British Journal of Pharmacology (2002) 137, 369–374

www.nature.com/bjp

Pharmacological characterization of the novel nociceptin/orphanin FQ receptor ligand, ZP120: *in vitro* and *in vivo* studies in mice

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1 This study reports on the pharmacological characterization of ZP120, a novel ligand of the nociceptin/orphanin FQ (N/OFQ) peptide receptor, NOP. ZP120 is a structure inducing probes modified NOP ligand: Zealand Pharma proprietary SIP technology was used to increase the enzymatic stability and half-life of peptide.

2 *In vitro*, ZP120 mimicked the inhibitory effects of N/OFQ in the electrically stimulated mouse vas deferens, showing however higher potency (pEC_{50} 8.88 *vs* 7.74), lower maximal effects (E_{max} $69 \pm 5\%$ *vs* $91 \pm 2\%$), and slower onset of action. Like N/OFQ, the effects of ZP120 were not modified by 1 μM naloxone, but they were antagonized by the NOP receptor selective antagonist J-113397 (pA_2 7.80 *vs* ZP120, 7.81 *vs* N/OFQ).

3 *In vivo*, ZP120 mimicked the effects of N/OFQ, producing pronociceptive effects in the tail withdrawal assay and decreased locomotor activity after i.c.v., but not after i.v. administration in mice. ZP120 elicited similar maximal effects as N/OFQ, but it was about 10 fold more potent and its effects lasted longer.

4 In conclusion, the novel NOP receptor ligand ZP120 is a highly potent and selective partial agonist of the NOP receptor with prolonged effects *in vivo*.
British Journal of Pharmacology (2002) **137,** 369–374. doi:10.1038/sj.bjp.0704894

Keywords: ZP120, Ac-RYYRWK-NH_2, nociceptin/orphanin FQ; NOP receptor; *in vitro* and *in vivo* assays, mice

Abbreviations: i.c.v., intracerebroventricular; i.v., intravenous; N/OFQ, nociceptin/orphanin FQ; NOP receptor, nociceptin/orphanin FQ peptide receptor; SIP, structure inducing probes; ZP120, Ac-RYYRWKKKKKKK-NH_2.

Introduction

Nociceptin/orphanin FQ (N/OFQ) (Meunier *et al*., 1995; Reinscheid *et al*., 1995), is the endogenous ligand of the N/OFQ peptide receptor (NOP), the fourth member of the opioid receptor family (Cox *et al*., 2000). *Via* NOP receptor activation N/OFQ modulates several biological actions both at central and peripheral levels (Calo *et al*., 2000b). Among these actions, the diuretic and antinatriuretic effect of N/OFQ appears to be of interest since no marketed drugs can induce this pattern of renal effects (Kapusta, 2000). The unique combination of water diuretic and antinatriuretic properties are considered particularly beneficial in the treatment of oedema-forming states with hyponatrimia (e.g., congestive heart failure and liver cirrhosis).

Ac-RYYRWKKKKKKK-NH_2 (ZP120) is a novel ligand for the NOP receptor (Larsen *et al*., 2001) which has been designed in accordance with the structure inducing probes (SIP) technology (Larsen, 1999) in order to improve the metabolic stability of Ac-RYYRWK-NH_2, a NOP selective hexapeptide identified by screening of synthetic peptide combinatorial libraries (Dooley *et al*., 1997). The SIP technology is based on the use of structure inducing probes which constrain the parent peptide into a more ordered conformation based on intramolecular hydrogen bonds (Larsen, 1999). SIP is represented by a short peptide sequence, i.e. $(Lys)_6$, added to the C-terminal of the parent peptide, which generally results in an increase in the enzymatic stability of the peptide while the biological activity at the same time is maintained. The SIP-approach has been successfully applied to the enkephalines: [Leu^5]enkephalin-$(Lys)_6$ was found to be 1000 fold more stable than the native peptide [Leu^5]enkephalin to the action of leucine aminopeptidase (Larsen *et al*., 2000). Similar results were obtained more recently by applying the SIP-approach to the peptide sequence glucagon-like peptide 1 (7–39) (J.S. Petersen, unpublished results).

The aim of the present study was to investigate and pharmacologically characterize the actions of ZP120 *in vitro* and *in vivo* in the mouse. *In vitro*, the effects of ZP120 were evaluated and compared with those of the natural ligand N/OFQ and Ac-RYYRWK-NH_2, in the electrically stimulated mouse vas deferens, a N/OFQ sensitive pharmacological preparation (Calo′ *et al*., 1996). *In vivo*, ZP120 was investigated for its ability to induce pronociceptive effects in the tail withdrawal assay (Calo′ *et al*., 1998) and to depress locomotor activity (Rizzi *et al*., 2001a) after intracerebroventricular (i.c.v.) as well as intravenous (i.v.) administration in mice.

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Methods

In vitro *studies*

Tissues from male Swiss mice (25–30 g) were prepared as previously described (Calo′ *et al*., 1996) and suspended in 5 ml organ baths containing Krebs buffer oxygenated with 95% O_2 and 5% CO_2. The mouse vas deferens was continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.05 Hz frequency. Electrically evoked contractions were measured isotonically with a strain gauge transducer (Basile 7006) and recorded with the PC based acquisition system Autotrace 2.2 (RCS, Florence, Italy). Following an equilibration period of about 60 min, the contractions induced by electrical field stimulation (twitches) were stable. At this time, cumulative concentration response curves to agonists were recorded (0.5 log unit steps). When N/OFQ or Ac-RYYRWK-NH_2 were tested as agonists three concentration response curves were performed in the same tissue at 60 min intervals, while when ZP120 was used as an agonist a single concentration response curve per tissue was performed. In some experiments the non-selective classical opioid receptor antagonist naloxone (1 μM) or the selective NOP antagonist J-113397 (0.1 μM) (Ozaki *et al*., 2000) were added to the Krebs solution 15 min before performing the concentration response curves to the agonists.

In vivo *studies*

Male Swiss mice weighing 20–25 g were used. All experimental procedures adopted for *in vivo* studies were as humane as possible and complied with the standards of the European Communities Council directives (86/609/EEC) and national regulations (D.L. 116/92). Animals were housed under standard conditions (22°C, 12-h light–dark cycle) with food and water *ad libitum* for at least 2 days before experiments began. Each animal was used once. I.c.v. injections (2 μl) were given into the left ventricle, according to the procedure described by Laursen and Belknap (1986). In few experiments the effects of ZP120 were evaluated after i.v. administration: the injection (100 μl) was performed in the tail ventral vein.

Tail withdrawal assay All experiments began at 10:00 h and were performed according to the procedure described by Calo′ *et al*. (1998). Briefly, the animals were placed in a holder and the distal half of the tail was immersed in water at 48°C. Withdrawal latency time was measured by an experienced observer blind to drug treatment. A cut off time of 20 s was chosen to avoid tissue damage. Four mice were randomly assigned to each experimental group and the same experiment was repeated at least six times. Tail withdrawal time was determined immediately before (time 0) and 5, 15, 30, 60 and 90 min after i.c.v. injection of 2 μl of saline or ZP120 (0.01, 0.1 or 1 nmol) or N/OFQ (1 nmol). In few experiments, the effects of 10 nmol ZP120 were evaluated after i.v. administration.

Locomotor activity assay Experiments were performed between 14:00–18:00 h, following the procedure described by Rizzi *et al*. (2001a). Briefly, the animals were routinely tested 3 min after i.c.v. injection of saline, N/OFQ (1 nmol), or ZP120 (1 nmol). Locomotor activity was assessed using Basile activity cages. The total number of impulses were recorded every 5 min for 30 min. In a separate series of experiments, locomotor activity was assessed 1 h after i.c.v. injection of peptides. Animals were not accustomed to the cages before drug treatments and the experiment was performed in a quiet and dimly illuminated room. Three mice were randomly assigned to each experimental group and the same experiment was repeated at least five times. In few experiments, the effects of 10 nmol ZP120 were evaluated after i.v. administration.

Drugs

N/OFQ, Ac-RYYRWK-NH_2 and J-113397 were synthesized and purified at the University of Ferrara as previously described (De Risi *et al*., 2001; Guerrini *et al*., 1997). ZP120 was synthesized according to solid phase procedure using the Fmoc-strategy as previously described (Larsen & Holm, 1998). The crude peptide was purified using preparative RP-HPLC and finally characterized using mass spectrometry. Naloxone was from Tocris Cookson (Bristol, U.K.). For *in vitro* experiments, the compounds were dissolved in physiological buffers and stock solutions (1 mM) were kept at −20°C until use. For *in vivo* studies, the substances were dissolved in saline just before performing the experiment.

Data analysis and terminology

All data are expressed as means ± s.e.mean of n experiments. For potency values the 95% confident limits are given. The pharmacological terminology adopted in this paper is consistent with the IUPHAR recommendations (Jenkinson *et al*., 1995). Agonist potencies were measured as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. The E_{max} is the maximal effect that an agonist can elicit in a given tissue and is expressed as per cent of the control twitch response to electrical stimulation. Antagonist potencies are expressed in terms of pK_B, which is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original submaximal response. pK_B values were determined by applying the Gaddum Schild equation ($pK_B = -\log((CR-1)/[\text{Antagonist}])$), assuming a slope equal to unity.

In vivo data were analysed as follows: raw data from tail withdrawal experiments were converted to the area under the time x withdrawal latency curve and the data expressed as area under the curve were used for statistical analysis; locomotor activity data were analysed using the data expressed as cumulative impulses over the 30 min observation period. Data have been analysed statistically using the Student's t-test or one-way ANOVA followed by the Dunnett's test, as specified in table and figure legends. P values lower than 0.05 were considered to be significant.

Results

In vitro *studies*

N/OFQ, Ac-RYYRWK-NH_2 and ZP120 inhibited in a concentration-dependent manner the electrically induced

contraction of the mouse vas deferens (Figure 1, left panel); however, the natural peptide produced maximal effects significantly higher than those evoked by Ac-RYYRWK-NH_2 and ZP120. Moreover, ZP120 was about 10 fold more potent than N/OFQ and Ac-RYYRWK-NH_2. The values of potency and efficacy of the three agonists are summarized in Table 1. The kinetics of action of N/OFQ and Ac-RYYRWK-NH_2 were similar: in fact, the effects of both peptides took place immediately after adding the peptide to the bath, were rapidly reversible after washing, and could be repeated in the same tissue. On the contrary, the effects elicited by ZP120 were slow to develop, slowly reversible after washing, and could not be repeated in the same tissue. Figure 2 displays typical tracings obtained with equieffective concentrations of N/OFQ, Ac-RYYRWK-NH_2 and ZP120 in the electrically stimulated mouse vas deferens.

The inhibitory effects of ZP120 in the mouse vas deferens were evaluated in the presence of the non-selective classical opioid receptor antagonist naloxone (1 μM) and of the selective NOP receptor antagonist J-113397 (0.1 μM). As shown in the right panel of Figure 1, naloxone did not modify the inhibitory effects of ZP120, while J-113397 produced a rightward shift of the concentration–response curve to the agonist without significantly modifying its maximal effects: a pK_B value of 7.80 was derived from these experiments. Similar experiments were performed using N/OFQ and Ac-RYYRWK-NH_2 as agonists and the obtained results are summarized in Table 2.

In vivo *studies*

ZP120 was administered i.c.v. in mice at doses ranging from 0.01 to 1 nmol. I.c.v. injections of ZP120 at 0.01 nmol did not induce any obvious behavioural effects. In contrast, mice injected with 0.1 and 1 nmol showed decrease in locomotor activity and muscular tone (especially in the hindpaws), ataxia, and loss of the righting reflex; however, a normal tail-pinch reflex was maintained. These behavioural effects of ZP120 were clearly evident about 30 min (1 nmol) or 1 h (0.1 nmol) after the i.c.v. injection. This behaviour was qualitatively similar but quantitatively more pronounced

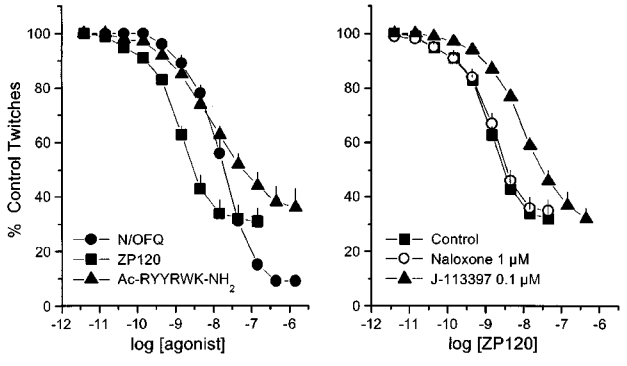

Figure 1 Left panel: concentration–response curve to N/OFQ, ZP120, and Ac-RYYRWK-NH_2 obtained in the electrically stimulated mouse vas deferens. Right panel: concentration–response curve to ZP120 measured in the absence (control) and in presence of 1 μM naloxone or 0.1 μM J-113397. Data are mean ± s.e.mean of at least five separate experiments. For statistical analysis of these data see Table 1.

Table 1 Effects of N/OFQ, Ac-RYYRWK-NH_2 and ZP120 in the electrically stimulated mouse vas deferens

	pEC_{50} ($CL_{95\%}$)	$E_{max} \pm$ s.e.mean
N/OFQ	7.74 (7.54–7.94)	91 ± 2%
Ac-RYYRWK-NH_2	8.07 (7.77–8.37)	64 ± 7%*
ZP120	8.88 (8.75–9.01)*	69 ± 5%*

Data are mean of at least five separate experiments. $CL_{95\%}$ confidence limits. *$P<0.05$ *vs* the corresponding value of N/OFQ, according to ANOVA followed by the Dunnet test for multiple comparison.

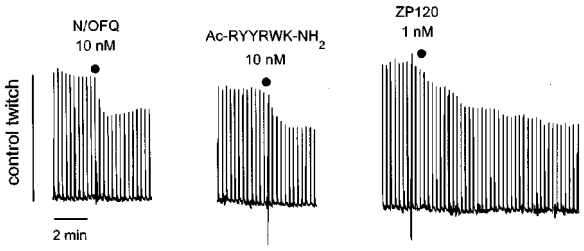

Figure 2 Typical tracings showing the effects of equieffective concentrations of N/OFQ, Ac-RYYRWK-NH_2 and ZP120 in the electrically stimulated mouse vas deferens.

Table 2 pK_B ($CL_{95\%}$) values of J-113397 and naloxone vs N/OFQ, Ac-RYYRWK-NH_2 and ZP120 in the electrically stimulated mouse vas deferens

	J-113397	*Naloxone*
N/OFQ	7.81 (7.56–8.06)	<6
Ac-RYYRWK-NH_2	7.89 (7.65–8.14)	<6
ZP120	7.80 (7.69–7.91)	<6

Data are the mean of at least five separate experiments. $CL_{95\%}$: 95% confidence limits.

than that evoked by high doses (i.e. 10 nmol) of N/OFQ, as reported by several investigators (Calo′ *et al*., 1998; Devine *et al*., 1996; Noble & Roques, 1997; Reinscheid *et al*., 1995). Moreover, four out of 60 animals treated with 1 nmol ZP120 developed convulsions: these animals were excluded from the study. No obvious behavioural effects were recorded after the i.v. injection of ZP120 at doses as high as 10 nmol/mouse.

Results summarized in Figure 3 show that tail-withdrawal reaction time of saline injected mice did not differ from the pre-injection value and was stable at values around 4–5 s over the time course of the experiment. ZP120 at 0.01 nmol produced a slight reduction of tail-withdrawal latencies 15–30 min after i.c.v. injection; however this reduction did not reach the level of significance. ZP120 elicited statistically significant pronociceptive effects both at 0.1 and 1 nmol which peaked at 60–90 min after injection. Few animals (five out of 36) treated with ZP120 1 nmol displayed a profound neurological impairment and did not respond at all to the nociceptive stimulation reaching the cut off time (20 s): these animals were excluded from the study. In some parallel experiments of this series, we tested the effects 1 nmol N/OFQ and confirmed previous findings (Calo′ *et al*., 1998): N/OFQ caused a significant reduction of tail-withdrawal

latencies only in the first 15 min after injection (data not shown).

As shown in Figure 4 left panel, mice treated with saline (2 μl mouse^{-1}, i.c.v.) displayed a progressive reduction in spontaneous locomotor activity during the 30 min of the experiment. N/OFQ at 1 nmol caused a significant reduction of locomotor activity in the first 15 min after i.c.v. administration. These results are in line with previously published findings (Rizzi *et al*., 2001a). ZP120 tested at 1 nmol produced a slight reduction of locomotor activity which, however, lasted for the whole observation period (30 min). On the basis of the results obtained with ZP120 in the tail withdrawal assay where the peptide produced delayed and long lasting effects, we decided to perform a novel series of experiments in which the effects of N/OFQ and ZP120 were evaluated 1 h after i.c.v. injection. As shown in the right panel of Figure 4, 1 h after the i.c.v. injection, N/OFQ

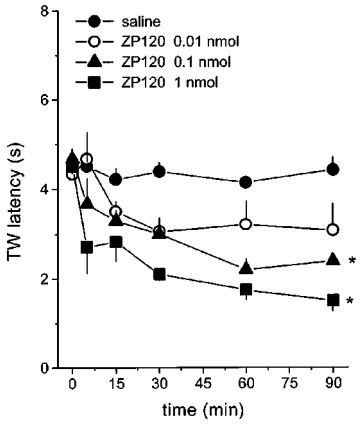

Figure 3 Dose response curve of ZP120 (0.01 – 1 nmol, i.c.v.) evaluated in the tail-withdrawal assay in mice. Data are mean ± s.e.mean of at least six separate experiments. Raw data were converted to the area under the time x withdrawal latency curve and the area under the curve data were used for statistical analysis. *$P<0.05$ *vs* saline, according to ANOVA followed by the Dunnett's test.

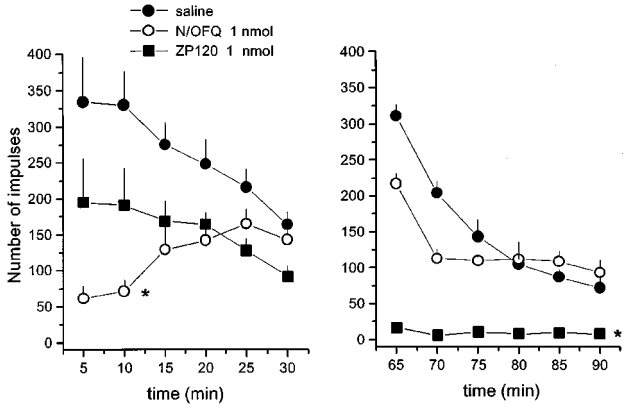

Figure 4 Effects of N/OFQ and ZP120 on spontaneous locomotor activity in mice. Locomotor activity was assessed 5 (left panel) or 65 (right panel) min after the i.c.v. injection of the peptides. Data are mean ± s.e.mean of at least five separate experiments. Cumulative impulses over the 30 min observation period were used for statistical analysis. *$P<0.05$ *vs* saline, according to ANOVA followed by the Dunnett's test.

treated animals displayed a motor behaviour similar to that of saline injected mice; on the contrary, locomotor activity was virtually suppressed in animals treated with ZP120.

In separate series of experiments, the actions of ZP120 given i.v. at the dose of 10 nmol mouse^{-1} were evaluated in the tail withdrawal and, 1 h after the injection, in locomotor activity assay. As shown in Figure 5, the i.v. administration of ZP120 did not produce any significant modification of the behaviour of the mice in both the assays.

Discussion

The findings of the present study demonstrated that ZP120, designed by applying the SIP technology to the selective NOP partial agonist Ac-RYYRWK-NH_2 (Dooley *et al*., 1997; Thomsen *et al*., 2000), is a novel highly potent and selective ligand for the NOP receptor; this peptide behaved as a partial agonist at NOP receptors but produced *in vivo* long lasting effects compared with the natural ligand N/OFQ.

In the mouse vas deferens ZP120 mimicked the inhibitory effects of N/OFQ and Ac-RYYRWK-NH_2 showing, however, about 10 fold higher potency. The maximal effects induced by ZP120 were similar to those of Ac-RYYRWK-NH_2 but significantly lower than those elicited by the natural peptide. Moreover, the actions of the novel peptide are solely due to the activation of the NOP receptor since they are resistant to naloxone while antagonized by the selective NOP receptor antagonist J-113397 (Ozaki *et al*., 2000) that displayed similar pK_B values (range 7.80 – 7.89) when tested against N/OFQ, Ac-RYYRWK-NH_2 or ZP120. In *in vivo* assays, ZP120 behaved as a highly potent agonist eliciting statistically significant pronociceptive effects at the dose of 0.1 nmol, while 10 fold higher doses were required with N/OFQ (Calo′ *et al*., 1998; Rizzi *et al*., 2001a) or Ac-RYYRWK-NH_2 (A. Rizzi, unpublished observation). In addition, the *in vivo* effects of ZP120 were found to be long lasting, since both the pronociceptive and motor depressant actions were present 90 min after i.c.v. injection while those of the natural ligand (at 1 nmol) lasted for no more than 15 – 20 min (Calo′ *et al*., 1998; Rizzi *et al*., 2001a). Therefore, ZP120 should be considered as a successful example of the application of SIP technology to a peptide ligand (Ac-

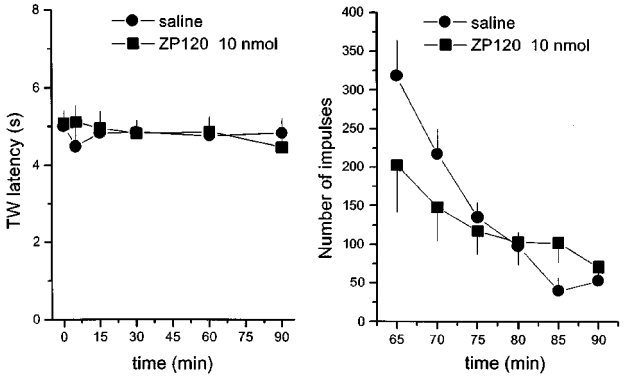

Figure 5 Lack of effect of 10 nmol ZP120 given i.v. in the tail-withdrawal assay (left panel) and in the locomotor activity assay (right panel) in mice. Data are mean ± s.e.mean of three separate experiments.

RYYRWK-NH_2) for increasing its potency and duration of action without modifying its pharmacological activity (partial agonism) and profile of action (NOP selectivity). Data obtained by testing the peptide half-life in human and mouse plasma corroborate this interpretation: in fact, ZP120 displayed approximately 20 and 50 fold higher half-life than Ac-RYYRWK-NH_2, when incubated in human and mouse plasma, respectively (Kapusta *et al*., 2002b).

It is worthy to mention that similar results (increased agonist potency and duration of action) have been obtained by introducing Arg and Lys in position 14 and 15 in the sequence of the natural ligand N/OFQ and thus generating [Arg^{14},Lys^{15}]N/OFQ (Okada *et al*., 2000). This NOP ligand behaves as a potent and selective full agonist at human recombinant receptors (Okada *et al*., 2000) as well as at native receptors of the rat, guinea-pig and mouse (Rizzi *et al*., 2002). In addition, similar to ZP120, [Arg^{14},Lys^{15}]N/OFQ displayed high potency and long duration of action *in vivo* in mice (Rizzi *et al*., 2002). These findings are in line with the idea that positively charged residues added at the C-terminal of NOP peptide ligands increase their potency and duration of action.

On the other hand, some characteristics of ZP120 (slow onset of action both *in vitro* and *in vivo*, delayed peak effects, non reversible effects after washing *in vitro*) could not be interpreted exclusively in terms of higher metabolic stability; they rather suggest that other mechanisms, for instance stronger binding to the receptor, might be involved. For investigating this possibility, ZP120 should be tritiated and used as a radioligand for comparing its association and dissociation constants with those of the already available NOP ligands [^{3}H]-N/OFQ and [^{3}H]-Ac-RYYRWK-NH_2 (Thomsen *et al*., 2000). Independently from the molecular mechanism involved, the slow onset of action both *in vitro* and *in vivo*, delayed peak effects, non-reversible effects after washing *in vitro* of ZP120 are quite unique among NOP peptide ligands whose effects take place almost immediately and are rapidly reversible (see for a review Calo′ *et al*., 2000a). The only example of a NOP ligand producing similar kind of effects is represented by the non-peptide agonist Ro 64-6198 (Dautzenberg *et al*., 2001; Jenck *et al*., 2000). In fact, the kinetic of action of this compound in electrically stimulated tissues (including the mouse vas deferens) is very similar to that of ZP120 (present data and Rizzi *et al*., 2001b). It is therefore suggested that ZP120 and Ro 64-6198 bind to the NOP receptor in a different way compared with N/OFQ, N/OFQ derivatives and Ac-RYYRWK-NH_2. Further studies are, however, required for validating this hypothesis.

N/OFQ is able to elicit a number of effects in the central nervous system, among which supraspinal pronociceptive and spinal antinociceptive actions are the best described (Mogil & Pasternak, 2001). The present study demonstrated that ZP120 is able to produce both pronociceptive and motor depressant effects when given i.c.v. While maximal effects was obtained at 1 nmol i.c.v., even a 10 fold higher i.v. dose did not produce any changes in pain threshold or locomotion. This demonstrated that ZP120 has a poor penetration into the central nervous system after i.v. administration. In line with these results, recent studies in the rat demonstrated that i.v. infusion of ZP120 mimicked the renal effects of N/OFQ, producing selective free-water diuresis without modifying any central function (food intake, locomotor activity) (Kapusta *et al*., 2002b).

In conclusion, this paper demonstrated that ZP120 is a potent and selective NOP partial agonist whose *in vivo* effects are long lasting and, after i.v. administration, confined to the periphery. This pharmacological profile makes ZP120 an interesting drug candidate, especially for those indications (i.e. aquaresis, Kapusta, 2000), for which NOP partial agonists that produce renal but not cardiovascular effects are more selective than full agonists which are known to elicit both renal and cardiovascular actions (Kapusta *et al*., 2002a).

We would like to thank Prof S. Salvadori and R. Guerrini of the Department of Pharmaceutical Sciences, University of Ferrara, for the kind gift of N/OFQ and Ac-RYYRWK-NH_2, and Prof P. Pollini and C. De Risi of the Department of Pharmaceutical Sciences, University of Ferrara, for that of J-113397. This work was financially supported by grants from the University of Ferrara and from Zealand Pharma.

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(Received April 10, 2002
Revised May 31, 2002
Accepted July 26, 2002)