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Spinal antinociceptive effects of AA501, a novel chimeric peptide with opioid receptor agonist and tachykinin receptor antagonist moieties

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Abstract

The use of "multimodal" combination analgesic therapies or novel single molecules possessing multiple analgesic targets is becoming increasingly attractive. In previous experiments we showed that a substance P antagonist injected intrathecally potentiated the antinociceptive effects of potent opioid receptor agonist, biphalin. Based on examination of the biphalin structure–activity relationship, we designed and synthesized a novel chimeric peptide, termed AA501 (*N′*(Tyr-D-Ala-Gly-Phe), *N″*(Z-Trp) hydrazide, Z = benzyloxycarbonyl). AA501 consists of an opioid receptor agonist pharmacophore related to biphalin and a substance P receptor antagonist pharmacophore, both linked by a hydrazide bridge. The present study evaluates the ability of a novel chimeric peptide, AA501, to bind to opioid and substance P receptors and to produce antinociception in tail-flick and formalin tests, and in a neuropathic pain model when administered intrathecally to rats.

Keywords: Novel chimeric peptide; Opioids; Substance P; Antinociception; Neuropathic pain; (Rat)

1. Introduction

The use of "multimodal" combination analgesic therapies (Walker et al., 2002) or therapies with novel single molecules possessing multiple analgesic targets is becoming increasingly attractive. Biphalin, a novel opioid tetrapeptide dimer (Tyr-D-Ala-Gly-Phe-NH-)$_2$, binds to multiple opioid receptors with a strong affinity for μ- and δ-opioid receptors and weaker, but still significant, affinity for the κ-opioid receptor. When biphalin is administered intrathecally to rats, it produces potent and long-lasting analgesia (Silbert et al., 1991). In addition, we have previously demonstrated that biphalin, given intrathecally (i.t.) together with a peptide substance P receptor antagonist ([D-Pro^2, D-Trp^{7,9}]substance P) produced more potent spinal antinociceptive effects in rats than biphalin given alone (Misterek et al., 1994). The potentiation of the antinociceptive effect of biphalin by substance P receptor antagonist was not dose-dependent in the range tested. However, an ability to test the effect of the substance P receptor antagonist at higher doses was limited by severe side effects and neurotoxicity.

Many physiological and pharmacological studies have indicated that substance P and opioids systems are functionally antagonistic in the mediation of nociception and antinociception in vivo (Maszczynska et al., 1998). In clinical practice it is simpler and hence more practical to inject a single drug than to co-administer multiple drugs. Thus, simultaneous activation of opioid receptors and inhibition of substance P by a single chimeric peptide should result in potentiation of opioid analgesic effects. We therefore sought to design a molecule with both opioid receptor agonist and tachykinin receptor antagonist properties. Theoretically, such a novel chimeric peptide should have a broad spectrum of high affinity to opioid receptors and lower but significant affinity to tachykinin-1 (NK_1) receptors, the latter with antagonistic properties to prevent

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compensatory hyperactivation of the substance P system (Hu et al., 1997). The structure–activity relationship study of biphalin showed that to achieve full antinociceptive activity only one of its "arms" is needed (Lipkowski et al., 1999). This finding implies first, that the sequence Tyr-D-Ala-Gly in one "arm" is essential for analgesia, and second, that during peptide-receptor binding the opposite arm is without any influence on the receptor-ligand complex (Lipkowski et al., 1999). Based upon the latter conclusion, the second arm might be replaced by a different pharmacophore with similar size and hydrophobity, and the chimeric peptide would still be analgesic. The structure–activity relationship study of ligands for tachykinin receptors led to development of a group of chemical derivatives of tryptophan with an affinity to substance P receptors and antagonistic properties (Boyle et al., 1994). Extension of these studies in our laboratory led to the conclusion that a corresponding diacylohydrazide should also possess affinity for the tachykinin NK_1 receptor. Hybridization of the fragment of biphalin and Trp-diacylohydrazide fragment resulted in a chimeric peptide, AA501 (*N′*(Tyr-D-Ala-Gly-Phe), *N″*(Z-Trp) hydrazide, Z = benzyloxycarbonyl), whose size is approximately that of biphalin. The respective molecular weights of these two agents are 439 and 321. The structure of AA501 is shown on Fig. 1.

To prove that AA501 binds to opioid and substance P (tachykinin) receptors, a series of receptor binding assays were conducted (see below). For the evaluation of antinociceptive properties of the AA501 we used an acute pain model (tail-flick test) and two chronic pain models (the formalin test and neuropathic pain model).

The formalin test provides a more valid model for clinical chronic pain and hyperalgesia than other models. Injection of formalin under the skin on the plantar surface of the hind paw causes an immediate and intense increase in the spontaneous activity of C fiber afferents (Wheeler-Aceto et al., 1990) and evokes quantifiable pain behavior: flinching/shaking and licking/biting of the injected paw (Wheeler-Aceto et al., 1990). The behavioral response to subcutaneous injection of formalin is biphasic: an initial acute phase starting immediately after injection and lasting for 5–10 min, followed by a prolonged tonic phase (15–60 min). It is suggested that the early phase is due to direct stimulation of nociceptors by formalin, whereas the late phase involves inflammation and central nervous system sensitization induced by neural activity generated during the first phase (Dallel et al., 1995). The windup and hyperalgesic component of the second phase of the formalin test are predominantly mediated by substance P receptors of tachykinin NK_1 type and glutamate receptors of the *N*-methyl-D-aspartate (NMDA) type (Coderre and Yashpal, 1994; Chaplan et al., 1997). Two principally different modalities of stimulation are active during the first and second phases of the formalin test, so the analgesic effects of a drug can be assessed separately in the two phases of this test. By virtue of its diversity of mechanisms, the formalin test offers a significant advantage over other pain models in which only one mechanism is evident.

Neuropathic pain caused by peripheral nerve injury is often characterized by combinations of spontaneous burning pain, sensory loss, hyperalgesia and allodynia. It is considered essential to begin treatment early on, although different components of neuropathic pain reflect various mechanisms and may require diverse treatments (Woolf et al., 1998).

A number of animal models have been developed to replicate some of the symptoms of human neuropathic pain conditions, to study mechanisms of their development and to test various agents (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992). The effectiveness of opioid analgesics in the treatment of neuropathic pain is controversial. Inconsistencies exist among results from numerous studies employing animal models to assess the antinociceptive effects of morphine in neuropathic pain. Systemically administered morphine has been reported to reverse both allodynia and hyperalgesia (Kayser et al., 1995; Koch et al., 1996), yet also to be relatively ineffective against thermal hyperalgesia in the chronic constriction model of nerve injury (Mao et al., 1995). In another study of spinal L5/L6 ligation in the rat, morphine administered via systemic and supraspinal routes was effective in attenuating allodynia, but spinal morphine had little effect (Lee et al., 1995). In

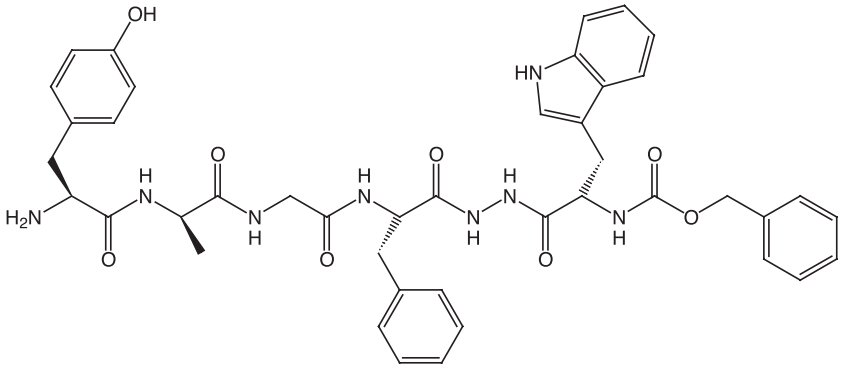

Fig. 1. Chemical structure of AA501.

contrast, still other investigators found that intrathecal morphine had antinociceptive and antihyperalgesic effects, but did not decrease allodynia in rats with spinal nerve ligation (Wegert et al., 1997). Thus, the apparent efficacy of morphine in attenuating allodynia and hyperalgesia in preclinical models is dependent on the specific model, the timing of assessment of opioid responsivity, and also on the route of administration of the drug. One possible explanation for inconsistencies in published studies is that peripheral nerve injury produces downregulation of opioid receptors localized presynaptically on the primary afferents and postsynaptically on dorsal horn neurons (Besse et al., 1990; Woolf and Mannion, 1999). Preclinical studies by Suzuki et al. (1999), however, suggest that early and aggressive opioid intervention can be effective.

Peripheral nerve injury often induces A fiber sprouting into the lamina II of the dorsal horn of the spinal cord. It is suggested that Aβ fiber sprouting is dependent on injury to the peripheral axons of C fibers and apoptosis with atrophy of their central connections (Mannion et al., 1996). The functional importance of Aβ fiber sprouting is that lamina II normally receives only nociceptive information from C fibers, and begins to receive information about non-noxious stimuli which can be misinterpreted as noxious. Substance P and other peptides involved in pain transmission (calcitonin gene-related peptide, CGRP) are normally expressed by primary nociceptive afferent C fibers and Aβ fibers. After peripheral nerve injury, the expression of these peptides on these fibers is downregulated (Woolf and Mannion, 1999). Phenotypic changes have also been observed, in which substance P and CGRP appear in the large myelinated Aβ fibers (Miki et al., 1998). Thus, low amplitude stimuli activate the Aβ fibers, and cause release of substance P in the dorsal horn, thereby generating responses that normally occur only after nociceptive stimulation (Woolf and Mannion, 1999).

2. Material and methods

2.1. Intrathecal catheterization

Experiments were carried out according to a protocol approved by the Tufts University Animal Research Committee. Adult male Sprague–Dawley rats (225–250 g) were housed in groups of three in cages and maintained on a 12 h light/12 h dark cycle. Animals had free access to food and water at all times. For spinal drug administration, rats were implanted with chronic indwelling intrathecal (i.t.) catheters according to a modification of the method described by Yaksh and Rudy (1976). Briefly, through an incision in the atlanto-occipital membrane, silastic tubing (i.d. = 0.30 mm, o.d. = 0.64 mm, length 11 cm) was inserted for a distance of 8.5 cm, thereby positioning the tip at the T_{13}-L_1 spinal level. To facilitate threading the catheter through the intrathecal space, a stylet was inserted into the silastic tubing. After placement, the stylet was removed and the catheter was secured by sutures. After surgery, animals were housed individually. Rats were given 5–7 days to recover from surgery, during which time they were habituated daily to the laboratory environment, testing chambers for formalin test, and analgesic testing apparatus. Each experimental group consisted of six rats.

2.2. Tail-flick assay

For measurement of thermal antinociception, a tail-flick apparatus was utilized. Light intensity was adjusted to yield a mean baseline latency of approximately 3 s with automatic cutoff at 10 s to avoid tail damage. Analgesic measurements were performed before drug administration and 5, 10, 15, 30, 45, 60, 75, 90, and 120 min after drug or vehicle solution (control group) administration. Antinociceptive responses were expressed as % maximum possible effect (%MPE) and calculated according to the following formula:

$$\%\mathrm{MPE} = \frac{\text{post-treatment latency} - \text{baseline latency}}{\text{cut off time} - \text{baseline latency}} \times 100$$

2.3. Formalin test

Formalin solution was prepared from 37% formaldehyde stock solution which was further diluted to 5% using 0.9% saline. Immediately prior to formalin injection rats were anesthetized with 3% halothane (v/v). When there was a loss of spontaneous movement but preservation of deep spontaneous respiration, 50 μl of 5% formalin was injected subcutaneously into the plantar surface of the right hind paw with a 27-gauge needle connected to a Hamilton microsyringe. Immediately after injection, rats were placed for observation in an open Plexiglas chamber with a mirror placed underneath and positioned at a 45° angle to the floor to allow for clear observation for the formalin injected paw. Pain behavior was quantified by counting the incidence of spontaneous flinches/shakes of the injected paw. Flinches were counted for 1 min periods at 1–2, 3–4 and 5–6 min, and at 5 min intervals during the interval from 10 to 65 min after formalin injection. AA501 was administered intrathecally 15 min before formalin injection (pretreatment study) or 9 min after formalin injection (posttreatment study). A control group of rats injected with formalin received only the vehicle (saline with dimethyl sulfoxide (DMSO) but no AA501). All intrathecal injections were made in the same volume (10 μl followed by 10 μl of saline for flushing the dead volume of the catheter).

2.4. Neuropathic pain model

Unilateral nerve injury was produced according to the method of Kim and Chung (1992). The surgical procedure was performed on all animals on the left side, and no

surgical intervention was performed on the right side. Surgical procedures were performed under light halothane anesthesia. The left paraspinal muscles were separated from the spinous processes at the L_4-S_2 levels. The L_6 transverse process was carefully removed with a small rongeur to identify visually the L_4-L_6 spinal nerves. The left L_5 and L_6 spinal nerves were isolated and tightly ligated with 3–0 silk thread. After all bleeding was controlled, the wound was sutured.

Injections of peptide AA501 (1 μg) and/or saline were given in a blinded fashion i.t. 30 min prior to ligation and 6 h later.

Animals were allocated into four treatment groups. Rats received: (1) saline before and after surgery i.t. (saline/saline group); (2) AA501 before and saline after surgery i.t. (AA501/saline group); (3) saline before and AA501 after surgery i.t. (saline/AA501 group); or (4) AA501 before and AA501 after surgery i.t. (AA501/AA501 group). To quantify mechanical sensitivity of the hind paws, the threshold for paw withdrawal in response to normally innocuous mechanical stimuli was measured. Mechanical sensitivity of the hind paws was assessed in all experimental animals before surgery (baseline, day 0) and once daily for the first 10 postoperative days. Mechanical thresholds were measured by using von Frey filament (“Electronic von Frey” anesthesiometer, model 1601, IITC-Life Science Instruments, Woodland Hills, CA). The Electronic von Frey anesthesiometer is a sensitive force transducer and recorder with one, fixed diameter probe. The probe was applied to the paw 10 times and the recordings were averaged.

Data were expressed as mean ± standard error of the mean (S.E.M.) for each measurement time, and as mean ± standard error (S.E.M.) of the area under the curve (AUC) of response versus time. Differences over time were analyzed using two-way non-parametric analysis of variance (ANOVA) and one-way ANOVA for comparison between groups at each time point. The paired *t*-test was used to compare the AUCs between each treatment group. Significance was defined as $P < 0.05$.

2.5. Receptor binding assay

The affinity of AA501 for the mu and tachykinin NK_1 receptors was determined using rat membrane preparations according to protocols of Zadina and Kastin (1984) and Charlton and Helke (1985) with some modifications. To prepare μ-opioid and substance P receptors, fresh frozen rat brains were homogenized in 40 volumes of standard buffer which consisted of 50 mM Tris HCl (pH 7.4), 0.2 mg/ml bovine serum albumin (BSA), 2.5 mM ethylenediaminetetraacetic acid (EDTA), 40 μg/ml bacitracin, 30 μg/ml bestatin and 5 mM $MgCl_2$ (5 mM $MnCl_2$ for the substance P receptor binding protocol). Next, brain homogenates were centrifuged at $15{,}000 \times g$ for 20 min. In order to remove endogenous ligands, 100 mM NaCl (μ-opioid receptor preparation only) was added to the standard buffer, and centrifugation was repeated. After another wash with standard buffer, the membrane preparation was resuspended in a final 10 volumes of incubation buffer, which consists of the standard buffer with 4 μg/ml leupeptin and 2 μg/ml chymostatin. Binding assays for μ-opioid and substance P receptors were performed at 4 °C for 90 min and at room temperature for 75 min, respectively. Each assay for opioid receptor binding was done in triplicate and contained incubation buffer, brain membrane (5 mg), 1.85 nM [^{3}H](D-Ala^2,N-Me-Phe^4,Gly^5-ol)enkephalin (DAMGO) (Dupont, New England Nuclear, Boston, MA) and increasing concentrations of AA501 (1, 10, 100, 500 nM, 1, 10 μM). Nonspecific binding was determined by co-incubation with 10 μM DAMGO. The triplicate reactions for substance P receptor binding contained incubation buffer, brain membrane (5 mg), 23 fmol of [^{125}I]substance P (Bolton-Hunter reagent, DuPont, New England Nuclear, Boston, MA) and increasing concentrations of AA501 (10, 100 nM, 1, 5, 10 μM). Nonspecific binding was determined by co-incubation with 10 μM substance P. After incubation, the samples were filtered and washed (for tachykinin NK_1 receptors with 50 mM Tris HCl (pH 7.4) and 3 mM $MnCl_2$) on a Brandell-Harvester apparatus using GF/B filters pre-soaked in 50 mM TrisHCl (pH 7.4) and 0.05% polyethyleneimine (PEI). Following filtration and wash, a gamma-counter was used to measure the radioactivity of each sample. All binding data were analyzed and presented as a % of binding versus log of used concentrations of standard and AA501. K_i was calculated for DAMGO, substance P and AA501.

3. Results

3.1. Binding affinities of AA501 for μ and tachykinin NK_1 receptors

The hypothesized ability of AA501 to interact with both opioid and substance P receptors was confirmed by in vitro binding assays using rat brain membranes preparations. The K_i value for AA501 for μ-opioid receptors measured in the presence of [^{3}H]DAMGO is 80 nM. AA501 displayed a K_i of 5 μM for inhibition of binding of radiolabeled substance P to the tachykinin NK_1 receptors (data not shown). The in vivo analgesic testing in rats followed completion of the in vitro binding assays.

3.2. Intrathecal administration of AA501

Intrathecal (i.t.) administration of AA501 (range 0.25–25 μg) produced dose-dependent antinociception as measured by the tail-flick assay. The time course of antinociceptive responses to lower doses of AA501 (0.25 and 0.5 μg) peaked at approximately 40% MPE at 15–30 min and declined to 20–30% MPE at 60 min. Intermediate doses (1 and 4 μg) produced antinociception that reached 60–80%

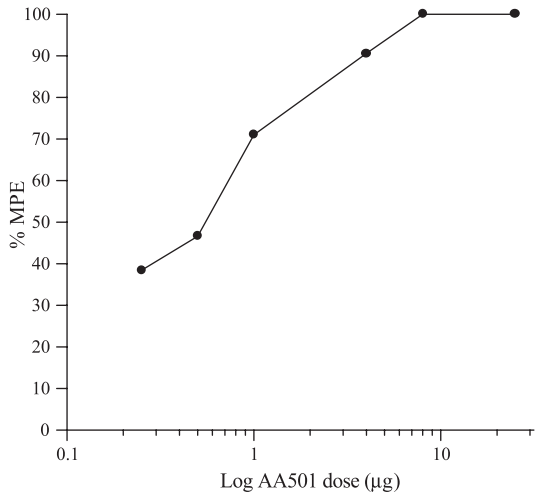

Fig. 2. Dose–response curve for antinociceptive effect of AA501 administered intrathecally to rats as measured in the tail-flick test 15 min after peptide injection. Each group consisted of six rats.

MPE at 5–45 min. Higher doses (8 and 25 µg) produced a maximal antinociceptive response (100% MPE) of long duration (30–75 min) (Fig. 2).

The antinociceptive action of AA501 (1 µg i.t.) was reversible by naloxone (10 µg i.t.) and diminished by substance P (150 ng) (Fig. 3). Naloxone administered alone in dose of 10 µg i.t. had no analgesic effect (data not shown).

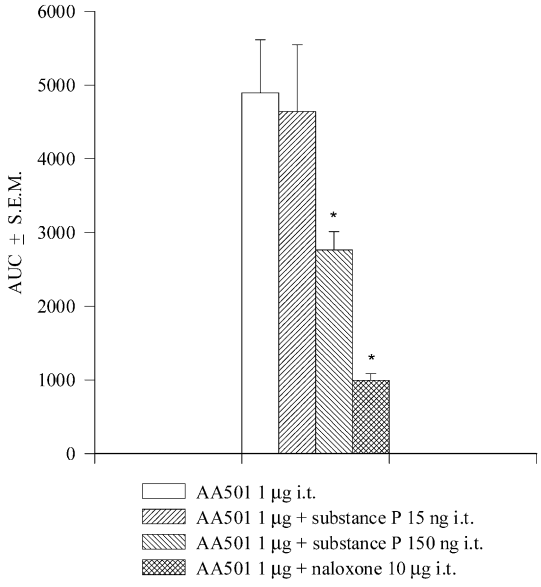

Fig. 3. The area under the curve (AUC) of % MPE versus time for AA501 injected at a dose of 1 µg, AA501 preceded 10 min earlier with 10 µg of naloxone, and AA501 co-administered with substance P at doses of 15 and 150 ng. All drugs given intrathecally. There is a statistically significant difference (*$P<0.05$) between group injected with AA501 and group co-administered with substance P (150 ng) and group pretreated with naloxone.

The development of tolerance to repeated daily doses of i.t. AA501 (1 µg per daily dose) was relatively slow. On day 5 the antinociceptive response was only 50% lower than the response on day 1 ($t_{1/2}=5$ days). In the animals receiving the AA501 together with a low dose of substance P (15 ng), the antinociceptive response on day 5 was approximately 65% lower than on day 1. There was slower development of tolerance in rats injected with AA501 alone than in those receiving both AA501 and substance P (Fig. 4).

3.3. Inhibition of behavioral response to formalin by AA501

Injection of formalin (5%, 50 µl) into the rat hind paw caused biphasic paw-flinching behavior with the maximum response at 1 and 40 min for the first and second phases, respectively. AA501 administered intrathecally 15 min before and 9 min after formalin injection into the rat hind paw blocked the biphasic response to formalin (Fig. 5). Comparison of AUC values of effects of AA501 administered before or after formalin injection revealed that AA501 administered 15 min before formalin injection produced significantly greater antinociceptive effect (Fig. 6). Naloxone (10 µg) administered intrathecally 10 min before injection of AA501 reversed the analgesic effects of the peptide. Naloxone injected alone did not have any significant effect in this test (data not shown).

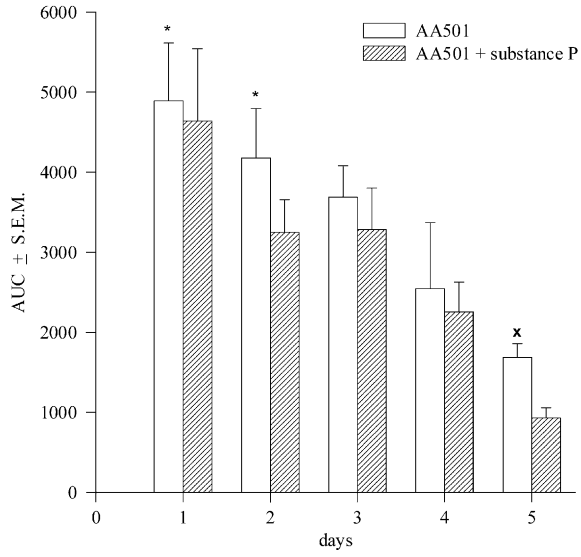

Fig. 4. Development of tolerance to 1 µg AA501 and 1 µg AA501 + 15 ng substance P over 5 days. Both drugs given intrathecally. There is a statistically significant difference (*$P<0.05$) in antinociceptive effect in the group injected with AA501 between day 1 and days 4 and 5, and also between day 2 and day 5. There is also a significant difference ($^{x}P<0.05$) between antinociceptive effect observed in group injected with AA501 and group injected with AA501 + substance P on day 5. Statistically significant ($P<0.05$) decrease of AUC values occurs in group AA501 + substance P on day 5 as compare with days 1, 2 and 3, and on day 4 as compare with value on day 1.

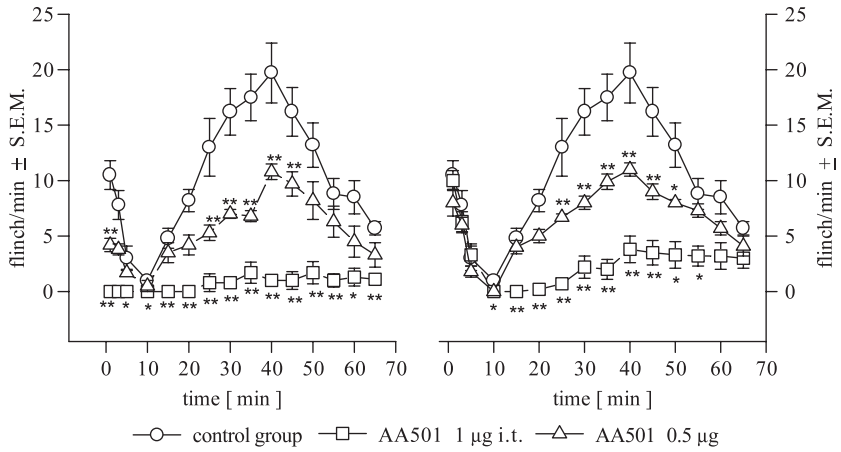

Fig. 5. Antinociceptive effects of AA501 injected at doses of 0.5 and 1 µg in the formalin test. AA501 was administered before phase 1 response (15 min before formalin injection, left panel) and after phase 1 but before phase 2 response (9 min after formalin injection, right panel). All experimental groups consisted of six rats. *$P<0.05$ and **$P<0.01$ group injected with AA501 compared to control group.

3.4. Effects of AA501 in the neuropathic pain model

Preoperative (baseline) testing indicated that rats were insensitive to mechanical stimuli applied with von Frey filament to the hind paws. Rats often did not react to probing and it was possible for investigator to elevate the rat's paw with the von Frey probe. Following nerve ligation, the left hind paw became markedly sensitive to mechanical stimulation in all experimental groups. Mechanically stimulated rats often lifted their hind paw, licked it and held it in the air for many seconds. We observed that the affected hind paw was everted and often rats remained in a postures as to guard the paw. There was a statistically significant decrease in mechanical threshold obtained by electronic von Frey filament on postoperative days 1–10 as compared to baseline values on the left (ligated) side in all four experimental groups. There was a statistically significant elevation of mechanical allodynia threshold in group AA501/AA501 compared to the control group (saline/saline) on postoperative days 1 through 6, and 8 and 9. There was also a significant difference on day 1 between group AA501/saline and the control saline/saline group (Fig. 7). Analysis of AUC values showed a statistically significant decrease in paw withdrawal threshold on the left (ligated) side com-

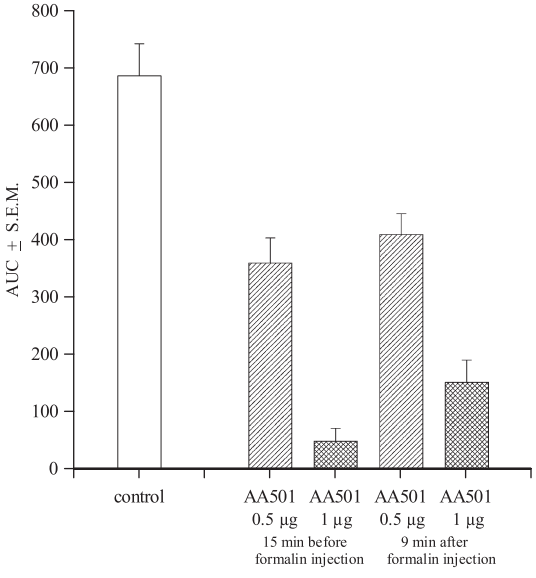

Fig. 6. The area under the curve (AUC) of number flinches/min versus observation time for the control group and groups treated intrathecally with AA501 doses of 0.5 and 1 µg administered 15 min before and 9 min after formalin injection.

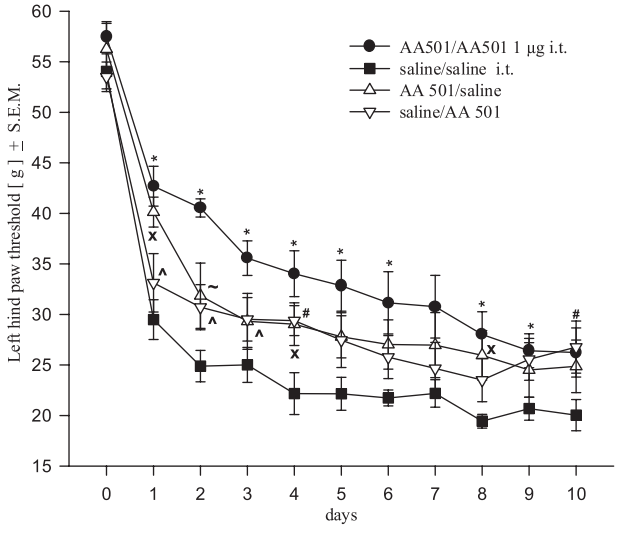

Fig. 7. Changes in the pain threshold on the left side after mechanical stimulation (electronic von Frey filament) estimated before surgery (control) and daily on postoperative days 1–10 after ligation of L_5 and L_6 nerves on the left side. Groups are labeled as to the treatment given 30 min before and 6 h after the surgery (before/after). There were four groups of rats ($n=6$/group): control group (saline/saline); saline/AA501; AA501/saline; and AA501/AA501. AA501 (1 µg) was injected i.t. *$P<0.05$ AA501/AA501 versus saline/saline; $^{x}P<0.05$ AA501/saline versus saline/saline; $^{\#}P<0.05$ saline/AA501 versus saline/saline; ^$P<0.05$ saline/AA501 versus AA501/AA501; $^{\sim}P<0.05$ saline/AA501 versus AA501/AA501.

pared to the control (right) side. Responses on the right side were unaffected by the nerve ligation on the left side (data not shown).

4. Discussion

Accumulated evidence suggests that tachykinin receptors may play an opposite role to μ-opioid receptors in the modulation of pain transmission (Maszczynska et al., 1998). We have previously shown that a hybrid peptide containing β-casomorphin-like and substance P-like structural characteristics possessed an antinociceptive effect in the mouse hot plate test after intrathecal administration (Lipkowski et al., 1994).

AA501 was designed to concurrently activate opioid receptors and block tachykinin NK_1 receptors. The binding assay results indicate that AA501 has high affinity for μ-opioid receptors attributable to the portion of AA501 taken from the peptide dimer, biphalin. The second part of the chimeric peptide AA501, designed as a tachykinin receptor antagonist, displayed weak affinity for substance P receptors.

The antinociceptive effects of AA501 in the tail-flick test after intrathecal administration are dose-dependent and result from the co-activation of opioid receptors and the inhibition of the substance P receptors in the dorsal horn of the rat spinal cord. Activation of presynaptic opioid receptors inhibits release of substance P from the primary afferent neurons, and activation of the postsynaptic opioid receptors decreases the responsivity of the postsynaptic cells to nociceptive neurotransmitters. Additionally, the tachykinin receptor antagonist portion of AA501 may block the release of substance P from primary afferent neurons and the binding of substance P to the postsynaptic tachykinin NK_1 receptors. The weak affinity of the AA501 for tachykinin NK_1 receptors may also prevent compensatory hyperactivation of substance P autoreceptors localized on the presynaptic membrane (Hu et al., 1997).

The antinociceptive effects of AA501 were reversible in a dose-dependent fashion by naloxone administered 10 min prior to injection of AA501 in the tail-flick test. This is consistent with observations of others (Matsumura et al., 1985; Vaught, 1988) that substance P receptor antagonists possess transient and weak, or no antinociceptive activity when given alone. Substance P in the tail-flick test diminished the analgesic effects of AA501 in a dose-dependent manner, and isolated the analgesic effects produced by the opioid receptor agonist portion of AA501.

Repeated administration of AA501 for 5 days caused the development of tolerance to its analgesic activity. The antinociceptive activity observed on the fifth day of administration was approximately 50% of that on day 1. Co-administration of AA501 with substance P led to a more rapid development of tolerance. Our results seem to indicate that co-release of anti-opioid peptides such as substance P during prolonged nociceptive stimulation may contribute to the development of opioid tolerance (South and Smith, 2001). Many hypotheses have been proposed to explain tolerance to the analgesic effects of opioids. After neutralization of the tachykinin receptor antagonist portion of AA501 by co-administration of substance P, tolerance to the analgesic action of AA501 developed more rapidly, and in a similar time course as is known to take place after repeated administration of "pure" μ-opioid receptor agonists (Ibuki et al., 1997; Ness and Follett, 1998).

We also tested the antinociceptive activity of AA501 in the formalin test. Formalin injected subcutaneously into the rat paw results in an immediate and intense increase in the spontaneous activity of C fiber afferents (first phase of response), followed by the second phase which reflects inflammation and central sensitization (Malmberg and Yaksh, 1992; Hong and Abbott, 1995). These two phases of response to formalin are differentially sensitive to various analgesic agents (Cowan et al., 1989). Intrathecal opioids administered before phase 1 completely suppress, in a dose dependent manner, the first and second phase responses. Injection of opioids after phase 1 but before phase 2, also blocks the phase 2 response (Malmberg and Yaksh, 1992). Administration of a substance P receptor antagonist after phase 1 but before phase 2, was observed to have no effect (Yamamoto and Yaksh, 1991). The antinociceptive effects of AA501 in the formalin test were dose-dependent and reversible by naloxone. We tested AA501 by injecting it before phase 1 (15 min before formalin injection) and after phase 1 but before phase 2 (9 min after formalin injection). We hypothesized that because of the unique combined properties of opioid receptor agonist and tachykinin receptor antagonist, AA501 should have better analgesic activity when administered prior to formalin injection. Indeed, analysis of AUC values calculated from the number of flinches/minute versus time, revealed that AA501 did produce stronger antinociceptive effects when administered prior to formalin injection. The analgesic effects of AA501 administered 9 min after formalin injection reflected only the effects produced by the opioid receptor agonist portion of AA501, the substance P receptor antagonist part of peptide remained without effect.

There is a need for effective drugs to prevent and treat painful neuropathies. The multiple analgesic target approach is attractive, especially in neuropathic pain states that are developed and sustained through complex mechanisms. The results from tail-flick and formalin tests encouraged us to test AA501 in a neuropathic pain model. We investigated only the potential neuroprotective effects of AA501 injected 30 min before and/or 6 h after the spinal nerve ligation, i.e. during the initial phases of development of allodynia and hyperalgesia. Unfortunately, in our initial experiments, AA501 injected at a dose of 1 μg i.t. failed to prevent development of hyperalgesia and mechanical allodynia in rats with spinal nerve ligation. However, statistically significant differences were evident in mechanical hyperalgesia between rats treated with AA501 before and after surgery

versus saline controls. Also, administration of AA501 before nerve ligation surgery was better able to block development of mechanical allodynia than the same dose given after surgery. We concluded that higher doses and/or more frequent AA501 administration may be required to avert the development of neuropathic pain. There also remains the unanswered question of whether AA501 repeatedly administered at higher doses can attenuate or reverse allodynia and hyperalgesia in pre-existing neuropathies. These potential effects of AA501 await further testing in neuropathic pain models.

Pain is a complex phenomenon involving a variety of receptors and neurotransmitters so pain management should be directed at more than one target (Besson, 1999). Prime "candidates" for combined pain therapy may be opioids and substance P because of their important roles in pain transmission (Maszczynska et al., 1998). Our chimeric peptide, AA501, comprising an opioid receptor agonist portion derived from biphalin, and a tachykinin receptor antagonist derivative of tryptophan, has strong affinity for μ-opioid receptors and weaker affinity for substance P receptors. Behavioral tests showed that AA501 possesses antinociceptive activity which depends on the injected dose, and also on the relative activation of receptors targeted by each component of AA501. AA501 and other multireceptor agents could find a prominent place in pain treatment and management, and/or as useful tools for pain research.

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