoxyphenyl)-2-(4-(2-methylbenzyl)piperazin-1-yl)-1*H*-indene-1,3(2*H*)-dione; **4b**: 1-cinnamyl-3-(diphenylphosphorothioyl)-2-methylimidazo[1,2-*a*]pyridin-1-ium; **5c**: *N*-(3-methyl-1-morpholinopentan-3-yl)-*N*-((1-methyl-2-oxo-1,2-dihydroquinolin-3-yl)methyl)cyclohexanecarboxamide; **6c**: 3-(5-(4-chlorophenyl)-6-methoxy-5*H*-imidazo[2,1-*a*]isoindol-5-yl)-*N*,*N*-diethylpropanamide; **8**: 5-phenyl-2-(2-(piperidine-1-carbonyl)phenyl)-2,3-dihydro-1*H*-pyrrolo[1,2-*c*]imidazol-1-one.

Table 1. *In vivo* activity of NPS and NPSR antagonists.

Assay	NPS effects	Antagonist	Antagonist effects	References
Locomotor activity				
Mouse locomotor activity	Stimulant (0.1 nmol)	SHA-68 (50 mg/kg)	Inactive per se. Antagonizes NPS effects. Inactive against caffeine stimulant effects.	[25, 54, 56]
		RTI-118 (50 mg/kg)	Inactive per se. Antagonizes NPS effects .	[57]
		QA1 (30 mg/kg)	Inactive per se or against NPS.	[59]
		PI1 (30 mg/kg)	Inactive per se or against NPS.	[59]
Sleep / wake				
Mouse righting reflex	Arousal promoting (0.1 nmol)	SHA-68 (50 mg/kg)	Inactive per se. Antagonizes NPS effects.	[56]
		QA1 (30 mg/kg)	Inactive per se. Poorly antagonizes NPS effects.	[59]
		PI1 (30 mg/kg)	Inactive per se or against NPS.	[59]
Emotional states				
Mouse open field	Anxiolytic (0.1 nmol)	SHA-68 (50 mg/kg)	Inactive per se. Antagonizes NPS effects.	[56]
		SHA-68 (2 nmol, intra-amigdala)	Anxiogenic.	[16]
Mouse conditioned fear behavior	Reduction of freezing (0.5 nmol)	SHA-68 (2 nmol, intra-amigdala)	Increases freezing.	[16]
Mouse conditioned fear extinction	Facilitation of fear-extinction in stressed mice	SHA-68	Impairment of fear-extinction in stressed mice	[18]

	(10 pmol, intra-amygdala)	(10 pmol, intra-amigdala)		
Rat elevated plus maze	Anxiolytic (1 nmol)	SHA-68 (50 mg/kg)	Inactive per se. Antagonizes NPS effects.	[56]
Rat defensive burying	Anxiolytic (1 nmol)	SHA-68 (50 mg/kg)	Inactive per se. Antagonizes NPS effects.	[56]
Mouse resident / intruder test	Reduces aggressiveness (0.1 nmol)	SHA-68 (50 mg/kg)	Inactive per se.	[27]
Rat learned helplessness	Antidepressant (10 pmol, NAc shell)	SHA-68 (100 pmol, bed nucleus of the stria terminalis)	Antidepressant when injected.	[85]
		SHA-68 (100 pmol, NAc shell)	Inactive per se. Antagonizes NPS effects.	[85]
Addiction				
Rat cocaine self-administration	Not tested	SHA-68 (50 mg/kg)	Reduction.	[47]
		QA1 (30 mg/kg)	Inactive.	[46]
Rat alcohol self-administration	Not tested	NCGC84 (1 mg/kg)	Reduction.	[6]
Rat reinstatement of cocaine seeking	Increases (1 nmol)	SHA-68 (30 mg/kg)	Reduction.	[45]
		RTI-118 (10 mg/kg)	Reduction.	[47]
		QA1 (15 mg/kg)	Reduction.	[46]
Rat intracranial self-stimulation	Not tested	RTI-118 (32 mg/kg)	Reduces the cocaine-facilitated intracranial self-stimulation.	[63]

Rat ERK phosphorylation in central amygdala and NAc shell induced by alcohol	Not tested	NCGC84 (1 mg/kg)	Reduction.	[6]
Food intake				
Rat palatable food intake	Anorectic (1 nmol)	SHA-68 (50 mg/kg)	Inactive per se. Poorly antagonizes NPS anorectic action.	[56]
		NCGC84 (18 nmol, icv)	Antagonizes NPS effects.	[58]
Rat food self-administration	Not tested	QA1 (30 mg/kg)	Reduction.	[46]
Memory				
Mouse inhibitory avoidance paradigm	Increases aversive memory (1 nmol)	SHA-68 (50 mg/kg)	Antagonizes NPS effects. No effects per se.	[34]
Mouse novel object recognition	Facilitates memory (0.1 nmol)	SHA-68 (50 mg/kg)	Inactive per se. Antagonizes NPS effects.	[32]
Pain				
Rat spinal nerve ligation	Analgesic, reduces microglia and astrocyte reactivity and TNF-α, IL-1β, and IL-6 production in the spinal cord (100 pmol, intra-amygdala)	SHA-68 (100 pmol intra-amygdala)	Increases microglia and astrocyte reactivity and TNF-α, IL-1β, and IL-6 production in the spinal cord. Antagonizes NPS effects.	[35]
Rat arthroses	Analgesic (14 nmol intranasal)	SHA-68 (50 µM, intra-amygdala)	Antagonizes NPS effects.	[39]

When not specified, NPS was given i.c.v. (intracerebroventricular) and NPSR antagonists were given i.p. (intraperitoneal).

Table 2. Behaviour and NPS sensitivity of NPSR(-/-) mice

Assay	Phenotype of NPSR(-/-) mice	NPS effects		References
		NPSR(+/+)	NPSR(-/-)	
Locomotor activity				
Locomotor activity	No phenotype	Stimulant	Inactive	[41, 86-88]
Hyperlocomotion induced by stimulants	No phenotype	Not tested	Not tested	[86, 87]
Rotarod	Improved motor performance	Not tested	Not tested	[88]
Sleep / wake				
Circadian activity	Decreased during the dark phase	Arousal promoting	Not tested	[86, 88]
Righting reflex	No phenotype	Arousal promoting	Inactive	[41]
	Less sensitive to hypnotic effects of diazepam and ethanol			[89]
Emotional states				
Elevated plus maze	No phenotype	Anxiolytic	Inactive	[41, 86]
	More anxious			[88]
Elevated zero maze	No phenotype	Anxiolytic	Inactive	[87]
Elevated T maze	No phenotype	Panicolytic	Inactive	[14]
Open field	No phenotype	Anxiolytic	Inactive	[41, 87]
	More anxious			[88]
Light-dark box	More anxious	Not tested	Not tested	[88]
Stress induced hyperthermia	No phenotype	Not tested	Not tested	[41]
Murble buryng	No phenotype	Not tested	Not tested	[88]
Conditioned freezing	No phenotype	Not tested	Not tested	[86]
Plasma corticosterone levels	No phenotype	Increases	Inactive	[87]
Resident / intruder test	Increased aggressiveness	Reduction	Inactive	[27]
Forced swimming test	Depressive behavior (male)	Not tested	Not tested	[87]
	No phenotype	Not tested	Not tested	[41]
Tail suspension test	No phenotype	Not tested	Not tested	[87]
Memory				

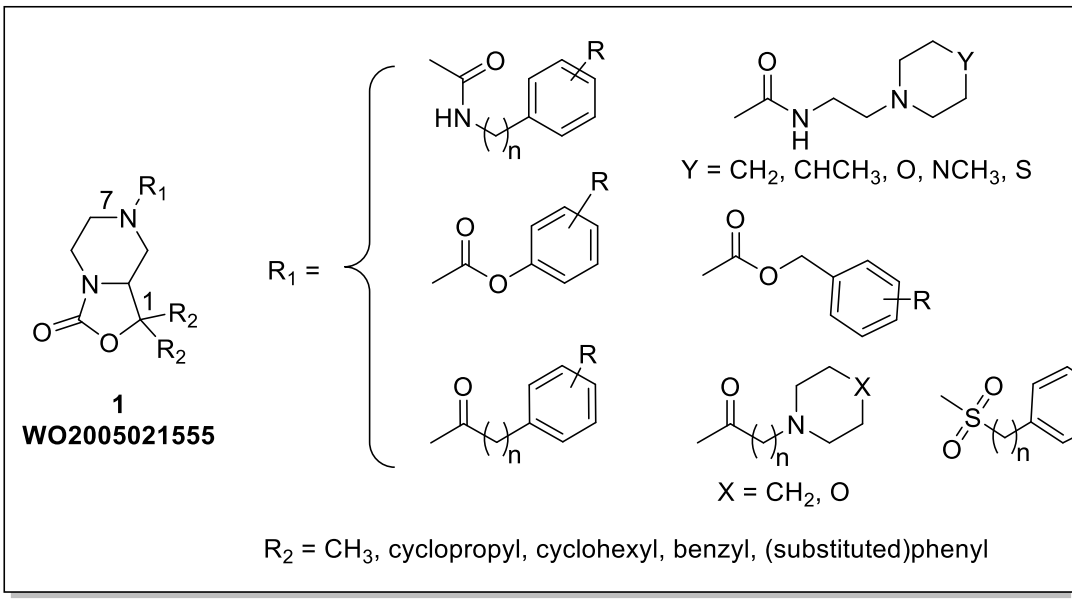
	Morris water maze	No phenotype	Not tested	Not tested	[87]
	Inhibitory avoidance	Impairment	Facilitation	Not tested	[34]
	Novel object recognition	Impairment	Facilitation	Not tested	[34]
		No phenotype	Not tested	Not tested	[41]
Pain					
	Formalin test	No phenotype	Not tested	Not tested	[41]
Sensorimotor gating					
	Acoustic startle response	No phenotype	Not tested	Not tested	[88]
	Prepulse inhibition	Decreased reactivity	Not tested	Not tested	[86, 87]
		No phenotype			[88]

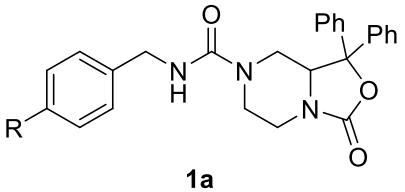
Table 3. *In vitro* potency, normalized to SHA-68, of representative NPSR antagonists of each chemotype derived from Ca^{2+} mobilization assays

Compound code	Compound name	Concentration Ratio	References
Oxazolo[3,4-a]pyrazine derivatives			
R-**1a**	*R*-SHA-68	0.5	[55]
S-**1a**	*S*-SHA-68	> 150	[55]
1b	SHA-66	1.3	[54]
1c	RTI-118	9	[57]
R-**1c**	*R*-RTI-118	2	[57]
S-**1c**	*S*-RTI-118	> 150	[57]
1d		5	[57]
1e		1.4	[57]
1f		1	[57]
Furo[3,4-c]pyridine derivatives			
2a	R06039-447	106[1]	[64]
2b	R06039-478	0.6[1]	[64]
2c	R06039-286	13[1]	[64]
Imidazo[1,2-a]pyridines			
4a	MLS001018695	0.13	[6]
4b	NCGC84	0.06	[6]
Quinoline derivatives			
5a		0.01[2]	[68]
5b		0.006[2]	[68]
5c	QA01	0.02	[59]
5c (enantiomer 1)		0.01[2]	[68]
5c (enantiomer 2)		0.7[2]	[68]
5H-imidazo[2,1-a]isoindoles			
6a (racemate)		1.7[3]	[70]
6a (enantiopure)		0.8[3]	[70]
6b		0.1[3]	[70]
6c (enantiopure)	PI1	0.2	[59]

Concentration Ratio = potency of the compound / potency of SHA-68 tested under the same experimental conditions. In these studies the potency of SHA-68 was in the range (7.28 – 8.16);
[1] potency of SHA-68 from [57]; [2] CR was extrapolated from CR of **5c** calculated from [59]; [3]CR was extrapolated from CR of **6c** calculated from [59]; indanone, indandione derivatives, naphto-pyrano-pyrimidine derivatives, and pyrrolo-imidazole derivatives have not been included in this table because the data needed to compare their *in vitro* pharmacological activity to those of the other chemical classes of NPSR antagonists are not available.

Figure 1.





1a

SHA-68 (R = F)

hNPSR Asn^{107} Ca^{2+} pA_2 = 7.28-8.16

hNPSR Ile^{107} Ca^{2+} pA_2 = 7.55-8.03

mNPSR Ca^{2+} pA_2 = 7.74-8.16

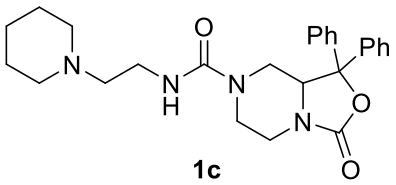
[^{125}I]Tyr^{10}-hNPS pK_i = 7.32

1b

SHA-66 (R = H)

hNPSR Asn^{107} Ca^{2+} pA_2 = 7.66

hNPSR Ile^{107} Ca^{2+} pA_2 = 7.48

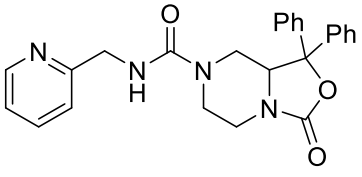


1c

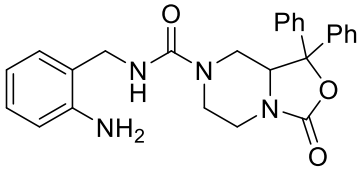
RTI-118

hNPSR Asn^{107} Ca^{2+} pA_2 = 6.31

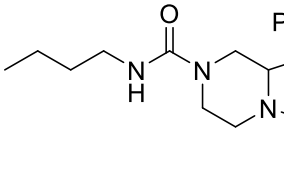
hNPSR Ile^{107} Ca^{2+} pA_2 = 6.96



1d

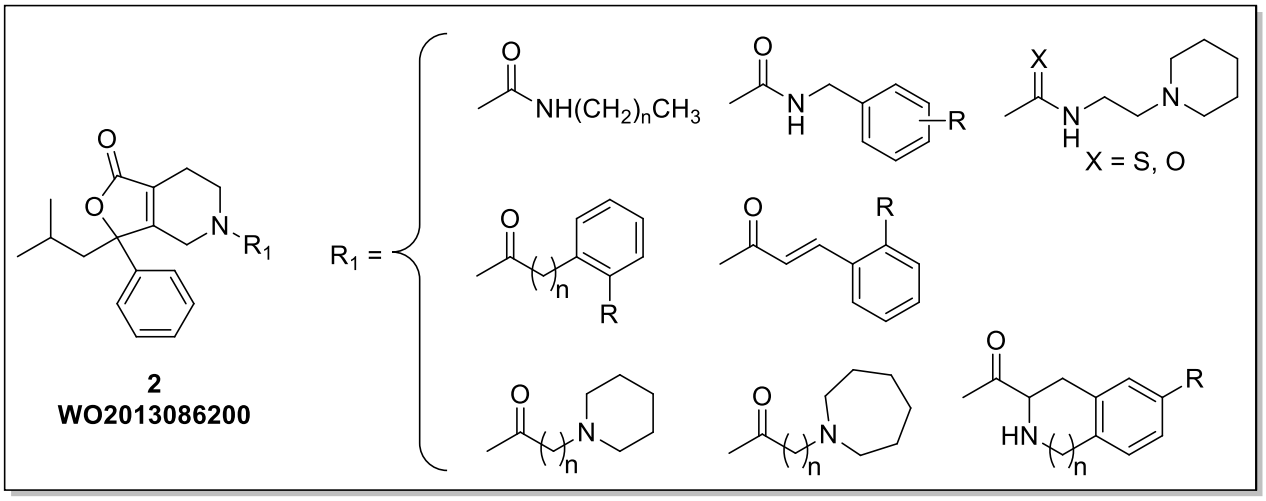


1e

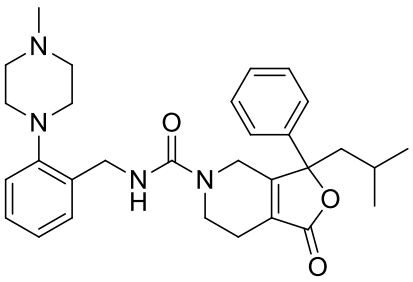


1f

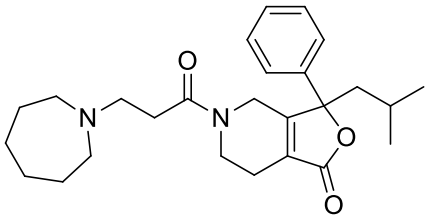
Figure 2.



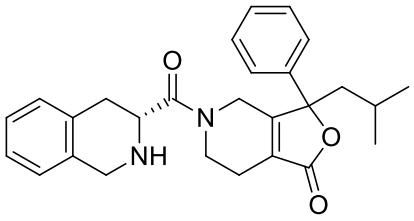
2
WO2013086200



2a
R06039-447
hNPSR Asn^{107} Ca^{2+} pA_2 = 5.83

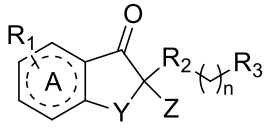


2b
R06039-478
hNPSR Asn^{107} Ca^{2+} pA_2 = 7.51



2c
R06039-286
hNPSR Asn^{107} Ca^{2+} pA_2 = 6.18

Figure 3.



3
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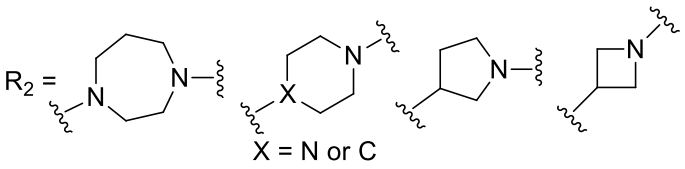
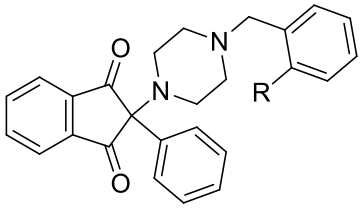
A = pyridine or benzene.
Y = CH_2 or C=O.
R_1 = halogen, (cyclo)alkyl, alkoxy, fluoroalkyl, fluoroalkoxy

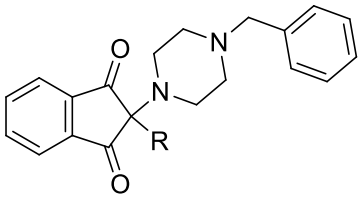


X = N or C

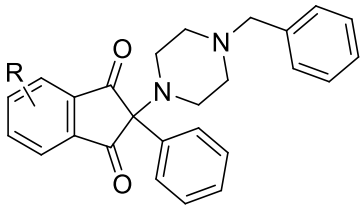
R_3 = (substituted)aryl or (substituted)heteroaryl
Z = (substituted)aryl or (substituted)heteroaryl
$1 \leq n \leq 4$



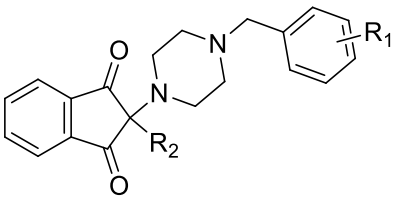
3a, R = H, pIC_{50} = 7.57
3b, R = CH_3, pIC_{50} = 8.52
3c, R = OCH_3, pIC_{50} = 8.22



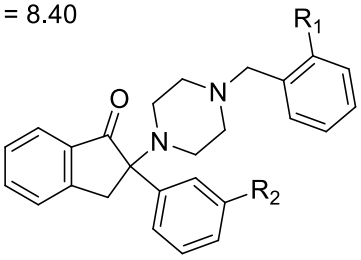
3d, R = 3-CH_3-Ph, pIC_{50} = 8.70
3e, R = 3-OCH_3-Ph, pIC_{50} = 8.40
3f, R = 3-morpholin-4-yl-Ph, pIC_{50} = 8.70
3g, R = thiophen-3-yl, pIC_{50} = 7.25
3h, R = pyridin-2-yl, pIC_{50} = 6.28
3i, R = pyridin-4-yl, pIC_{50} = 5.89
3j, R = pyrazin-2-yl, pIC_{50} = 5.38
3k, R = 2-iPr-6-CH_3-pyridin-4-yl, pIC_{50} = 9.00
3l, R = 6-CH_2CH_3-pyridin-2-yl, pIC_{50} = 8.40



3m, R = 4-CH_3, pIC_{50} = 6.97
3n, R = 4-Cl, pIC_{50} = 7.06
3o, R = 5-CH_3, pIC_{50} = 6.23
3p, R = 5-Cl, pIC_{50} = 5.83



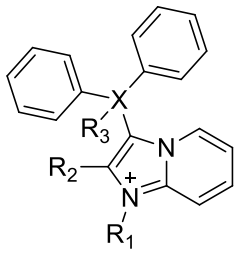
3q, R_1 = 2-CH_3, R_2 = 3-CH_3-Ph, pIC_{50} = 8.70
3r, R_1 = 2-CH_3, R_2 = 3-OCH_3-Ph, pIC_{50} = 9.00
3s, R_1 = 2-CH_3, R_2 = 6-CH_2CH_3-pyridin-2-yl, pIC_{50} = 9.00



3t, R_1 = H, R_2 = H, pIC_{50} = 7.77
3u, R_1 = CH_3, R_2 = H, pIC_{50} = 8.70
3v, R_1 = OCH_3, R_2 = H, pIC_{50} = 8.30
3w, R_1 = CH_3, R_2 = CH_3, pIC_{50} = 8.40

The reported pIC_{50} values were calculated from the Ca^{2+} assay at the hNPSRAsn[107]

Figure 4.



4

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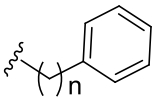
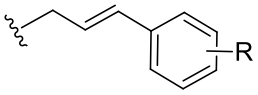
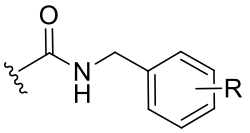
R_1 = CH_3,



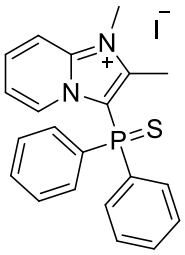




R_2 = H, CH_3, (substituted)phenyl

R_3 = thiocarbonyl, carbonyl, OH

X = P, C



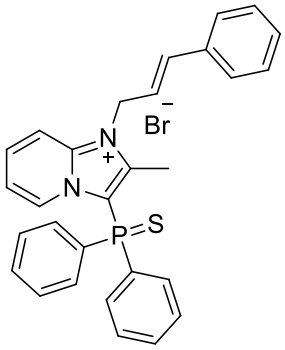
4a, **MLS001018695**

hNPSR Ca^{2+} pIC_{50} = 7.11-7.29

hNPSR cAMP pIC_{50} = 5.77-6.13

hNPSR ERK pIC_5 = 7.52

[^{125}I]Tyr^{10}-hNPS pIC_5 = 6.97-7.07



4b, **NCGC84**

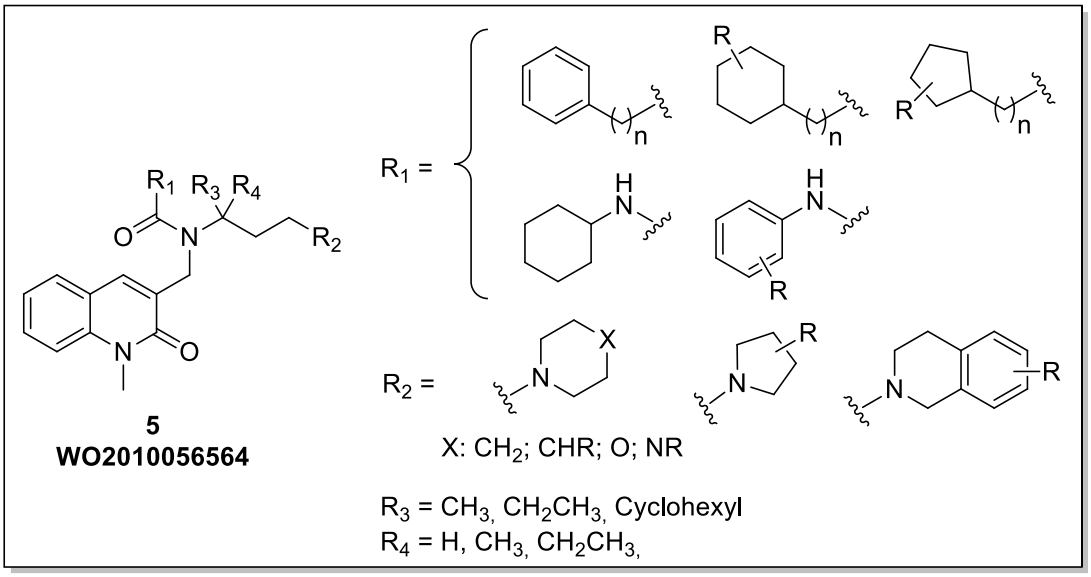
hNPSR Ca^{2+} pIC_{50} = 7.44-8.02

hNPSR cAMP pA_2 = 8.98

hNPSR ERK pIC_5 = 8.03-8.89

[^{125}I]Tyr^{10}-hNPS pIC_5 = 8.30-8.46

Figure 5.



5
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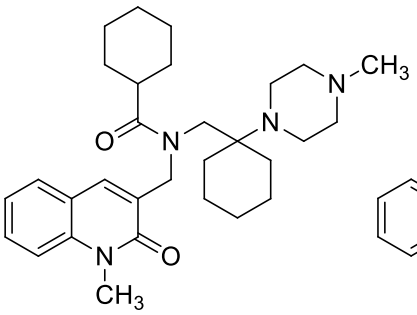
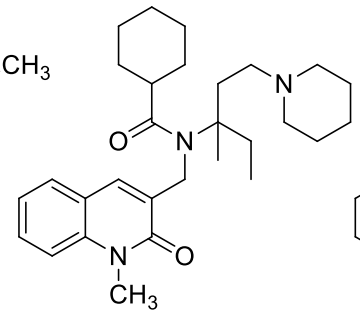
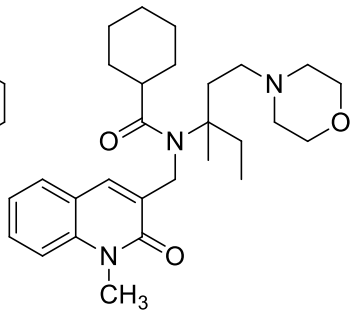


5a
hNPSR Ca^{2+} pIC_{50} = 7.74

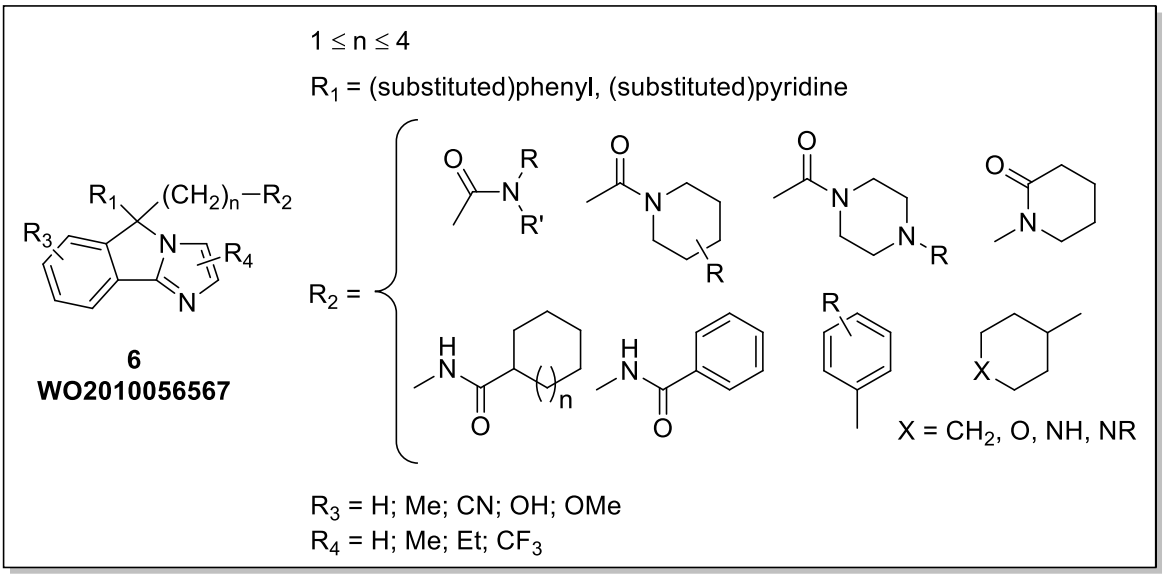


5b
hNPSR Ca^{2+} pIC_{50} = 8.15



5c, **QA1**
hNPSR Asn^{107} Ca^{2+} pK_B= 9.60
hNPSR Ile^{107} Ca^{2+} pK_B = 9.82
hNPSR $cAMP^{+}$ pIC_{50} = 7.26
mNPSR Ca^{2+} pK_B = 9.63

Figure 6.



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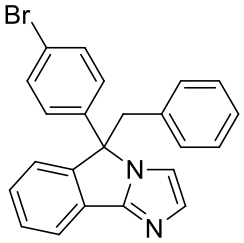
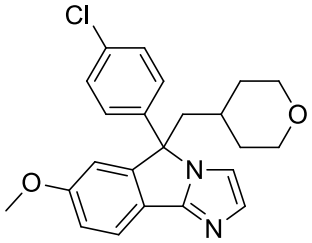
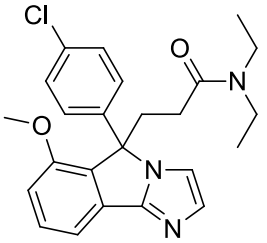


6a
hNPSR Ca^{2+} pIC_{50} = 6.42

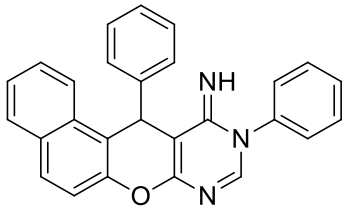


6b
hNPSR Ca^{2+} pIC_{50} = 7.09



6c (**PI1**)
hNPSR Asn^{107} Ca^{2+} pK_B= 8.45
hNPSR Ile^{107} Ca^{2+} pA_2 = 7.94
mNPSR Ca^{2+} pA_2 = 7.74

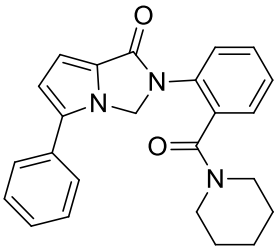
Figure 7.



7

hNPSR Ca^{2+} pIC_{50} = 6.16

hNPSR cAMP pIC_{50} = 5.71



8

hNPSR Ca^{2+} pIC_{50} = 8.00

Figure 8.