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The Pharmacology of Ro 64-6198, a Systemically Active, Nonpeptide NOP Receptor (Opiate Receptor-Like 1, ORL-1) Agonist with Diverse Preclinical Therapeutic Activity

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ABSTRACT

The NOP receptor (formerly referred to as opiate receptor-like 1, ORL-1, LC132, OP_4, or NOP_1) is a G protein–coupled receptor that shares high homology to the classic opioid MOP, DOP, and KOP (mu, delta, and kappa, respectively) receptors and was first cloned in 1994 by several groups. The NOP receptor remained an orphan receptor until 1995, when the endogenous neuropeptide agonist, known as nociceptin or orphanin FQ (N/OFQ) was isolated. Five years later, a group at Hoffmann-La Roche reported on the selective, nonpeptide NOP agonist Ro 64-6198, which became the most extensively published nonpeptide NOP agonist and a valuable pharmacological tool in determining the potential of the NOP receptor as a therapeutic target. Ro 64-6198 is systemically active and achieves high brain penetration. It has subnanomolar affinity for the NOP receptor and is at least 100 times more selective for the NOP receptor over the classic opioid receptors. Ro 64-6198 ranges from partial to full agonist, depending on the assay. Preclinical data indicate that Ro 64-6198 may have broad clinical uses, such as in treating stress and anxiety, addiction, neuropathic pain, cough, and anorexia. This review summarizes the pharmacology and preclinical data of Ro 64-6198.

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INTRODUCTION

The NOP receptor (formerly referred to as opiate receptor-like 1, ORL-1, LC132, OP_4, or NOP_1) is a seven-transmembrane G protein–coupled receptor that shares high homology to the classic opioid MOP, DOP, and KOP (mu, delta, and kappa, respectively) receptors and was first cloned as an orphan receptor in 1994 by several groups. Like the classic opioid receptors, the NOP receptor is negatively coupled to adenylate cyclase, activates potassium channels, and inhibits calcium channels (Meunier et al. 2000). The NOP receptor remained an orphan receptor for only 1 year, when the endogenous neuropeptide agonist known as nociceptin or orphanin FQ (N/OFQ) was isolated (Meunier et al. 1995; Reinscheid et al. 1995). Five years later, a group at Hoffmann-La Roche reported on the selective, nonpeptide NOP receptor agonist Ro 64-6198 (Fig. 1) (Wichmann et al. 2000), which became the most extensively published selective nonpeptide NOP receptor agonist and a valuable pharmacological tool in determining the potential of the NOP receptor as a therapeutic target.

DEVELOPMENT OF Ro 64-6198

N/OFQ as a peptide, while very valuable in NOP receptor research, is subject to certain limitations. Because N/OFQ is a peptide, it undergoes metabolism by peptidases (Montiel et al. 1997; Sakurada et al. 2002) and may have a relatively short half-life (Gunduz et al. 2006). Complicating the matter further are the facts that N/OFQ metabolism produces bioactive peptide fragments, some of which may act at sites other than the NOP receptor (Chen et al. 2002; Inoue et al. 2001; Suder et al. 1999) or behave as functional NOP receptor antagonists (Sakurada et al. 1999; Sakurada et al. 2000), and that N/OFQ is inherently nonselective by virtue of being a competitive inhibitor of its peptidases as well as a NOP receptor agonist. For example, both N/OFQ and substance P are metabolized by endopeptidase-24.11 in the spinal cord (Sakurada et al. 2002; Sakurada et al. 2004) and, therefore, N/OFQ administration could increase substance P levels by competing for metabolism. In addition, the use of peptide NOP receptor agonists prevents discerning the effects of simultaneous activation of NOP receptors across a complete physiological system. Because peptide agonists are not practical for treating CNS diseases, peptide agonists cannot accurately predict whether NOP receptor agonists will have real therapeutic value for CNS diseases. Not only are peptide agonists not practical in treating CNS diseases clinically, but

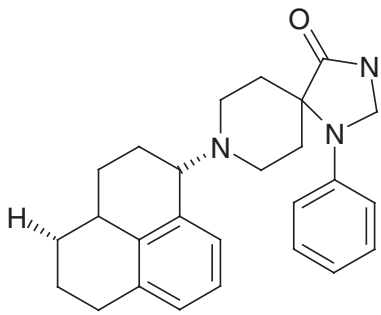

FIG. 1. The chemical structure of the NOP agonist Ro 64-6198.

even preclinically the use of intracerebroventricular (i.c.v.) injections can be impractical, complicating NOP receptor research. The use of i.c.v. injections of N/OFQ resulted in some early debate as to whether N/OFQ produced hyperalgesia (Meunier et al. 2000; Reinscheid et al. 1995) or a reversal of stress-induced analgesia following the stress of an i.c.v. injection (Mogil et al. 1996; Suaudeau et al. 1998); see also (Calo et al. 1998; Mogil et al. 1999).

Hoffmann-La Roche then patented the nonpeptide 8-substituted-1,3,8-triaza-spiro [4.5]decan-4-one derivatives as ligands at the NOP receptor for therapeutic treatments related to anxiety, stress, pain, addiction, and several other areas (Adam et al. 2001). These compounds, based on the high throughput screening (HTS) hit, 8-(5,8-dichloro-1,2,3,4-tetrahydro-naphthalen-2-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one, were somewhat similar in structure to the MOP agonist lofentanil, which also has some affinity for the NOP receptor (Rover et al. 2000). Structure-activity relationship (SAR) studies of the HTS hit at the 8-position of the triazaspirodecanone, or at the amide nitrogen, led to a profile of compounds exhibiting high affinity for the NOP receptor, ranging from partial to full agonist, but with only moderate selectivity (Rover et al. 2000). These studies also demonstrated that the chlorine substituents on the tetralinyl ring did not contribute to binding while lipophilic substituents were well tolerated, and that variations at the amide nitrogen did not influence selectivity, indicating that hydrogen binding at this location with the NOP receptor was not vital. Because diverse substitutions at the amide position did not dramatically alter NOP affinity, it was hypothesized that this part of the molecule did not interact with the binding pocket, perhaps being instead exposed to the water layer; therefore, further modifications at this position were not pursued. SAR studies also suggested that the lipophilic binding pocket could tolerate larger substitutions, leading to the 8-acenaphthenyl derivative, which had high potency and an agonist activity similar to N/OFQ, but still only moderate selectivity (Rover et al. 2000). SAR studies on this compound began with variations at the 1 phenyl-ring position (Wichmann et al. 1999). However, the larger substitutions at this position yielded a decrease in NOP affinity, indicating steric limitations at this part of the binding pocket. The 3-F substitution did yield a slight increase in NOP affinity, but also a concurrent increase in classical opioid receptor affinity (Wichmann et al. 1999). Furthermore, because the high affinity compounds of this series were chiral, requiring a larger number of synthesis steps that retards the SAR process, Hoffmann-La Roche turned to a series of simpler 8-cycloalkyl-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-ones (Rover et al. 2000). These studies confirmed that moderately large lipophilic substitutions at the 8-position were well tolerated and revealed that NOP affinity increased with small increases in ring size, consistent with an interaction of this part of the ligand with the hydrophobic binding pocket. Selectivity for the NOP receptor was improved in this series, up to a moderate 40-fold preference for NOP over MOP, by realizing that the NOP receptor is more discriminating than the classical opioid receptors against large substituents and stereochemical centers (Rover et al. 2000). This led to an introduction of more stereochemical information into the molecules and the 1-(2,3,3a,4,5,6)-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one derivatives, which confirmed that the NOP receptor is more sensitive than the classical opioid receptors to such stereochemical information. Of the stereoisomers produced in this series, (1S,3aS) had the highest affinity for the NOP receptor, and thus the best selectivity over the classical opioid receptors (100-fold), concluding the development of Ro 64-6198 or (1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one (Wichmann et al. 2000).

CHEMISTRY AND RECEPTOR BINDING

A detailed description of the synthesis of Ro 64-6198 was reported by Wichmann et al. (2000). Briefly, 3-(3,4-dihydro-naphthalen-1-yl)-propionic acid, synthesized from starting materials, was used to form Ro 64-6198 in a multiple step process, beginning with its transformation to (S)-3-(1,2,3,4-tetrahydro-naphthalen-1-yl)-propionic acid, followed by (S)-2,3,3a,4,5,6-hexahydro-phenalen-1-one to (1S,3aS)-1-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-4-phenylamino-piperidine-4-carbonitrile. This was then transformed to the final product (1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one hydrochloride or Ro 64-6198, an off-white solid with a melting point of 262°C (Wichmann et al. 2000).

Molecular modeling studies have been used to illustrate the probable binding interaction of Ro 64-6198 with the NOP receptor (Broer et al. 2003). The protonated nitrogen of the piperidine in Ro 64-6198 most likely represents an anchoring point to the receptor, forming a salt bridge with the negatively charged Asp-130 in the transmembrane III region. This allows the amide nitrogen to form a hydrogen bond with the oxygen of Thr-105 (Broer et al. 2003), although SAR studies revealed that this hydrogen bond was not vital for binding affinity (see preceding text). These studies also revealed that the lipophilic moiety at the piperidine nitrogen interacts with a hydrophobic cavity consisting of Ile-127, Tyr-131, Met-134, Phe-135, Ile-204, Phe-215, Ile-219, Phe-220, Phe-224, Phe-272, Trp-276, Val-279, and Val-283 (Broer et al. 2003). In addition, these studies confirmed the importance of the lipophilic moiety in gaining selectivity over the classic opioid receptors. As hypothesized in the SAR studies (see preceding text), this hydrophobic region of the binding pocket in the classic opioid receptors does allow for larger hydrophobic substituents, due to the smaller size of the amino acids and the greater flexibility of the aliphatic residues. It has also been hypothesized, based on the structures of several agonists and antagonists of the NOP receptor, that the lipophilic moiety at the piperidine nitrogen of Ro 64-6198 is also important in determining agonist activity, such that agonist responses are produced because this lipophilic moiety occupies a binding site in the receptor that is located near the protonated piperidine (Zaveri et al. 2005).

In addition, it was reported that Ro 64-6198 has very slow kinetics of on and off binding, much slower than N/OFQ (Chiou et al. 2004; Rizzi et al. 2001). Ro 64-6198 also produced rapid desensitization of the NOP receptor *in vitro* and *in vivo* (Dautzenberg et al. 2001). *In vitro* studies showed that treatment with Ro 64-6198 resulted in a functional desensitization of the receptor, a loss in binding sites, and an apparent decrease in binding affinity. The desensitization produced by Ro 64-6198 was not reversed by acidic washes, whereas the desensitization produced by N/OFQ was, leading to the hypothesis that Ro 64-6198, but not N/OFQ, produces internalization of the NOP receptor (Dautzenberg et al. 2001). However, another study using green fluorescence protein-tagged NOP receptors and hypertonic sucrose preincubation did demonstrate N/OFQ-induced internalization of the NOP receptor (Corbani et al. 2004). *In vivo* treatment with Ro 64-6198 resulted in a loss of binding sites 30 min after injection, with recovery beginning 60 min after injection. Binding sites were still substantially decreased at 3 hours after injection, but fully recovered by 24 hours. Chronic treatment with Ro 64-6198 did not alter the desensitization or recovery of NOP receptors (Dautzenberg et al. 2001).

Thus, in agreement with the SAR studies (see preceding text), the piperidine nitrogen and its lipophilic substituents are involved in both selectivity and agonist activity, apparently

influencing the binding of Ro 64-6198 the most. Moreover, Ro 64-6198 has slower binding kinetics than N/OFQ and produces a desensitization that is not readily reversed by acidic washes.

PHYSIOCHEMICAL PROPERTIES, PHARMACOKINETICS, AND TOXICOLOGY

The molecular weight of Ro 64-6198 (empirical formula $C_{26}H_{31}N_{3O}$) is 401.6 (free base) or 438.0 (hydrochloride). Very little has been published on the physiochemical properties of Ro 64-6198, except that it is hydrophobic (Dautzenberg et al. 2001), hence it is usually dissolved in 0.3% Tween 80 for *in vivo* experiments (Jenck et al. 2000; Le Pen et al. 2002; Recker and Higgins, 2004). The software-predicted LogP of Ro 64-6198 is approximately 5 (VCCLAB, 2006). Ro 64-6198 is also a base, and the piperidine nitrogen, the strongest base nitrogen on the molecule, has a software-predicted pK_a of 9.2 (VCCLAB, 2006); therefore, it is most likely ionized at a physiological pH. The aniline nitrogen may be ionized at lower gut pH values, whereas the amide nitrogen is not a base. With its relatively low molecular weight and LogP, four hydrogen bond acceptors, and one hydrogen bond donor, Ro 64-6198 has no "Rule of Five" violations (Lipinski et al. 2001). In addition, Ro 64-6198 has a relatively low software-calculated TPSA of 36 square angstroms (Molinspiration, 2006). Based on these physiochemical properties and published equations of permeability, it would be predicted that Ro 64-6198 has high caco-2 permeability and gastrointestinal absorption (Hou et al. 2004) and high brain penetration, with a calculated LogBB of slightly less than 0 (Hou and Xu, 2003). Indeed, Ro 64-6198 was reported to have high brain penetration, with levels reaching up to 1000 ng/g at 15 min after an intraparitoneal (i.p.) injection of the drug, 10 mg/kg (Jenck et al. 2000). However, despite these good physiochemical properties and good predicted cellular permeability, Ro 64-6198 has a low oral bioavailability of about 4% (Jenck et al. 2000). This could possibly be due to extensive first pass metabolism. Fentanyl, somewhat similar in structure to Ro 64-6198, also undergoes extensive metabolism in the intestine and liver (Labroo et al. 1997). Additional software-calculated properties of Ro 64-6198 include that it is a relatively rigid molecule, with two rotational bonds, and has a calculated volume of 387 cubic angstroms (Molinspiration, 2006). Because Ro 64-6198 is a hydrophobic base, it may undergo protein binding to alpha-1-acid glycoprotein (Kremer et al. 1988; Urien et al. 1991).

Very little has been reported on the pharmacokinetics of Ro 64-6198. Ro 64-6198 is most likely absorbed relatively quickly following i.p. injection, based on brain levels at 15 min (Jenck et al. 2000) and the fact that at 1–3 mg/kg Ro 64-6198 blocked morphine antinociception within 5 min of i.p. injection (Kotlinska et al. 2003b). It has been mentioned in a discussion that Ro 64-6198 has reasonable or good bioavailability following i.p. or subcutaneous (s.c.) injection (Higgins et al. 2001; Varty et al. 2005); however, no bioavailability data has been reported in the literature. The NOP agonist W-212393 (2-{3-[1-((1R)-acenaphten-1-yl)piperidin-4-yl]-2,3-dihydro-2-oxo-benzimidazol-1-yl}- N-methylacetamide), which is very similar in structure to Ro 64-6198, has an oral bioavailability of approximately 4%, and 13% after i.p. injection (Teshima et al. 2005). The metabolism of Ro 64-6198 is also unclear. It was reported that Ro 64-6198 has "slow" elimination from the brain (Jenck et al. 2000). In rats, at 10 mg/kg i.p., Ro 64-6198 is active

at producing hypothermia for up to 2 hours after injection (Higgins et al. 2001) and in guinea pigs, at 3 mg/kg, it inhibits cough for up to 4, but not 6 hours. Additionally, it was reported that at 3 mg/kg, i.p., b.i.d., Ro 64-6198 did not accumulate to toxic levels, which was predicted by its pharmacokinetics, and that repeated injections of the drug at 3 mg/kg for 14 days did not result in tolerance on day 15 (Dautzenberg et al. 2001). It was also reported that when Ro 64-6198 at 1 or 27 mg/kg i.p. was administered to rats, its plasma levels at 30 min after injection reached 0.06 and 0.67 μM, respectively (Varty et al. 2005).

In terms of toxicology, again few data are available. It was reported that at 3 mg/kg Ro 64-6198 i.p., given b.i.d. for 5 days, was well tolerated by rats (Dautzenberg et al. 2001). When the drug was administered to rats at 3.2 mg/kg i.p., once daily for 21 days, no changes in weight gain or body temperature were detected, and there were no changes in liver weight or cytochrome P450 enzyme activity (Dautzenberg et al. 2001).

In summary, Ro 64-6198 generally has good physiochemical properties with high cellular permeability and brain penetration. However, the bioavailability of Ro 64-6198 following oral administration is very low, and its bioavailability by i.p. injection is unknown. In addition, little has been published on the pharmacokinetics and metabolism of Ro 64-6198. The brain levels at which the drug is effective *in vivo* are also unknown.

PHARMACOLOGY

Affinity, Agonist Activity, and Selectivity

Ro 64-6198 was introduced as a full agonist at the NOP receptor, with an affinity of approximately 0.4 nM and more than 100-fold selectivity over the classic opioid receptors (Jenck et al. 2000; Wichmann et al. 2000). The binding affinity, selectivity, and agonist activity of Ro 64-6198 has been confirmed in other studies (Dautzenberg et al. 2001; Hashiba et al. 2002; McDonald et al. 2003b; McLeod et al. 2004). However, there is some discrepancy in the literature as to whether Ro 64-6198 is a full or partial agonist. In a study examining the actions of Ro 64-6198 in rat periaqueductal gray slices, it was found that Ro 64-6198 was only a partial agonist, displaying only about 60% the efficacy of N/OFQ. Ro 64-6198 was also found to have slow action kinetics in that study and only affects a subset of the neurons affected by N/OFQ (Chiou et al. 2004). The implications of these differences between Ro 64-6198 and N/OFQ are discussed in the following.

Ro 64-6198 binds to the MOP, KOP, and DOP receptors with affinities of approximately 50, 90, and 1380 nM, respectively (Jenck et al. 2000; Wichmann et al. 2000). At the MOP receptor, Ro 64-6198 acts as a partial or full agonist, dependent on the assay (Dautzenberg et al. 2001). However, the degree to which Ro 64-6198 acts as a MOP agonist *in vivo* may be limited. In drug discrimination studies in rats, Ro 64-6198, although producing its own internal stimulus, only weakly generalized at high doses to a morphine stimulus (20%), and did not generalize to either kappa or delta agonists. Similarly, morphine only partially generalized (40%) to the Ro 64-6198 cue (Recker and Higgins, 2004). On the other hand, in a guinea pig ileum preparation, the response of Ro 64-6198 was only blocked by a combination of MOP and NOP antagonists, and in the mouse vas deferens the response of Ro 64-6198 was not blocked by either MOP or NOP antagonists (Rizzi et al. 2001). Thus, the apparent selectivity of Ro 64-6198 probably varies by species or tissue. Ro 64-6198 also has micromolar affinity for sodium-site 2 channels and histamine H_2, sigma, and dopamine D_2

receptors (Jenck et al. 2000; Wichmann et al. 2000). Ro 64-6198 does not have significant affinity for at least 44 other sites, including serotonin $5HT_{1D\alpha}$, $5HT_{2A}$, $5HT_{2C}$, $5HT_6$, and $5HT_7$, dopamine D_1, D_3, and D_4, CRF_1, $CRF_{2\alpha}$, benzodiazepine, adenosine A_1, A_{2a}, A_3, somatostatin, NPY, galanin, cannabinoid, histamine H_1, muscarine, CCK_A, and central nicotine receptors, norepinephrine, dopamine, and GABA transporters, and calcium and potassium channels (Jenck et al. 2000; Wichmann et al. 2000).

The affinity, selectivity, and agonist activity of Ro 64-6198 determined from various experiments are summarized in Table 1. Interestingly, there is a wide range in potency values from functional assays. Ro 64-6198 is more potent in cAMP assays than in GTPγS binding assays (Table 1), possibly due to signal amplification. It is least potent in the assays where Ro 64-6198 was reported to be a partial or nonselective agonist (Chiou et al. 2004; Rizzi et al. 2001).

In summary, Ro 64-6198 has high sub-nanomolar affinity for NOP, behaving as either a full or partial agonist. Although Ro 64-6198 has high selectivity as a NOP agonist, its affinity for other sites should not be ignored *in vivo*, especially since the pharmacokinetic profile of Ro 64-6198 at target tissues following injection is usually unknown. Levels of Ro 64-6198 in the plasma were demonstrated to be 60 nM in the rat at the single time-point of 30 min after a 1 mg/kg injection (Varty et al. 2005). This suggests that Ro 64-6198, with a MOP affinity of 50 nM, could activate MOP receptors *in vivo*, especially in areas with a high ratio of MOP to NOP expression. Furthermore, Ro 64-6198 produces rapid desensitization of the NOP receptor, accompanied by an apparent decrease in NOP agonist affinity (Dautzenberg et al. 2001), which could also encourage nonselective actions. Therefore, *in vivo* experiments with Ro 64-6198 should make use of NOP or MOP antagonists, NOP knockout mice, or complimentary NOP agonists.

Possible Functional Heterogeneity of NOP Receptors

It has been suggested in the literature that Ro 64-6198 and N/OFQ may activate a different functional subset of NOP receptors. As mentioned previously, in rat periaqueductal gray matter Ro 64-6198 activates only a subset of the neurons activated by NOP. It is, therefore, conceivable that two subtypes of NOP receptor exist, with only one of them being sensitive to Ro 64-6198 (Chiou et al. 2004). In the mouse vas deferens, Ro 64-6198 mimicked the effects of N/OFQ, but the effects of Ro 64-6198 were not blocked by either NOP or MOP antagonists (Rizzi et al. 2001). Although this could reflect differences in selectivity, with Ro 64-6198 acting at some unknown receptor, it has been hypothesized that this difference could also be due to Ro 64-6198 activating a functional subset of NOP receptors, one that the NOP antagonists tested do not bind to (Chiou et al. 2004). In an *in vivo* study, it was shown that N/OFQ affects locomotor activity in a biphasic manner, with hyperlocomotion at lower doses and hypolocomotion at higher doses (Kuzmin et al. 2004). Ro 64-6198 failed to fully reproduce the effect of N/OFQ, instead inhibiting locomotor activity at all doses (Kuzmin et al. 2004). In addition, although both phases of N/OFQ's effects on locomotor activity were blocked by all of the NOP antagonists tested, only one of the NOP antagonists tested was able to block the hypolocomotor effects of Ro 64-6198 (Kuzmin et al. 2004). It was suggested that Ro 64-6198 lacks action at the functional subtype responsible for hyperlocomotion and that only one of the antagonists tested could competitively antagonize Ro 64-6198 at this subtype

TABLE 1. Binding affinity, selectivity, and functional potency of Ro 64-6198.

	Binding affinity					Functional assays			
Reference	NOP	MOP	KOP	DOP (pK_i)	Lowest selectivity	Tissue	pEC_{50}	Assay	Agonist activity
Wichmann et al. (2000)	9.41	7.33	7.05	5.86	120	hNOP transfected cells	7.4	GTPγS	Full
Jenck et al. (2000)	9.41	7.33	7.05	5.86	120	hNOP transfected cells	7.4	GTPγS	Full
Jenck et al. (2000)						hNOP transfected cells	9.5	cAMP	Full
Chiou et al. (2004)						rat PAG tissue	5.6	GIRK channels	Partial
McDonald et al. (2003b)	9.06					hNOP transfected cells	8.1	GTPγS	Full
Hawes et al. (1998)						hNOP transfected cells	7.6	GTPγS	Full
Hawes et al. (1998)						hNOP transfected cells	8.5	cAMP	Full
Dautzenberg et al. (2001)	9.41	7.28	6.97	5.85	134	hNOP transfected cells	9.6	cAMP	Full
McLeod et al. (2004)	9.52	7.44	6.67	5.42	120	hNOP transfected cells	8.4	GTPγS	Full
Rizzi et al. (2001)						Rat vas deferens	7.2	Stimulated twitch	Full
Rizzi et al. (2001)						Mouse vas deferens	6.8	Stimulated twitch	Full
Rizzi et al. (2001)						Guinea pig ileum	6.2	Stimulated twitch	>N/OFQ

The pK_i binding affinities (the negative log of the affinity Ki, which is measured in M) of Ro 64-6198 for the hNOP receptor are listed. Binding affinities and lowest selectivity for MOP, KOP, and DOP are also given. Lowest selectivity is the highest affinity (K_i) for one of the classical opioid receptors divided by the K_i affinity of Ro 64-6198 for the NOP receptor. See references for details. Potencies (pEC_{50}, or negative log of the effective concentration, which is measured in M) and agonist activity (partial or full) of Ro 64-6198 in several functional assays are also listed. Ro 64-6198 demonstrated efficacy greater than N/OFQ in the guinea pig ileum assay, possibly due to nonselective, additive effects at the MOP receptor. Values left blank were not determined. Abbreviations: hNOP (human NOP), GTPγS (Guanosine 5'-(3-O-thio)triphosphate), cAMP (cyclic adenosine monophosphate), GIRK (G protein–activated inwardly rectifying potassium channel).

(Kuzmin et al. 2004). In addition, it was reported that Ro 64-6198 attenuated the expression of morphine sensitization, an effect not blocked by a NOP antagonist. The authors suggested that Ro 64-6198 could activate a subset of NOP receptors that the antagonist tested does not bind to (Kotlinska et al. 2005). Of course, there are other possible explanations as well for the results of these last two experiments, including differences in selectivity between Ro 64-6198 and N/OFQ or the antagonists tested, and possibly inadequate doses of antagonists being tested. Furthermore, there is some biochemical evidence for heterogeneity based on binding (Mathis et al. 1997), and splicing variants of NOP (Mogil and Pasternak 2001) as well as *in vitro* heterodimerization of the NOP receptor (Wang et al. 2005) have been reported. Taken together, these results could suggest the possible existence of functional NOP subtypes. Of course, differences in selectivity, receptor coupling, agonist efficacy, receptor desensitization, and susceptibility to varying receptor level, among other things, could be responsible for the tissue or experiment dependent differences between N/OFQ and Ro 64-6198. But the possibility that functional subtypes at the NOP receptor exist raises the exciting possibility of using subtype selective NOP agonists to gain specific clinical efficacy or reduce specific side effects.

Potential Side Effects

The main side effects of Ro 64-6198 include its effects on motor activity, learning, and memory. At doses of 3–6 mg/kg it impaired operant performance on a variable interval 20-second schedule in rats (Higgins et al. 2001). Starting at 10 mg/kg in rats, Ro 64-6198 produced motor impairments on a rotarod, fixed ratio responding, and traction tests, and effected grip strength, grasping reflex, and pad removal, while producing hypolocomotion, ataxia, catalepsy, spontaneous jerks, and an abnormal body posture (Higgins et al. 2001; Jenck et al. 2000; Kamei et al. 2004). At 30 mg/kg, deficits in beam walking were also produced (Varty et al. 2005). However, when handled, the animals regained some muscle tone and locomotor activity (Higgins et al. 2001; Varty et al. 2005). The motor side effects in mice were similar, but were generally observed at lower doses. Catalepsy and deficits in beam walking became apparent at 0.3 mg/kg (Higgins et al. 2001). At 1 mg/kg Ro 64-6198 affected swim behavior and at 3 mg/kg decreased rearing, locomotor activity, and rotarod performance. Catalepsy and effects on fixed ratio performance, body posture, grip strength, and righting reflex were observed at 10 mg/kg, i.p.(Higgins et al. 2001; Varty et al. 2005). These side effects are NOP-mediated and not due to nonselective or off-target effects of Ro 64-6198. When tested in NOP knockout mice, Ro 64-6198 did not produce hypolocomotion (although a slight reduction may occur at 10 mg/kg, depending on the methods), catalepsy, or changes in rotarod or fixed ratio performance or grip strength (Higgins et al. 2001; Varty et al. 2005). At a higher dose, 6 mg/kg, the drug was shown to produce short-term memory impairment in rats using a delayed matching or delayed nonmatching to position task. In mice, Ro 64-6198 mildly impaired spatial learning in a Morris water maze (Higgins et al. 2002). Ro 64-6198 may also have undesirable effects on feeding. Although at 3.2 mg/kg, given once daily for 21 days, it did not affect weight gain in rats (Dautzenberg et al. 2001), at 10 mg/kg Ro 64-6198 produced bouts of feeding (Jenck et al. 2000). In addition, another study found that at 2.5 mg/kg Ro 64-6198 increased feeding in rats (Ciccocioppo et al. 2002). Finally, Ro 64-6198 produces hypothermia, again with greater potency in mice than in rats. In rats, at 30 mg/kg it decreased body temperature by 2.7°C (Varty et al. 2005),

whereas in mice at 10 mg/kg it reduced body temperature by about 2.5–5.5°C. These hypothermic effects were absent in NOP knockout mice (Higgins et al. 2001; Varty et al. 2005).

In summary, Ro 64-6198 has major side effects on motor activity and coordination and also affects memory and learning, feeding, and body temperature. Importantly, the potency at which Ro 64-6198 produces these side effects varies by species. In the rat, a therapeutic window exists between anxiolytic doses and doses that produce side effects (Varty et al. 2005). In the mouse, however, motor disturbances became apparent at doses as low as 0.3 mg/kg (Higgins et al. 2001), and anxiolytic doses cannot be separated from doses with side effects (Jenck et al. 2000; Varty et al. 2005). Therefore, behavioral experiments using mice will need careful controls in order to eliminate the possibility that motor impairments influenced the results.

Stress and Anxiety

Perhaps the most well-studied therapeutic target for Ro 64-6198 is anxiety (Table 2). The NOP receptor and its endogenous agonist are expressed in several brain areas related to stress and anxiety, such as the amygdala, hypothalamus, and locus coeruleus (Mogil and Pasternak 2001). The NOP receptor appears to be involved in stress responses, since N/OFQ administration either increases levels of stress hormones under normal or mildly stressed conditions (Devine et al. 2001; Fernandez et al. 2004; Leggett et al. 2006) or attenuates the increase in stress hormones caused by the stress of the injection (Le Cudennec et al. 2002), and stressful events alter levels of N/OFQ measured by radioimmunoassay (Devine et al. 2003; Ploj et al. 2002). NOP receptors in anxious mice couple to G proteins less efficiently than in nonanxious mice (Le Maitre et al. 2006), and N/OFQ knockout mice show increased anxiety and deficits in stress adaptation (Koster et al. 1999; Ouagazzal et al. 2003). Although NOP receptor knockout mice do not appear to show changes in basal levels of anxiety (Mamiya et al. 1998; Varty et al. 2005), this could reflect developmental or compensatory changes. Rats injected with NOP antisense oligonucleotides, which resulted in a significant decrease in NOP expression in the brain, did show enhanced anxiety (Blakley et al. 2004). In line with the NOP receptor being involved in anxiety and stress, Ro 64-6198 was found to be anxiolytic in several animal models (see subsequet text). This is particularly exciting, because Ro 64-6198 may lack the side effects of traditional anxiolytics, such as tolerance and abuse potential (Dautzenberg et al. 2001; Jenck et al. 2000; Le Pen et al. 2002).

At low doses Ro 64-6198 was found to be anxiolytic in several neophobic tests, including the marble burying test in mice (Nicolas et al. 2006b), the elevated plus maze in rats (Dautzenberg et al. 2001; Jenck et al. 2000; Wichmann et al. 2000) and the open field test in rats (Wichmann et al. 2000). In the marble burying test, at 1 mg/kg, i.p., Ro 64-6198 produced a decrease in the number of marbles buried, without altering locomotor activity, indicating a decrease in neophobia and anxiety (Nicolas et al. 2006b). Ro 64-6198 selectively increased the number of open arm transitions and time spent in the open arms of the elevated plus maze at doses of 0.32–3 mg/kg, i.p., without affecting closed arm transitions or locomotor activity in the closed arms (Dautzenberg et al. 2001; Jenck et al. 2000; Wichmann et al. 2000). An increase in time spent in the open arms and number of open arm transitions is considered to reflect a decrease in anxiety, as rodents normally prefer the closed arms to the stressful

TABLE 2. *Effects of Ro 64-6198 and N/OFQ in animal models of anxiety.*

Reference	Test	Result	Subjects	Treatment	Effects of antagonist, partial agonist, functional antagonist, or NOP knockout	Locomotor activity
Nicolas et al. (2006b)	Marble burying	Anxiolytic	Male C57BL/6J mice	1 mg/kg Ro 64-6198, i.p., 30 min	Not determined	No effect at 1 mg/kg during test
Leggett et al. (2006)	ACTH, corticosterone	Increase	Male Sprague-Dawley rats	0.05–5.5 nmol N/OFQ, i.c.v., 30–60 min	Blockade of hormone increase with UFP-101, 1 μg	
Vitale et al. (2006)	Elevated plus maze	Anxiolytic following 1st injection, anxiolytic following 2nd injection	Male Wistar rats	0.3–1.5 nmol N/OFQ, i.c.v., 5 min	Blockade of anxiolytic effects with 10 nmol UFP-101	Hypolocomotion following 1st injection. No effect following 2nd injection.
Vitale et al. (2006)	Conditioned defensive burying test	Anxiolytic	Male Wistar rats	0.3–1.5 nmol N/OFQ, i.c.v., 5 min	Blockade of anxiolytic effects with 10 nmol UFP-101	
Vitale et al. (2006)	Corticosterone	Anxiolytic following 1st injection, trend at increase following 2nd injection	Male Wistar rats	0.3–1.5 nmol N/OFQ, i.c.v., >20 min	Not determined	

Continued

TABLE 2. Continued

Reference	Test	Result	Subjects	Treatment	Effects of antagonist, partial agonist, functional antagonist, or NOP knockout	Locomotor activity
Varty et al. (2005)	Vogel lick-suppression test	Anxiolytic	Male CD rats	1–10 mg/kg Ro 64-6198, i.p., 30 min	Attenuation of anxiolytic effect with 10 mg/kg J-113397	
Varty et al. (2005)	Isolation-induced vocalizations	Anxiolytic	Male CD rat pups	0.3–3 mg/kg Ro 64-6198, i.p., 30 min	Not determined	
Varty et al. (2005)	Isolation-induced vocalizations	Anxiolytic	Male and female Hartley guinea pigs pups	0.1 – 1 mg/kg Ro 64-6198, i.p., 30 min	Not determined	
Varty et al. (2005)	Geller-Seifter conflict	Anxiolytic	Mixed C57BL/6 129 × 1Sv background	1–3 mg/kg Ro 64-6198, i.p., 30 min	No effect of Ro 64-6198 in NOP knockout mice	
Kamei et al. (2004)	Hole-board test	Anxiolytic at low doses, anxiogenic at high doses	Male ICR mice	0.01–5 nmol N/OFQ, i.c.v., 30 min	Blockade of anxiolytic effects with 5 nmol nocistatin	No effect on locomotor activity
Fernandez et al. (2004)	Open field	Anxiogenic	Male Long-Evans rats	0.001–1 nmol N/OFQ, I.c.v., 6 min	Not determined	
Fernandez et al. (2004)	Elevated plus maze	Anxiogenic	Male Long-Evans rats	0.001–1 nmol N/OFQ, i.c.v., 5 min	Not determined	1 nmol N/OFQ decreased closed arm entries

Continued

TABLE 2. Continued

Reference	Test	Result	Subjects	Treatment	Effects of antagonist, partial agonist, functional antagonist, or NOP knockout	Locomotor activity
Fernandez et al. (2004)	Light-dark box	Anxiogenic	Male Long-Evans rats	0.01–1 nmol N/OFQ, i.v., 6 min	Not determined	0.1 nmol N/OFQ had no effect in a separate experiment in a 'dark-dark' box
Fernandez et al. (2004)	Corticosterone	Increase	Male Long-Evans rats	0.001–1 nmol N/OFQ, i.c.v., 30 min	Not determined	
Bauer (2004)	Open field	Anxiogenic	Male Long-Evans rats	0.1 nmol N/OFQ, i.c.v., 6 min	No effect of 1 nmol J-113397. J-113397 tended to be anxiogenic.	
Le Cudennec et al. (2002)	Corticosterone	Attenuation of the injection-induced stress related increase in corticosterone	Male Swiss albino mice (CD1)	0.005–0.55 nmol N/OFQ (converted from μg), i.c.v., 0–120 min	Blockade of decrease with [Nphe1]N/OFQ(1-13)NH2, [Phe1-ψ(CH2-NH)Gly2]N/OFQ(1-13)NH2, and N /OFQ(1-13)NH	
Gavioli et al. (2002)	Elevated plus maze	Inverted-U shaped curve (anxiolytic and no effect)	Male Swiss mice	0.0001–1 nmol N/OFQ, i.c.v., 5 min	Blockade of anxiolytic effects with 1 pmol nocistatin	

Continued

TABLE 2. Continued

Reference	Test	Result	Subjects	Treatment	Effects of antagonist, partial agonist, functional antagonist, or NOP knockout	Locomotor activity
Dautzenberg et al. (2001)	Elevated plus maze	Anxiolytic	Male Sprague-Dawley rats	3 mg/kg Ro 64-6198, i.p., 30 min	Not determined	
Devine et al. (2001)	ACTH, corticosterone	Increase	Male Sprague-Dawley rats	0.01–1 nmol N/OFQ, i.c.v., 15–30 min	Similar effect of [Phe1ψ(CH2–NH)Gly2]nociceptin(1–13)NH2	
Wichmann et al. (2000)	Elevated plus maze	Anxiolytic	Male Sprague-Dawley rats	0.3–3.2 mg/kg Ro 64-6198, i.p., 30 min	Not determined	No effects on closed door transitions or locomotor activity in closed area
Wichmann et al. (2000)	Open field	Anxiolytic	Male Sprague-Dawley rats	0.3–3.2 mg/kg Ro 64-6198, i.p., 30 min	Not determined	
Jenck et al. (2000)	Elevated plus maze	Anxiolytic	Male Sprague-Dawley rats	0.3–3.2 mg/kg Ro 64-6198, i.p., 30 min	Not determined	No effect on closed transitions or distance traveled in closed arms
Jenck et al. (2000)	Fear-potentiated Auditory Startle	Anxiolytic	Male Wistar rats	1–10 mg/kg Ro 64-6198, i.p., 30 min	Not determined	No effect on startle response during habituation
Jenck et al. (2000)	Geller-Seifter conflict	Inverted-U shaped curve (anxiolytic and no effect)	Male Wistar rats	0.3–3.2 mg/kg Ro 64-6198, i.p., 30 min	Not determined	

Continued

TABLE 2. Continued

Reference	Test	Result	Subjects	Treatment	Effects of antagonist, partial agonist, functional antagonist, or NOP knockout	Locomotor activity
Jenck et al. (2000)	Panic-Like Anxiety Test	No selective effect	Male Wistar rats	0.3–3.2 mg/kg Ro 64-6198, i.p., 30 min	Not determined	10 mg/kg decreased escape latency
Wichmann et al. (1999)	Modified open field	Anxiolytic	Not specified	0.32–3 mg/kg (R)-8-acenaphthen-1-yl-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one, i.p., 30 min	Not determined	
Wichmann et al. (1999)	Elevated plus maze	Anxiolytic	Not specified	0.32–3 mg/kg (R)-8-acenaphthen-1-yl-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one, i.p., 30 min	Not determined	No effect on locomotor activity in closed arms
Griebel et al. (1999)	Mouse defense test battery	Anxiolytic	Male Swiss mice	0.1–3 nmol N/OFQ, i.c.v., 15 min	Not determined	Determined in testing apparatus before anxiety testing. No effect, but trend at decrease at 3 nmol N/OFQ

Continued

TABLE 2. Continued

Reference	Test	Result	Subjects	Treatment	Effects of antagonist, partial agonist, functional antagonist, or NOP knockout	Locomotor activity
Jenck et al. (1997)	Light-dark box	Inverted-U shaped curve (anxiolytic and no effect)	Male MORO mice	0.1–3 nmol N/OFQ, i.c.v., 5 min	Not determined	
Jenck et al. (1997)	Geller-Seifter conflict	Anxiolytic	Male MORO mice	3 nmol N/OFQ, i.c.v., 5 min	Not determined	
Jenck et al. (1997)	Open field with urocortin-induced hypolocomotion	Inverted-U shaped curve (anxiolytic and no effect)	Male BALB/c mice	0.1–1 nmol N/OFQ, i.c.v., 10 min	Not determined	
Jenck et al. (1997)	Elevated plus maze	Inverted-U shaped curve (anxiolytic and no effect)	Male Wistar RoRo rats	0.3–1 nmol N/OFQ, i.c.v., 10 min	Not determined	No change in locomotion in non-open areas of the maze

The effects of Ro 64-6198 and N/OFQ in several animal models of anxiety and on stress hormones are listed. Pretreatment times refer to dosing time before the start of the test or blood collection. Dosing ranges refer to doses tested, not effective doses. In tests where locomotor side effects may interfere with interpretation of the results, data on locomotion is listed (if reported). Abbreviations: ACTH (adrenocorticotropic hormone), UFP-101 ([Nphe(1),(pF)Phe(4),Aib(7),Aib(11),Arg(14),Lys(15)]N/OFQ-NH(2)), J-113397 (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H benzimidazol-2-one).

conditions of the open, elevated, and lit arms of the plus maze (Pellow et al. 1985). In the open field test, Ro 64-6198, at doses of 0.32–3 mg/kg, attenuated the inhibition of exploration that results from the stress of a novel environment (Wichmann et al. 2000).

In addition, Ro 64-6198 was anxiolytic in conflict tests, disinhibiting punished responding in both the Vogel lick-suppression test in rats (Varty et al. 2005) and Geller-Seifter conflict test in mice and rats (Jenck et al. 2000; Varty et al. 2005). In the lick-suppression test Ro 64-6198, at 3–10 mg/kg, increased punished responding (Varty et al. 2005). In the Geller-Seitfer conflict test, Ro 64-6198 elicited an inverted-U shaped dose response curve in rats, with anxiolytic activity at 1 but not 3.2 mg/kg, i.p. (Jenck et al. 2000), while being effective at 3 mg/kg in mice (Varty et al. 2005). However, there should be some caution in interpreting these results. As stated previously, Ro 64-6198 can produce motor impairments that could decrease operant responding. NOP agonists appear to regulate food intake as well, since at 2.5 mg/kg, i.p., Ro 64-6198 increased feeding in rats (Ciccocioppo et al. 2002). In the Geller-Seifter conflict test in mice, Ro 64-6198 did not increase punished responding *per se*, but decreased unpunished responding, resulting in an increase in the percentage of punished responses (Varty et al. 2005). Therefore, it is possible that the effects of Ro 64-6198 in conflict tests are a combination of anxiolytic and sedative effects on operant responding.

Ro 64-6198 was also reported to be effective in other emotional tests of stress and anxiety. In the fear-potentiated auditory startle test in rats, Ro 64-6198 (3.2–10 mg/kg) decreased the startle response to an auditory stimuli that was potentiated by a light conditioned to shock, without affecting the startle response to the auditory stimuli alone during habituation (Jenck et al. 2000). Ro 64-6198 also decreased isolation-induced vocalizations in rat (at 1–3 mg/kg) and in guinea pig pups (at 0.3–1 mg/kg) (Varty et al. 2005). However, Ro 64-6198 had no effect on escape thresholds in the panic-like anxiety test in rats at doses that did not affect escape latencies (Jenck et al. 2000). Interestingly, N/OFQ also had no selective effect on flight responses in the mouse defense test battery (Griebel et al. 1999). This suggests that despite a diverse anxiolytic profile, Ro 64-6198 is less effective than traditional anxiolytics in animal models that involve a greater component of panic (Griebel et al. 1999; Jenck et al. 2000; Nicolas et al. 2006a).

Because Ro 64-6198 is a partial agonist with approximately 50 nM affinity at the MOP receptor (Dautzenberg et al. 2001), and the concentration of free drug in the brain following injection of an anxiolytic dose has not been reported, it is possible that the MOP receptor is responsible for some of Ro 64-6198's anxiolytic effects. However, some limited data suggest that this is unlikely. First, the NOP antagonist J-113397, 10 mg/kg, attenuated the effects of 3 mg/kg Ro 64-6198 in the lick-suppression test, whereas the MOP antagonist naltrexone (3 mg/kg) had no effect (Varty et al. 2005). Furthermore, no anxiolytic effects of Ro 64-6198 were seen at 1–3 mg/kg in the Geller-Seifter test in NOP knockout mice (Varty et al. 2005), although as stated previously, side effects could have interfered with this test. In addition, morphine, at 1–10 mg/kg was reported to have no anxiolytic effect in the elevated plus maze using the same protocol in which Ro 64-6198 was active (Wichmann et al. 2000). And finally, it was reported that naloxone (dose was not stated) did not block the effects of Ro 64-6198 in the elevated plus maze (Jenck et al. 2000). However, a lack of blockade by a single dose of a MOP antagonist cannot prove a lack of MOP contribution, as high doses of antagonists may be needed depending on the concentration of agonist and level of spare receptors. Taken together these data suggest that the anxiolytic effects of Ro 64-6198 are due to selective agonist activity at the NOP receptor.

In general, the anxiolytic effects of Ro 64-6198 are in agreement with results from the literature using other NOP agonists (Table 2). N/OFQ was found to be anxiolytic in the elevated plus maze, the conditioned defensive burying test, the hole-board test at low doses, the mouse defense test battery in measures not related to panic, the light-dark box, the open field, and the Geller-Seifter conflict test. The synthetic nonpeptide NOP agonist (R)-8-acenaphthen-1-yl-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one, also from Hoffmann-La Roche, was found to be anxiolytic in the open field test and elevated plus maze (Wichmann et al. 1999). However, N/OFQ has also been reported to produce inverted-U shaped dose response curves, with an anxiolytic effect at low doses and either no effect (Gavioli et al. 2002) or an anxiogenic effect at higher doses (Kamei et al. 2004). Complicating the matter further is the fact that a study by Fernandez et al. (2004) reported pure anxiogenic effects of N/OFQ over a broad range of doses (0.001–1 nmol). In that study, N/OFQ increased anxiety in three different neophobic tests: the open field, the elevated plus maze, and the light-dark box. The reason for this discrepancy is not clear. As mentioned previously, both Ro 64-6198 and N/OFQ can produce inverted-U shaped dose–response effects on anxiety. Because variations in methodology, perhaps especially when i.c.v. dosing is involved, can lead to variations in the effective dose range between laboratories, it is possible that the anxiolytic doses of N/OFQ were missed in this latter study. In addition, as the authors point out, the discrepancy could be due to the subjects used (Fernandez et al. 2004). Long-Evans rats, which were used in this study, but not in any of the other reported studies using NOP agonists, have been shown to vary from other strains in characteristics related to stress and anxiety (Vaidya et al. 2005). However, treatment of Long-Evans rats with NOP antisense oligonucleotides enhances anxiety (Blakley et al. 2004), consistent with an anxiolytic profile for NOP agonists. Griebel et al. (1999) hypothesized that N/OFQ may only play a role in inescapable or extremely stressful situations, and Fernandez et al. (2004) pointed out that many of the studies in which NOP agonists were anxiolytic involved inescapable or extreme stress. Indeed, several of the studies that reported anxiolytic activity of Ro 64-6198 involved shock (Jenck et al. 2000; Varty et al. 2005) or an apparent high stress in the control group. For example, in the marble burying test in mice, the strain tested was chosen based on its highly stressful reaction to the marbles (Nicolas et al. 2006b) and in the elevated plus maze experiments, the control groups spent much less than a third of the test period in the open arms (Dautzenberg et al. 2001; Jenck et al. 2000). However, data opposing this hypothesis come from a recent report by Vitale et al. (2006). In that study, animals were tested in the elevated plus maze and control animals spent approximately a third of their time in the open arms, similar to the study by Fernandez et al. (2004). In this test, N/OFQ was anxiogenic, but also produced a decrease in closed arm entries, indicating that nonselective effects on locomotor activity could have interfered with the results. When rats were made tolerant to the hypolocomotor effects of N/OFQ with a prior injection, N/OFQ was anxiolytic (Vitale et al. 2006). Indeed, locomotor activity is highly integrated in behavioral tests of anxiety and can lead to nonselective interferences (Dawson and Tricklebank, 1995) and a decrease in closed arm entries was also found in the study by Fernandez et al. (2004) Although Fernandez et al. (2004) reported no change in transitions by N/OFQ in a control dark-dark shuttle box, suggesting that locomotor activity did not interfere with the light-dark box test, it is possible for stress and locomotor activity to interact, resulting in different locomotor effects under control conditions and during behavioral testing (Nicolas et al. 2006b). Finally, it should be mentioned that a preliminary report, while replicating the anxiogenic effects of N/OFQ in the open field test, failed to block these effects with the NOP antagonist J-113397

(Bauer, 2004). The reason for this is unknown, but it was suggested that J-113397 could have partial agonist effects. Although so far J-113397 seems to lack partial agonist activity at the NOP receptor in a variety of tissue, it does have affinity for the classical opioid receptors at very high concentrations (Bigoni et al. 2000) and has other non-NOP mediated effects *in vivo* (Koizumi et al. 2004). Future experiments will be needed to determine whether the lack of blockade was due to nonselective or partial agonistic actions of J-113397 or if N/OFQ does not increase anxiety through NOP receptors.

In summary, Ro 64-6198 produces anxiolytic effects, without tolerance, in several animal models, except those involving panic, with an effectiveness comparable to that of traditional anxiolytics. However, since the side effects of Ro 64-6198 are more pronounced in mice than in rats (see preceding text), it is often more difficult to demonstrate anxiolytic efficacy in this species. Currently, it has not been definitively shown whether the anxiolytic effects of NOP agonists depend on the state of the animal (with a reduction in anxiety observed only in highly stressed animals), the strain of the animal, the dose used, or are only apparent when nonselective locomotor interferences are removed. Interestingly, while N/OFQ has been shown to be anxiogenic under some conditions, Ro 64-6198 has not. Although this could be simply due to Ro 64-6198 not being tested under the specific conditions that are susceptible to anxiogenic effects, it raises the intriguing possibility that this could be due to differences in selectivity between Ro 64-6198 and N/OFQ or bioactive metabolites, or due to possible differences in the subset of NOP receptors that are activated between the two.

Pain, Cough, and Food Intake

There is evidence that N/OFQ is effective in a variety of neuropathic pain models and the NOP receptor and N/OFQ immunoreactive content is upregulated following neuropathic injury (Briscini et al. 2002; Ma et al. 2005; Sun et al. 2001). Ro 64-6198 was also shown to have efficacy in a neuropathic pain model. In a rat sciatic nerve injury model, Ro 64-6198, given by either intrathecal (i.t.) or intraplantar injections, was antiallodynic in mechanical and thermal (cold water) tests, and these effects were reversed by NOP antagonists (Obara et al. 2005). Ro 64-6198 was not effective by s.c. injection at a low dose of approximately 0.1 mg/kg (Obara et al. 2005). Besides being antiallodynic, Ro 64-6198 is also antianalgesic, similar to N/OFQ (Mogil and Pasternak 2001). At 1–3 mg/kg Ro 64-6198 was reported to block morphine analgesia in a tail withdrawal test in mice (Kotlinska et al. 2003b). While several studies have shown that N/OFQ by itself often produces analgesia by i.t. or hyperalgesia by i.c.v. administration (Mogil and Pasternak, 2001), Ro 64-6198 did not modulate basal levels of nociception in a number of assays. Ro 64-6198 did not alter responses in the tail flick, tail immersion, tactile, or cold water stimulation (in nonneuropathic rats), or foot shock tests (Jenck et al. 2000; Kotlinska et al. 2003b; Obara et al. 2005; Varty et al. 2005). Two likely reasons for this deviation are that Ro 64-6198 is usually given systemically and that Ro 64-6198 is only a partial agonist in the periaqueductal gray (Chiou et al. 2004), a major site in the NOP modulation of pain pathways (Bytner et al. 2001; Yang et al. 2003).

The commonly used antitussive drugs, codeine, and dextromethorphan, suffer from a large number of side effects (Chung, 2005; Miller, 2005), demonstrating the need for novel antitussive treatments. Ro 64-6198 displayed potent, moderately efficacious antitussive properties in the guinea pig capsaicin-induced cough model (McLeod et al. 2004). A maximum inhibition of 50% was achieved with 0.3 mg/kg, and its effect at 3 mg/kg was

completely blocked by the NOP antagonist J-113397, 12 mg/kg, but not by the MOP antagonist naltrexone at a high dose (10 mg/kg) (McLeod et al. 2004). The effects of Ro 64-6198 are probably due to both peripheral and central actions, since N/OFQ is antitussive when given by either i.c.v. or intravenous routes (McLeod et al. 2001).

N/OFQ is known to increase feeding (Mogil and Pasternak 2001), and thus may be useful in treating anorexia or cachexia. Ro 64-6198 (2.5 mg/kg, 20 min pretreatment time) increased food intake in a 30- and 60-min test in nondeprived rats tested during the light cycle (Ciccocioppo et al. 2002). Ro 64-6198, 1 mg/kg, tended to increase feeding in food-deprived rats, an effect that may have been limited by higher levels of food intake and a low dose of the drug. Ro 64-6198 was even more potent at reversing stress-induced hypophagia, at 0.3–1 mg/kg it increased feeding in food-deprived animals subjected to restraint stress or treated with CRF, i.c.v. The effect of Ro 64-6198 on CRF stress-induced hypophagia was blocked by a NOP antagonist (Ciccocioppo et al. 2002). The potent effects of Ro 64-6198 on stress-induced hypophagia are consistent with the putative antistress properties of this compound.

Addiction

The NOP receptor is expressed in several brain areas relevant to addiction, including the amygdala, nucleus accumbens, and ventral tegmental area. N/OFQ is known to modulate several neurotransmitter systems important to addiction, including dopamine (Ciccocioppo et al. 2000a). In line with this, N/OFQ has been shown to attenuate the rewarding or reinforcing properties of several drugs of abuse. N/OFQ pretreatment inhibited the acquisition of conditioned place preference (CPP) to cocaine, morphine, methamphetamine, amphetamine, and ethanol (Ciccocioppo et al. 2000b; Kotlinska et al. 2003a; Kuzmin et al. 2003; Murphy et al. 1999; Sakoori and Murphy, 2004; Zhao et al. 2003). In addition, N/OFQ was found to attenuate the expression of cocaine CPP (Kotlinska et al. 2002). That study also found that the systemically active, nonpeptide NOP agonist Ro 65-6570 did not block the expression of cocaine CPP, but this could have been due to the anxiolytic effects of Ro 65-6570 interfering with the time spent in the black versus white chambers or due to the higher MOP affinity of that drug (Kotlinska et al. 2002; Wichmann et al. 1999). N/OFQ was also found to inhibit the expression and drug-primed reinstatement of ethanol CPP (Kuzmin et al. 2003) and to decrease ethanol self-administration under either fixed or progressive ratios (Ciccocioppo et al. 2004). Furthermore, N/OFQ was reported to block cue- and stress-induced reinstatement of ethanol self-administration (Ciccocioppo et al. 2004; Martin-Fardon et al. 2000). In the same study that examined stress-induced reinstatement of ethanol self-administration, N/OFQ did not attenuate stress-induced reinstatement of cocaine (Martin-Fardon et al. 2000). Although this could indicate that N/OFQ is not effective in blocking stress-induced reinstatement of cocaine, it could also indicate that N/OFQ is less effective in this model due to conditioned anxiogenic effects of cocaine. In other words, if N/OFQ blocked stress-induced reinstatement of ethanol through anxiolytic effects, it could be expected that N/OFQ would be less effective in a model with cocaine. Finally, despite the effects of N/OFQ on morphine-conditioned reward, N/OFQ was found to have no effect on heroin self-administration (Walker et al. 1998). Overall, these data strongly suggest that NOP agonists could prove useful in the treatment of addiction to several different drugs of abuse. Ro 64-6198, similar to N/OFQ, has been

shown to be effective in several models of drug abuse as well. Ro 64-6198 has demonstrated efficacy at inhibiting the rewarding or reinforcing effects of either morphine or ethanol.

While Ro 64-6198 (0.3–1 mg/kg, 15-min pretreatment) did not affect the expression of morphine CPP in mice, daily pretreatment with Ro 64-6198 (1 mg/kg) blocked the acquisition of morphine CPP (Shoblock et al. 2005). Ro 64-6198 pretreatment was given 15 min before both control saline conditioning and morphine conditioning, in this case, to control for any potential slightly negative affective properties of NOP agonists in mice (Sakoori and Murphy, 2004). Daily treatment with Ro 64-6198 (1 mg/kg) 15 min prior to extinction sessions did not alter the rate of extinction to established morphine CPP, but Ro 64-6198 (1 mg/kg) prevented morphine from reinstating extinguished CPP (Shoblock et al. 2005). Ro 64-6198 pretreatment did not temporarily mask the effects of the morphine prime, because the mice treated with Ro 64-6198 still did not display CPP on the following day, when tested in a drug-free state, unlike the morphine-primed vehicle pretreated animals, which still displayed partial reinstatement (Shoblock et al. 2005). These results indicate that Ro 64-6198 is effective when given immediately before morphine (blocking both acquisition and morphine-primed reinstatement), but not otherwise, leaving expression and extinction of morphine CPP unaffected. This could suggest that Ro 64-6198 interferes with the acute rewarding properties of morphine. Although it was shown that Ro 64-6198 did not block the ability of morphine to substitute for morphine in a discriminative stimulus study; as the authors point out, positive affect could be just one component of the interoceptive stimuli of morphine (Recker and Higgins, 2004). The mechanism by which Ro 64-6198 could interfere with the acute rewarding properties of morphine has not yet been determined. NOP agonists have been shown to attenuate morphine's elevation in dopamine levels (Di Giannuario and Pieretti, 2000) and to also cross-desensitize MOP receptors *in vitro* (Hawes et al. 1998; Mandyam et al. 2002), both of which could functionally antagonize some of the effects of an acute injection of morphine. Although Ro 64-6198 does have high affinity for the MOP receptor (see above), it is unlikely that Ro 64-6198 competitively antagonized morphine, given the high morphine dose (20 mg/kg) used in the study (Shoblock et al. 2005). The effect of Ro 64-6198 on morphine dependence has also been examined. In a separate study, Ro 64-6198 was found to decrease naloxone precipitated morphine withdrawal, an effect that could have been due to a sedative effect (Kotlinska et al. 2003b). Ro 64-6198 (1–3 mg/kg) given four times a day for the 3 days of a morphine pellet implantation, did not affect the development of morphine dependence when measured by naloxone precipitated jumps (Kotlinska et al. 2003b).

Ro 64-6198 was also found to inhibit the acquisition, expression, and drug-primed reinstatement of ethanol CPP. Ro 64-6198 (0.1 mg/kg) blocked the acquisition of ethanol CPP in mice. The dose–response curve for this effect had an inverted-U shape, so that at 0.1 and 1 mg/kg Ro 64-6198 blocked the acquisition of ethanol CPP, while at 0.3 mg/kg it produced a place aversion to ethanol (Kuzmin et al. 2003). However, at 0.3 mg/kg the drug also decreased the number of crossings between the conditioning compartments. Starting at 0.1 mg/kg, Ro 64-6198 also blocked the expression of ethanol CPP, and at 1 mg/kg, the would-be preference for the previously conditioned ethanol compartment emerged as an apparent aversion. Locomotor sedative effects were seen at the 1 mg/kg dose, however (Kuzmin et al. 2003). Following extinction to CPP, a priming injection of ethanol reinstated CPP, an effect that was blocked by 0.3 mg/kg Ro 64-6198. However, Ro 64-6198 produced hypolocomotion in this test (Kuzmin et al. 2003). In a self-administration paradigm, where

rats could choose between ethanol plus saccharin or water, Ro 64-6198, with a 30-min pretreatment at doses of 0.1 mg/kg and higher, suppressed responding for ethanol, whereas responding for saccharin only or water was affected only at 1 mg/kg (Kuzmin et al. 2006). Responses on the ethanol lever were still inhibited at 24 hours after administration of Ro 64-6198, an effect which naltrexone did not share (Kuzmin et al. 2006). After 10 days of absence from the operant chambers, in a model (Spanagel and Holter, 2000) somewhat similar to behavioral contrast, animals self-administered greater amounts of ethanol on return to the chambers compared with pre-absence baseline levels (Kuzmin et al. 2006). However, treatment with 0.3 mg/kg Ro 64-6198 for the last 3 days of absence prior to test day (but not on test day) blocked this deprivation-induced enhancement of ethanol self-administration (Kuzmin et al. 2006). Therefore, Ro 64-6198 attenuates the acquisition of ethanol CPP, the expression of ethanol CPP and self-administration, ethanol primed reinstatement of CPP, and a deprivation enhancement of ethanol self-administration. In some cases, Ro 64-6198 treatment resulted in an apparent aversion to ethanol, but these doses also produced locomotor effects, so that the significance of these results is not clear. These results suggest that, in contrast to morphine, Ro 64-6198 has more generalized effects in blocking ethanol reward and reinforcement.

It is important to note that Ro 64-6198 is devoid of affective properties in and of itself. Ro 64-6198 does not produce conditioned place preference or aversion and does not alter intracranial self-stimulation (Jenck et al. 2000; Le Pen et al. 2002). However, it should be mentioned that the apparent anti-abuse efficacy of NOP agonists could be an artifact of motor or learning side effects (see earlier). For example, NOP agonists could decrease levels of operant responding in self-administration experiments through sedative effects or by disrupting the associations between the lever and the reinforcement. However, this is probably unlikely. N/OFQ did not affect self-administration of sucrose under fixed or progressive ratios and N/OFQ did not attenuate cue-induced reinstatement of water responding (Ciccocioppo et al. 2004). In addition, Ro 64-6198 did not affect saccharin or water self-administration at doses below 1 mg/kg (Kuzmin et al. 2006). The results of the CPP experiments are more difficult to interpret, however. Most CPP studies with N/OFQ or Ro 64-6198 examined the effects of the NOP agonist on acquisition of CPP. Because the NOP agonist was paired with the drug-conditioned chamber each day, it is possible that state-dependent learning took place. That is, since the animals learned the drug conditioning in the presence of the NOP agonist, that learning may only be expressed in the presence of the NOP agonist. This possibility was ruled out for Ro 64-6198's effects on morphine. In that study, when the animals pretreated with Ro 64-6198 during acquisition were re-tested for the expression of morphine CPP in the presence of Ro 64-6198, morphine CPP was still not observed for the Ro 64-6198 group (Shoblock et al. 2005). An interference of NOP agonists with learning during conditioning is a more difficult hypothesis to rule out. It was shown that N/OFQ did not affect learning in the Morris water maze at doses that blocked the acquisition of morphine CPP (Ciccocioppo et al. 2000b). However, the type of spatial learning required in this task may be different from that during conditioned learning. It was shown that N/OFQ did not affect acquisition of conditioned place aversion to naloxone (Sakoori and Murphy 2004), which could suggest that NOP agonists do not produce general impairments in conditioned learning. However, in that study N/OFQ itself produced a slight aversive effect, which could impede N/OFQ from blocking the conditioned aversion effects of other drugs (Sakoori and Murphy, 2004). In fact, N/OFQ tended to decrease naloxone CPA to the level of aversion produced by N/OFQ itself (Sakoori and Murphy, 2004). In addition, the effects of NOP agonists on the

CPP to nondrug rewards are currently unknown. It is likely that the effects of Ro 64-6198 on the expression of ethanol CPP are at least not due to interferences in memory retrieval, since at 0.1 mg/kg Ro 64-6198 inhibited the expression of ethanol CPP (Kuzmin et al. 2003), whereas at 1 mg/kg it did not affect the expression of morphine CPP (Shoblock et al. 2005). However, until further studies are completed, it remains possible that the effects of Ro 64-6198 on acquisition and reinstatement of CPP are due to interferences in condition learning. The effects of Ro 64-6198 and N/OFQ in addiction-based models are summarized in Table 3.

Summary of Therapeutic Targets

Ro 64-6198 has demonstrated efficacy in several animal models of anxiety, except those involving panic. In addition, Ro 64-6198 may be effective in neuropathic pain models, while having no effect on nociception (except blockade of morphine analgesia) in normal animals. Ro 64-6198 also has antitussive efficacy and, since Ro 64-6198 increases feeding, especially stress-inhibited feeding, Ro 64-6198 may be useful in treating anorexia or cachexia. Ro 64-6198 may have some usefulness in treating opiate addiction, since Ro 64-6198 blocked the acquisition and drug-primed reinstatement of morphine place preference. Ro 64-6198 has more broad effects on ethanol reward and reinforcement, decreasing the acquisition, expression, and drug-primed reinstatement of ethanol place preference and decreasing ethanol self-administration and deprivation-induced enhancement of ethanol self-administration. Finally, based on data with N/OFQ, Ro 64-6198 may have further clinical usefulness, including treating gastrointestinal disorders and overactive bladder, as well as an antiepileptic (Calo' et al. 2002).

Future of Ro 64-6198

The future of Ro 64-6198 is uncertain. At present, only limited information is available on its toxicology, pharmacokinetics, and therapeutic levels. Also, it has been tested only in a limited number of species: rats, mice, and guinea pigs. Ro 64-6198 has a poor oral bioavailability. This may not prevent its clinical use, since modern drug delivery methods may extend its use beyond oral dosing. However, Ro 64-6198 suffers from numerous target-based side effects, including impairments of motor activity, coordination, learning, and memory, as well as hyperphagic and hypothermic effects. Most importantly, the doses at which these side effects are produced depend on the species. Therefore, it is difficult to predict whether a therapeutic dose range with limited side effects will exist in humans, as it does not in mice. In addition, as with other NOP ligands, there appears to be some tissue-dependent variability in Ro 64-6198's agonist efficacy and selectivity (Calo et al. 1998; Gunduz et al. 2006; McDonald et al. 2003a; Okawa et al. 1999). Furthermore, the literature is filled with inconsistencies on the pain- and anxiety-modulating properties of NOP agonists. Indeed, the controversy surrounding all of this variation raises some questions about the therapeutic value of NOP agonists in general. It was pointed out that if these polymorphic responses were seen in humans, then NOP receptor-based drugs would be "highly idiosyncratic" (Mogil et al. 1999). In conclusion, several questions remain regarding Ro 64-6198 and NOP agonists in general, including whether there are subtypes of the NOP

TABLE 3. Efficacy of Ro 64-6198 and N/OFQ in animal models of drug abuse.

	Self-administration		Conditioned place preference			
Drug	Expression	Relapse models	Acquisition	Expression	Extinction	Relapse models
Alcohol	Blockade by N/OFQ (Ciccocioppo et al. 2004) and Ro 64-6198 (Kuzmin et al. 2006)	Blockade of cue- and stress-reinstatement by N/OFQ (Ciccocioppo et al. 2004; Martin-Fardon et al. 2000) and blockade of deprivation enhancement by Ro 64-6198 (Kuzmin et al. 2006)	Blockade by N/OFQ and Ro 64-6198 (Kuzmin et al. 2003)	Blockade by N/OFQ and Ro 64-6198 (Kuzmin et al. 2003)		Blockade of drug-prime reinstatement by N/OFQ and Ro 64-6198 (Kuzmin et al. 2003)
Opiates	No effect of N/OFQ on heroin (Walker et al. 1998)		Blockade of morphine by N/OFQ (Ciccocioppo et al. 2000b; Murphy et al. 1999; Sakoori and Murphy, 2004) and Ro 64-6198 (Shoblock et al. 2005)	No effect on morphine by Ro 64-6198 (Shoblock et al. 2005)	No effect on morphine by Ro 64-6198 (Shoblock et al. 2005)	Blockade of morphine drug-prime reinstatement by Ro 64-6198 (Shoblock et al. 2005)

Continued

TABLE 3. Continued

	Self-administration		Conditioned place preference			
Drug	Expression	Relapse models	Acquisition	Expression	Extinction	Relapse models
Cocaine		No effect of N/OFQ on stress-reinstatement (Martin-Fardon et al. 2000)	Blockade by N/OFQ (Sakoori and Murphy, 2004)	Blockade by N/OFQ (Kotlinska et al. 2002)		
Amphetamine			Blockade by N/OFQ (Kotlinska et al. 2003a)			
Methamphetamine			Blockade by N/OFQ (Zhao et al. 2003)			

The effects of Ro 64-6198 and N/OFQ on the drug self-administration and relapse models of self-administration, and on the acquisition, expression, extinction, and reinstatement of conditioned place preference to several drugs of abuse. Values left blank were not determined.

receptor and whether NOP agonists will have any true therapeutic value for humans. In any case, as a systemically active and selective NOP agonist, Ro 64-6198 will provide a valuable tool in answering these questions.

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