

## Accepted Manuscript

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PII: S0928-0987(16)30263-9  
DOI: DOI: [10.1016/j.ejps.2016.07.009](https://doi.org/10.1016/j.ejps.2016.07.009)  
REFERENCE: PHASCI 3632

TO APPEAR IN:

RECEIVED 29 APRIL 2016  
DATE:  
REVISED DATE: 5 JULY 2016  
ACCEPTED 10 JULY 2016  
DATE:

Please cite this article as: Kowalczyk, Agnieszka, Kleczkowska, Patrycja, Rekawek, Monika, Kulik, Kamila, Lesniak, Anna, Erdei, Anna, Borics, Attila, Martin, Charlotte, Pawlik, Karolina, Lipkowski, Andrzej W., Benyhe, Sándor, Makulska-Nowak, Helena, Ballet, Steven, Bujalska-Zadrozny, Magdalena, Biological evaluation and molecular docking studies of AA3052, a compound containing a  $\mu$ -selective opioid peptide agonist DALDA and D-PHE-PHE-D-PHE-LEU-LEU-NH<sub>2</sub>, A SUBSTANCE P ANALOGUE, (2016), DOI: [10.1016/j.ejps.2016.07.009](https://doi.org/10.1016/j.ejps.2016.07.009)

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**Biological evaluation and molecular docking studies of AA3052, a compound containing a  $\mu$ -selective opioid peptide agonist DALDA and *D*-Phe-Phe-*D*-Phe-Leu-Leu-NH<sub>2</sub>, a substance P analogue**

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## **Abstract**

The design of novel drugs for pain relief with improved analgesic properties and diminished side effect induction profile still remains a challenging pursuit. Tolerance is one of the most burdensome phenomena that may hamper ongoing opioid therapy, especially in chronic pain patients. Therefore, a promising strategy of hybridizing two pharmacophores that target distinct binding sites involved in pain modulation and transmission was established. Previous studies have led to the development of opioid agonist/NK1 agonist hybrids that produce sufficient analgesia and also suppress opioid-induced tolerance development. In our present investigation we assessed the antinociceptive potency of a new AA3052 chimera comprised of a potent MOR selective dermorphine derivative (DALDA) and an NK1 agonist, a stabilized substance P analogue. We have shown that AA3052 significantly prolonged responses to both mechanical and noxious thermal stimuli in rats after intracerebroventricular administration. Additionally, AA3052 did not trigger the development of tolerance in a 6 - day daily injection paradigm nor did it produce any sedative effects, as assessed in the rotarod performance test. However, the antinociceptive effect of AA3052 was independent of opioid receptor stimulation by the DALDA pharmacophore as shown in the agonist-stimulated G-protein assay. Altogether the current results confirm the antinociceptive effectiveness of a novel opioid/SP hybrid agonist, AA3052, and more importantly its ability to inhibit the development of tolerance.

## **Keywords**

DALDA; substance P; hybrid peptide; analgesia; anticancer activity; tolerance

## 1. Introduction

Opioids serve as mainstay analgesics in treatment of moderate to severe pain from a variety of origins. Despite beneficial analgesic properties, their use is limited by several burdensome side effects, such as tolerance and dependence that hamper the effectiveness of opioid therapy and lead to decreased quality of life (Collet, 1998; Kosten and George, 2002). Therefore, many different strategies have been developed to overcome the typical shortcomings of opioids. Among them is the strategy of engineering opioid/non-opioid hybrid compounds that show altered receptor binding and activate signaling pathways different from those triggered by two individual pharmacophores separately (George et al., 2002; Kleczkowska et al., 2013; Rosenfeld and Devi, 2007). The opioid subunit can include common peptide or non-peptide pharmacophores, while the non-opioid component often features an endogenous neurotransmitter or neuromodulator involved in pain signaling pathways.

Substance P (SP) is one of such natural peptide candidates that was extensively studied in relation to its regulation of multiple mechanisms related to pain transmission by activating a well-defined receptor, the neurokinin 1 (NK1) receptor (Henry, 1982; Zubrzycka and Janecka, 2000; Mantyh, 2002). The rationale behind the design of opioid/SP hybrids lie in numerous studies confirming that NK1 and opioid receptors are co-distributed in the brain and superficial dorsal horn of the spinal cord, which strongly implies common interactions of both systems (Kar and Quirion, 1995; Aicher et al., 2000; Pinto et al., 2008). It has been widely accepted that activation of NK1 and opioid receptors produce opposing effects in pain mediating pathways (Cridland and Henry, 1988). Pronociceptive signaling is attributed to SP release from primary afferents, whereas opioids secreted from second order spinal cord neurons and interneurons act as antinociceptive agents (Iversen, 1982; Yaksh, 1997). Previous co-administration studies indicate that low doses of SP may potentiate opioid analgesia probably by triggering endogenous opioid peptide release (Tang et al., 1983; Kream et al., 1993). Moreover, SP shows strong inhibitory effects on the development of morphine-induced tolerance (Maszczyńska et al., 1998). This observation sparked the development of opioid/SP chimeras in hopes of tackling the pressing problem of opioid-induced tolerance. Endomorphin-1 and -2 tetrapeptides were proposed as potent MOR-selective opioid pharmacophores that when conjugated with C-terminal fragments of SP (e.g. SP<sub>8-11</sub> or SP<sub>7-11</sub>) show enhanced metabolic stability and are able to sustain morphine analgesia under chronic treatment conditions (Foran et al., 2000a; Varamini et al., 2012).

Similar observations were made for a hybrid containing both morphine and SP<sub>3-11</sub> domains that showed no cross-tolerance in morphine-tolerant rats (Kream et al., 2007).

Taking into consideration the new possibilities offered by multiple target ligands composed of a potent MOR and NK1 receptor agonists, we aimed to characterize the *in vitro* and *in vivo* properties of a novel hybrid compound - AA3052 (H-Tyr-*D*-Arg-Phe-Lys-*D*-Phe-Phe-*D*-Phe-Leu-Leu-NH<sub>2</sub>). Its structure joins a frog-skin dermorphin derivative peptide - DALDA (Tyr-*D*-Arg-Phe-Lys-NH<sub>2</sub>) (Schiller et al., 1990) and SP (H-Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Me-NH<sub>2</sub>). DALDA was described previously to possess high affinity and selectivity at MOR. It also inhibits norepinephrine (NA) uptake and exhibits lower tolerance liability, when compared with morphine (Schiller et al., 1990; Usenko et al., 2002). Thus, DALDA together with its analogs are promising centrally-active drug candidates for pain relief that show long-lasting analgesia (Shimoyama et al., 2001). The activity of AA3052 was assessed *ex vivo* based on its ability to stimulate G protein activation. Furthermore, the antinociceptive action of intracerebroventricular administered AA3052 was determined *in vivo* in two distinct antinociceptive tests, involving mechanical and thermal stimulation. Additionally, the sedative effect of AA3052 was investigated in the rotarod test. In our current study we found AA3052 to produce antinociception to noxious thermal and mechanical stimuli after intracerebroventricular injection. Interestingly, chronic treatment with AA3052 did not produce tolerance as opposed to DALDA, confirming the modulatory role of SP in the modulation of tolerance development. This justifies the design of opioid/SP hybrids as potential drugs for pain management where opiate/opioid tolerance is a major issue.

## **2. Material and methods**

### **2.1 Molecular dynamic (MD) simulations**

Molecular dynamic simulations of AA3052 were performed using the GROMACS 5.0.4 program package and the AMBER ff99sb-ildn-nmr force field parameter set (Li and Brüschweiler, 2010). The simulation was started from an extended, energy minimized geometry of the peptide. The peptide was solvated by pre-equilibrated TIP4P-Ew (Horn et al., 2004) water molecules in a 50 Å × 50 Å × 50 Å cubic box. If the distance between any atom of the solute and solvent molecules was less than the sum of their van der Waals radii then the solvent molecules involved were removed from the box. The protonated N-terminal amino group of the peptide was neutralized by replacing solvent molecules by Cl<sup>-</sup> ions at a position

with the most favorable electrostatic potential. The system was subjected to 1000 steps of steepest descent, followed by 1000 steps conjugate gradient energy minimization with 0.001 kJ mol<sup>-1</sup> convergence criteria. A 0.5 ns long NVT MD simulation at 300 K was performed in order to allow the solvent density to equilibrate. During equilibration the position of the solute was fixed in the center of the box with a force constant of 1000 kJ mol<sup>-1</sup> Å<sup>2</sup> on each heavy atom. Subsequently, a 200.5 ns NPT production MD simulation was performed at constant temperature (300 K) and pressure (1 bar), with the following parameters: the time step was set to 2 fs, bond lengths were constrained using the LINCS algorithm, the v-rescale algorithm with a coupling constant of 0.1 ps was used for temperature regulation, constant pressure was maintained using isotropic scaling with a relaxation constant of 1.0 ps and 4.5 × 10<sup>-5</sup> bar<sup>-1</sup> isothermal compressibility. Non-bonded interactions were calculated using the PME method with all cut-off values set at 12 Å. The coordinates were stored after every 1000 steps to yield a total of 100000 sampled conformations for each trajectory, after excluding the first 0.5 ns of equilibration.

The evolution of secondary structure of AA3052 along the trajectory was analyzed with the STRIDE algorithm (Frishman and Argos, 1995) and Perl scripts written in-house.

## 2.2 Docking studies

The crystallographic structure of the active mu opioid receptor (PDB code: 5C1M) (Huang et al., 2015) was used as docking target after all missing side chains were added to the structure. Dockings were performed with the Autodock Vina software (Trott and Olson, 2010). Side chains within 5.0 Å distance of the bound ligand in the crystal complex of the mu opioid receptor and BU72 were kept flexible, as well as all  $\Phi$ ,  $\Psi$  and  $\chi^1$  ligand torsions. One thousand attempts of blind docking of AA3052 to the target receptor were performed in a 80 Å x 80 Å x 80 Å grid volume, large enough to cover the whole receptor region accessible from the extracellular side. The twenty lowest energy complex generations were ranked according to the corresponding binding free energies. *In silico* inhibitory constants were calculated according to the following equation:  $\Delta G = RT \ln K_i$ .

## 2.3 Displacement and [<sup>35</sup>S]GTP $\gamma$ S binding assay

### 2.3.1 Animals

In the displacement binding studies and [<sup>35</sup>S]GTP $\gamma$ S binding assays both male and female Albino Wistar rats (*Rattus norvegicus*) of 250–300 g body mass were utilized, which were

housed in the local animal keeping facility of the Medical University of Warsaw (Warsaw, Poland) and Biological Research Center (BRC, Szeged, Hungary), respectively. The animals were kept in groups of four, allowed free access to tap water and standard food, and maintained on a 12/12 hour light/dark cycle up until the time of sacrifice. The animals were kept and handled humanely, in complete accordance with the European Communities Council Directives (86/609/ECC) and Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§). Additionally, studies performed in Poland were approved by the 2<sup>nd</sup> Local Committee for Ethics in Animal Experiments (Permit No.: 15/2015). Both the number of rats and their suffering were minimized throughout our experiments.

### **2.3.2 Chemicals and radioligands**

DAMGO ([D-Ala<sup>2</sup>,NMePhe<sup>4</sup>,Gly<sup>5</sup>-ol]enkephalin), spantide I (*D*-Arg-Pro-Lys-Pro-Gln-Gln-*D*-Trp-Phe-*D*-Trp-Leu-Leu-NH<sub>2</sub>) and substance P were obtained from Bachem Holding AG (Bubendorf, Switzerland), while naloxone was a gift from ENDO Laboratories (Garden City, NY, USA), respectively. Guanosine-5-[ $\gamma$ -<sup>35</sup>S]-triphosphate (1000 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany), whereas [<sup>3</sup>H]DAMGO was purchased from Perkin Elmer (USA). EDTA, bovine serum albumin (BSA), polyethylenimine (PEI), tris(hydroxymethyl)amino-methane (Tris), guanosine 5'-diphosphate (GDP), unlabeled GTP $\gamma$ S, and peptidase inhibitors - bestatin, bacitracin and phosphoramidon were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### **2.3.3 Rat brain membrane preparation**

Forebrain membrane fractions were prepared according to a method previously described by Benyhe et al. (1997). Briefly, the rats were decapitated and the full brain (without cerebellum) was quickly removed then homogenized using a Teflon-glass homogenizer in 30 volumes (mL/g wet mass) of 50 mM Tris-HCl buffer pH 7.4. After that, the homogenate was centrifuged at 40,000  $\times$  g (18.000 rpm) for 20 min at 4 °C. The resulting pellet was resuspended in a fresh buffer (30 volume/weight, by use of a vortex) and then incubated for 30 minutes at a temperature of 37 °C. Subsequently, the centrifugation step was repeated. The final pellet was resuspended in 5 volumes of 50 mM Tris-HCl (pH 7.4) buffer containing 0.32 M sucrose and stored at -80 °C until future use. Membranes were rethawed for the binding assays and then resuspended in 50 mM Tris-HCl (pH 7.4) buffer and centrifuged at 40,000  $\times$  g for 20 min at 4 °C to remove the sucrose. The resulting pellets were suspended in an ice-cold

TEM buffer so that the desired protein concentration for the assay (~40 µg/ml) could be achieved.

#### **2.3.4 Displacement binding studies**

Brain membrane preparations (0.2 mg of protein/ml) were incubated with 0.5 nM [<sup>3</sup>H]DAMGO for 60 min. at 25.5 °C in 50 mM Tris-HCl (pH 7.4) buffer containing 0.1 mg/ml bovine serum albumin (BSA), 30 µg/ml bacitracin, 30 µM bestatin, 10 µM captopril and 10<sup>-10</sup> - 10<sup>-6</sup> M of AA3052. Non-specific binding was determined in the presence of 10 µM of unlabeled DAMGO. All experiments were performed in triplicate. Samples were rapidly filtered with 4 ml of ice-cold 50 mM Tris-HCl (pH 7.4) through 0.3 % PEI presoaked GF/B Whatman filter paper (Whatman, USA) in a M-24 Cell Harvester (Brandel, USA). Filter discs were placed in plastic scintillation vials and 3 ml of EcoScint A scintillation liquid was added (National Diagnostics, USA). Filter-bound radioactivity was assessed with a Beckman scintillation counter. The binding curve was fitted with non-linear regression and affinity was presented as an inhibitory constant (K<sub>i</sub>).

#### **2.3.5 Functional mu opioid and NK1 receptor assay**

Agonist induced-receptor mediated G protein activation was measured as described by Traynor and Nahorsky (1995) with slight modifications. Importantly, to distinguish between the receptors involved in the observed effects in brain membranes, different selective antagonists were used (e.g. spantide for NK1 and naloxone for mu opioid receptor).

Rat brain membrane fractions (~40 µg protein per sample) were incubated at 30 °C for 60 min in Tris-EGTA buffer. The buffer was composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, containing 0.05 nM [<sup>35</sup>S]GTPγS (20 MBq/0.05 cm<sup>3</sup>) and 30 mM GDP and increasing concentrations (10<sup>-10</sup> – 10<sup>-5</sup> M) of the peptides tested. The final volume was 1 ml. Total binding (T) was measured in the absence of the test ligands, while non-specific binding (NS) was determined in the presence of 100 µM unlabelled GTPγS and subtracted from the total binding value, to determine the specific binding. Throughout this paper, G protein activation is given as percentage over the specific [<sup>35</sup>S]GTPγS binding obtained in the absence of receptor ligands (basal activity). The difference of total binding (T) and non-specific binding (NS) represents the basal activity and was defined as 100%.

After incubation bound and free [<sup>35</sup>S]GTPγS were separated by vacuum filtration through Whatman GF/B glass fiber filters with a Brandel M24R Cell harvester. Filters were washed three times with 5 ml ice-cold buffer (pH 7.4), and the radioactivity of the filters was



measured in UltimaGOLD™ (Perkin Elmer) scintillation cocktail with a Packard TriCarb 2300TR counter.

### **2.3.6 Data Analysis**

Analysis of data collected from three independent experiments performed in duplicates is done using GraphPad Prism 5 software (GraphPad, San Diego, CA). Log IC<sub>50</sub> values for each test compound were determined from nonlinear regression. In the competition binding studies affinity was expressed as the equilibrium inhibition constant (K<sub>i</sub> value) and calculated from the IC<sub>50</sub> values according to the built-in Cheng-Prusoff equation module. Results from the [<sup>35</sup>S]GTPγS assay were expressed as efficacy (E<sub>max</sub>) and potency (pEC<sub>50</sub>) of G protein stimulation.

## **2.4 *In vivo* analgesic tests in acute pain model in rats**

### **2.4.1 Animals**

Adult male WAG rats (250-350 g) were housed in a temperature-controlled environment (22 ± 2 °C) under a 12-hour light/12-hour dark cycle (lights on at 7:00 a.m.). Free access to food and water was available throughout the entire study. After surgery rats were housed individually. Experimental groups consisted of at least 6 rats. The individual animals were used in only one experiment. All experimental procedures were conducted in accordance with the guidelines of the 2<sup>nd</sup> Local Ethical Committee for Experiments on Animals of the Medical University of Warsaw in Poland (Permit no. 35/2011) and adhered to guidelines published in the European directive 2010/63/EU on the protection of animals used for scientific purposes.

### **2.4.2 Drug administration**

For the intracerebroventricular (i.c.v) drug administration, rats were implanted with chronic cannula according to the method described by Noble and Wurtman (1967) with modifications done by Robinson et al. (1969) and Strada et al. (1970). Briefly, the rats were deeply anesthetized with a mixture of ketamine (70 mg/kg, i.p) and xylazine (6 mg/kg, i.p). The cannula was inserted into the right lateral ventricle. The i.c.v drug administration began on the fifth day after surgery.

All tested drugs (DALDA and AA3052) were administered i.c.v in a volume of 3 µl over a 60 sec period using a Hamilton syringe. Both tested substances were dissolved in distilled water immediately before injections. AA3052 was administered at a dose of 10, 100, 200 and 500

µg/rat, while DALDA at a dose of 0.1, 1, 10 and 100 µg/rat. Control animals were i.c.v infused with distilled water in a 3-µl volume.

Additionally to this, in order to determine a tolerance development, animals were injected i.c.v with a tested compounds (AA3052 at a dose of 100 µg/rat and DALDA at a dose of 10 µg/rat) once daily for 6 consecutive days and the behavioral tests were carried out 30 min after drug administration. Importantly, the dose and the time point for measuring analgesic responses (30 min) were selected based on the analgesic effects induced following a single administration of the compound.

### **2.4.3 Behavioral measurements**

Changes in nociceptive thresholds were estimated using a mechanical (the modification of the Randall-Selitto paw withdrawal test) (Randall and Selitto, 1957) and a thermal nociceptive stimulus (hot plate test) (Hosseini et al., 2011).

#### **2.4.3.1 Randal-Selitto test**

For mechanical stimulation a progressively increased pressure was applied to the dorsal surface of the rat's paw using an analgesimeter (Ugo-Basile, Comerio, Italy). The nociceptive threshold was defined as the force in grams to which the rat subjected to withdrawal its paw. An increase in the paw pressure threshold was interpreted as analgesia. The test was terminated if the rat did not withdrawal its paw by 500 g.

The nociceptive thresholds were determined before and at 15, 30, 45, 60, 90, 120 and 150 min. after acute i.c.v administration of tested substances as well as every day for 6 consecutive days before and 30 min. after i.c.v administration of the tested substances.

Changes in pain threshold were calculated as the percentage of the maximal possible effect (%MPE) according to following formula:  $\%MPE = 100 \times [(test\ score - baseline\ score) / (cut-off\ score - baseline\ score)]$ , where the baseline score indicates the score before drug administration, and the cut-off score was set up at 500 g.

#### **2.4.3.2 Hot plate test**

The hot plate test is designed for examination of acute pain in rodents. In this test pain was induced by thermal stimuli. This test consisted of placing the rat on an enclosed hot plate ( $52 \pm 1$  °C) and measuring the latency to lick a hind paw or attempt to jump out of the apparatus (i.e., the latency of the nociceptive reaction). Rats were removed from the plate if they did not respond within 40 seconds. The nociceptive thresholds were determined before and at 15, 30,

45, 60, 90, 120 and 150 min post-injections (i.c.v) administration of tested drugs. Changes in pain threshold were calculated as the percentage of the maximal possible effect (%MPE) according to the formula mentioned above, however, in this case the baseline score indicated the latency before drug administration and the cut-off score was set up at 40 sec.

### **2.4.3.3 Rotarod**

A rotarod motor skills assay is a frequently used test in rodents to screen out drugs that might cause side effects on motor coordination and balance.

Rats were trained to walk on an automated rotating rod at an accelerating speed of 2–20 rpm per 5 min for maximal cut off time of 300 s (Chu et al., 2011). Training comprised three sessions: one session with nonmoving rod and two sessions with moving rod. Following the training sessions the actual test was performed. Baseline values were recorded for each rat before i.c.v infusion of vehicle or a tested drug. At 15 and 30 min. following i.c.v administration of tested compounds, latencies for each animal to fall from the rotating rod were recorded. Each rat was subjected to 3 rotarod trials, five minutes apart.

### **2.4.4 Statistical Analysis**

The results of the experiments were statistically evaluated using two-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test for group comparisons.  $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$  indicate a significant difference compared to the control group treated with vehicle (water).

Data were plotted in GraphPad Prism 5 software for Macintosh (GraphPad Software, USA) and represent the mean  $\pm$  standard error of the mean (S.E.M.).

## **3. Results**

### **3.1 Computational analysis of the interaction between AA3052 and the mu opioid receptor**

The AA3052 opioid-NK1 hybrid peptide was investigated as a potential ligand of the mu opioid receptor utilizing molecular dynamics (MD) simulations and docking studies. Secondary structure analysis of MD trajectories revealed that the AA3052 peptide favors  $\beta$ -

turn backbone structure in aqueous environment which include both the residues of the NK1 pharmacophore and the opioid pharmacophore, although this latter with lower occurrence (Figure 2, Table 1). Docking results indicated that the binding pocket of the mu opioid receptor is spacious enough to accommodate the hybrid peptide regardless of its relatively large size compared to mu opioid peptide agonists in general (Keresztes et al., 2010). The lowest energy docked complex and the amino acid side chains of the mu opioid receptor in close contact with AA3052 are shown on Figure 3 and listed in Table 2. The bound ligand adopts a type IV  $\beta$ -turn spanning residues Phe<sup>3</sup>-Phe<sup>6</sup> (Figure 3). The conserved salt bridge between the protonated N-terminal amino group of the mu opioid pharmacophore and Asp<sup>147</sup> of the receptor (Pogozheva et al., 1998) was not present in the docked complexes of lowest binding free energy. Instead, Asp<sup>147</sup> was found to interact with the Lys<sup>4</sup> side chain and the amide NH of Phe<sup>3</sup> of AA3052. No polar interactions between the ligand and Tyr<sup>148</sup>, Lys<sup>233</sup> or His<sup>297</sup>, residues forming a water molecule assisted polar network in the active mu opioid receptor (Huang et al., 2015), were observed. Nevertheless, the predicted K<sub>i</sub> value for this ligand was 46.8 nM which prompted us to perform the synthesis and further analysis of AA3052 regarding its *ex vivo* activity upon interaction with G $\alpha$ -GTP and *in vivo* analgesic activity after central administration.

**Table 1.** Secondary structural elements of AA3052 identified by STRIDE analysis of MD trajectories.

	residues involved	population / %	comments
random	all	31.62	
type II' $\beta$ -turn	Lys <sup>4</sup> – D-Phe <sup>7</sup>	21.97	
type IV $\beta$ -turn	Lys <sup>4</sup> – D-Phe <sup>7</sup>	16.97	
type II' $\beta$ -turn + type IV $\beta$ -turn	Lys <sup>4</sup> – D-Phe <sup>7</sup> , D-Phe <sup>5</sup> – Leu <sup>8</sup>	6.63	overlapping turns
type II' $\beta$ -turn + type IV $\beta$ -turn	Lys <sup>4</sup> – D-Phe <sup>7</sup> , D-Phe <sup>5</sup> – Leu <sup>8</sup>	3.44	stabilized by
type IV $\beta$ -turn + type IV $\beta$ -turn	Lys <sup>4</sup> – D-Phe <sup>7</sup> , D-Phe <sup>5</sup> – Leu <sup>8</sup>	1.73	$\beta$ -bridge
type II' $\beta$ -turn	Tyr <sup>1</sup> – Lys <sup>4</sup>	1.72	
type IV $\beta$ -turn	Tyr <sup>1</sup> – Lys <sup>4</sup>	1.60	
type IV $\beta$ -turn	D-Phe <sup>5</sup> – Leu <sup>8</sup>	1.59	
type IV $\beta$ -turn	Phe <sup>3</sup> – Phe <sup>6</sup>	1.18	

<sup>a</sup>Only structures with occurrence higher than 1% of the whole structural ensemble are listed.

**Table 2.** Contacts between AA3052 and the mu opioid receptor observed by docking studies.

type of interaction	ligand residues	receptor residues
<b>polar</b>	Tyr <sup>1</sup>	Gln <sup>124</sup>
	Lys <sup>4</sup>	Asn <sup>150</sup>
	Phe <sup>3</sup>	Asp <sup>147</sup>
	Lys <sup>4</sup>	Asp <sup>147</sup>
	Leu <sup>9</sup>	Tyr <sup>128</sup>
<b>hydrophobic</b>	Tyr <sup>1</sup>	Tyr <sup>128</sup>
	Phe <sup>3</sup>	Ile144, Leu219
	<i>D</i> -Phe <sup>5</sup>	Inserted in a hydrophobic pocket formed by Met <sup>151</sup> , Val <sup>236</sup> , Phe <sup>237</sup> , Val <sup>300</sup> and Ile <sup>301</sup>

### 3.2 Activity of AA3052 in competition binding and functional assays

Binding affinity of AA3052 to MOR was studied in a competition binding assay with [<sup>3</sup>H]DAMGO. Whereas, efficacy ( $E_{\max}$ ) and potency ( $pEC_{50}$ ) of G protein stimulation was assessed in a functional, agonist-stimulated G protein activation assay ([<sup>35</sup>S]GTP $\gamma$ S). As shown in Fig. 4A the AA3052 hybrid displaced the highly selective, potent MOR agonist [<sup>3</sup>H]DAMGO with a limited affinity of 729.8 nM (Fig. 4A). Similarly, as compared with the full agonist DAMGO ( $E_{\max} = 152.3\%$ ,  $pEC_{50} = 6.84$ ), AA3052 stimulated G protein activation with very low affinity and with an intrinsic activity characteristic for partial agonists ( $E_{\max} = 130.9\%$ ,  $pEC_{50} = 4.95$ ). The  $E_{\max}$  value for AA3052 was comparable with that of SP, reported to be a partial agonist in this assay ( $E_{\max} = 118.2\%$ ). However, AA3052 exhibited much lower potency in activating G proteins, as compared to SP ( $pEC_{50} = 10.48$ ) (Fig. 4B).

In order to further confirm whether the activity of AA3052 was associated with MOR or NK1, highly selective MOR and NK1 receptor antagonists were used in high, 10  $\mu$ M concentrations. (Fig. 4B). The NK1 antagonist spantide I decreased the activity of AA3052-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding to baseline level (from 130.9% to 107.4%). Naloxone on the other hand had no effect on AA3052 efficacy (from 130.9% to 124.0%). This result indicates that the biological activity of AA3052 is not associated with its activation of opioid receptors.

### **3.3 Effects of acute administration of AA3052 and DALDA on thermal and mechanical nociceptive threshold**

As shown in Figure 5A, the paw withdrawal threshold to mechanical stimuli was increased significantly after both DALDA and AA3052 treatment in a time- and dose-dependent manner. As we anticipated, an intracerebroventricular administration of one of the highest doses of DALDA (100 µg/rat) induced strong analgesic effect in the Randall-Selitto test with a % MPE of  $30.14 \pm 8.54$  % at 30 min post-injection. A similar response was observed for a 200 µg/rat dose of AA3052 (% MPE =  $30.17 \pm 9.87$  %). However, administration of doses higher than 100 µg/rat (in both cases) resulted in visible abnormalities of motor behavior in rats, like repeated lateral rolling.

A different pharmacodynamic profile of each drug was observed in the hot plate test both in terms of time-course and analgesic magnitude (Fig. 5B). However, later studies such as tolerance development assay and locomotor coordination test described only the two highest doses of both peptides. In the Randall-Selitto test, the maximal pain-relieving action of DALDA was observed at 45 min. and of AA3052 occurred at 30 min. post-administration, respectively. Whereas in the hot-plate test, the strongest drug-induced effects were observed already at 15 min post-administrations (% MPE =  $63.14 \pm 15.06$  % for 100 µg/rat of DALDA and % MPE =  $36.60 \pm 15.22$  % for 100 µg/rat of AA3052). However, after this time point a gradual decrease of analgesia was reported (Fig. 5B).

### **3.4 Effects of repeated administration of AA3052 and DALDA on the nociceptive threshold after mechanical stimulation**

Intracerebroventricular administration of AA3052 at a dose of 100 µg/rat once daily for 6 consecutive days produced a long-lasting analgesic effect manifested by increased nociceptive thresholds to mechanical stimuli, as monitored in the Randall-Selitto test. As opposed to DALDA, no analgesic tolerance developed over the entire period of repeated compound injections. The pain-relieving action induced by AA3052 remained at approx. 20-30% MPE throughout the whole testing period (Fig. 6). Notably, a 10-fold lower dose of DALDA (i.e. 10 µg/rat) in comparison with AA3052, caused the development of analgesic tolerance (Fig. 6), starting from the third day of drug injection.

### 3.5 Effects of AA3052 and DALDA on motor coordination in naïve rats

Possible motor impairments of AA3052 and DALDA were tested at doses that produced a sufficient response in antinociceptive tests. Intracerebroventricular administration of AA3052 at a dose 10-fold higher (100 µg/rat) than its opioid pharmacophore DALDA did not produce motor impairments or sedation as tested using the rotarod device. Additionally, this lack of potency to depress motor activity was similar to that of vehicle (Fig. 7).

## 4. Discussion

Chemical hybridization is a promising and innovative approach that is mainly useful for patients facing problems related with a still common multidrug therapy. However, to design an effective and safe single entity that contains at least two distinct, but biologically active components seems to be a difficult task. This is because connected pharmacophores can lead to significant changes or reduction of binding to their molecular target. This problem may occur even after careful QSAR studies or molecular modeling and computational calculations.

In the pursuit for a new type of compound with dual activity, we developed a chimeric analogue of a previously synthesized NK1-antagonistic peptide AWL-60 (Tyr-Pro-*D*-Phe-Phe-*D*-Phe-*D*-Trp-MetNH<sub>2</sub>) (Lipkowski et al., 2006; Lipkowski and Misterek, 1992). The analogue of this peptide was combined herein with a MOR-specific agonist [D-Arg<sup>2</sup>,Lys<sup>4</sup>]dermorphin-(1,4)-amide (DALDA). Thus, in the present paper we anticipated to obtain a novel compound that would possess agonistic activity at MOR and antagonistic activity at NK1 receptors. Our assumption regarding the agonistic character of AA3052 toward the mu opioid receptor relied on the fact that our design contained the unmodified DALDA pharmacophore. MD simulations indicated that the mu opioid agonist moiety of the hybrid peptide is capable of forming bent structure, previously proposed to be important for mu opioid binding (Borics and Tóth, 2010; Eguchi et al., 2002; Gentilucci, et al., 2011; Keresztes et al., 2010; Piekielna et al., 2014; Tömböly et al., 2010). Docking studies predicted the binding affinity of AA3052 to the mu opioid receptor to be in the nanomolar range. The previously proposed native contacts (Huang et al., 2015) between opioid agonists and the mu opioid receptor were, however, only partially present in the low binding free energy docked complexes obtained in our studies. Hybridization brought about an unexpected loss of MOR affinity as AA3052 did not readily compete for the same site with the potent MOR-selective

agonist DAMGO. Moreover, a nonselective opioid receptor antagonist - naloxone failed to inhibit AA3052 - induced G protein activation, suggesting that its activity is mainly mediated by AA3052 binding to the opioid receptors. Interestingly, the effect of AA3052 seen in the [<sup>35</sup>S]GTPγS assay was comparable with SP and in addition, was reversed by a NK1 receptor antagonist - spantide I implying the role of NK1 receptors in intracellular secondary messenger system activation.

Though AA3052 was found not to possess agonistic activity at MOR, we have decided to determine its activity *in vivo*. This in turn was dictated by the knowledge that also substance P and other SP-like agonists at NK1 receptor induced antinociception (Kotani et al., 1981; Lin et al., 2012; Mousseau et al., 1994; Rosén et al., 2004).

The biological activity of AA3052 was confirmed in an acute and chronic treatment paradigm. Intracerebroventricular administration of AA3052 significantly increased pain thresholds in response to mechanical and thermal stimuli (Fig. 5B). However, the antinociceptive activity of AA3052 was more pronounced after the application of thermal stimuli, which implies the involvement of C-fibers rather than Aβ-fibers in the modulation of pain response. The hybrid peptide was however 10 times less potent than DALDA in both analgesic tests. This observation is not surprising given the lack of MOR agonism of AA3052. We therefore hypothesize that the antinociceptive effect of AA3052 is manifested by the C-terminal SP fragment that might activate other receptor systems involved in pain transmission and modulation. In fact, it was shown before that low-dose SP triggers the release of endogenous opioid peptides in the dorsal horn of the spinal cord thus facilitating an analgesic response (Tang et al., 1983; Kream et al., 1993). The involvement of other receptor systems can also be confirmed by the fact that rats administered with high doses of AA3052 (> 100 μg/rat, i.c.v) exposed a peculiar form of behavior, named barrel rotation. In fact, many neurotransmitters were found to induce animals' unidirectional turning on the spot (e.g., dopamine, somatostatin, bradykinin, bombesin, etc.), however only a few are related with the action of substance P and the observed effect(s) is similar to that reported in our studies. Following this, although barrel rotations resulting from the intracerebroventricular injection of substance P were reported to be strongly related with the stimulation/activity of dopaminergic pathway (Glowinski et al., 1980; James and Starr, 1979), in presented studies we did not report a development of tolerance after drug repeated administration (Fig. 6). This, in consequence, might exclude the involvement of dopamine system in the observed effects. Similarly to this, several groups (Hermansen, 1980; McIntosh et al., 1987) indicated substance P to stimulate secretion of somatostatin (SOM), which apart from its



antinociceptive effects (Chapman and Dickenson, 1992; Helyes et al., 2004; Pinter et al., 2006; Sandor et al., 2006) also induced barrel rotations (Van Wimersma Greidanus et al., 1987; Vécsei et al., 1989). This would fit well into our observations if not for the fact that SP inhibited the release and activity of SOM (McIntosh et al., 1987). Bradykinin and bombesin were additional peptides responsible for the induction of barrel rotation. However, while bombesin is an amphibian skin compound (Brown and Vale, 1979; Nagalla et al., 1995) and does not naturally exist in mammals, bradykinin is pronociceptive (Snyder et al. 1989; Steranka et al., 1988).

Taking this all into consideration and still searching for the possible involvement of other neuropeptide/neurotransmitter, we have found that vasopressin (VP) and its receptors might be strongly related to the actions of substance P that were observed in our experiments. This assumption may be further confirmed by the fact that vasopressin was proven to increase pain thresholds (Kordower and Bodnar, 1984) and induce barrel rotation (Kruse et al., 1977; Wurpel et al., 1986). Moreover, only SP antagonists were presented to inhibit vasopressin (Seckl et al., 1995), while substance P and SP-like agonists at NK1 receptor occurred to increase both the activity and release of VP (Boer and Gash, 2012).

On the other hand, AA3052's ability to induce pain-relieving effects could be also justified by its partial agonism at NK1, thus indicating the role of an antagonist component. Partial agonist includes ligands, which display both agonistic and antagonistic actions. Therefore, if we assume that the antagonist component has superiority over the agonistic one, it is obvious that AA3052-mediated analgesia resulted from the inhibition at NK1 receptors. This is in accordance with several studies revealing that SP antagonists play a great role as antinociceptive agents (Altier and Stewart, 1999; Lembeck et al., 1982; Muñoz and Coveñas, 2011).

Another observation indicating that AA3052 is indeed a partial agonist at NK1 receptors is the absence of grooming and scratching behavior. Indeed, these behaviors were assigned to effects elicited by spinal SP agonist, as presented by Revard et al. (1996) and others (Stoessl et al., 1995; Van Wimersma Greidanus and Maigret, 1988; Wilcox, 1988). This may, however, be highly dependent on the applied dose of such a compound.

Our current study however contributed by an interesting finding that the chimeric DALDA/SP analog did not evoke tolerance as opposed to DALDA administered alone. Low tolerance liability may be attributed to the agonistic activity at NK1 receptors. This observation is in accordance with previously described observations, that SP and other NK1R agonists inhibited the expression of morphine tolerance in the experimental animals (Kreeger

and Larson, 1996; Murtra et al., 2000). This was also reported for chimeras containing an opioid and different C-terminal SP fragments (Foran et al., 2000b; Kream et al., 2007).

In regard to the extraordinary result demonstrating, despite possessing unmodified DALDA pharmacophore, the lack of MOR agonism of AA3052, it is obvious that we cannot exclude DALDA's involvement, and thus any of its specific roles.

Firstly, due to DALDA capability of crossing the blood-brain barrier (Riba et al., 2002; Samii et al., 1994), it is possible that the hybridization of DALDA pharmacophore with SP one will result in an active compound after its systemic administration. Also, DALDA is well known from its great metabolic stability and long elimination half-life (Szeto et al., 2001). Therefore, it is more likely that the obtain hybrid compound (AA3052) will be characterized by an enhanced stability against enzymatic hydrolysis. This in turn, is of great importance as i) long peptides and several endogenous opioid peptides (e.g., endomorphin-2) have difficulties in penetrating blood-brain barrier, which highly depend on its lipophylicity (Banks et al., 1985; Hau et al., 2002) and ii) natural opioid peptides are found to have an increased susceptibility to rapid enzymatic degradation (Bausback and Ward, 1985; Beaumont and Hughes, 1979). Importantly, the absence of such properties would be problematic for further clinical use.

Secondly, DALDA can inhibit norepinephrine (NE) uptake (Schiller 2010) and can act at adrenergic receptors, particularly  $\alpha$ 2-adrenergic receptors ( $\alpha$ 2AR) (Reimann et al., 1999).

It is interesting to note that noradrenergic as well as serotonergic systems have been shown to be directly involved in descending inhibition of nociceptive transmission (Herradón et al., 2003, ; Jones 1991; Nakamura and Ferreira, 1988; Proudfit 1988); Wei et al., 2012). Considering this, we can hypothesize that the analgesic response observed after intracerebroventricular administration of a test compound AA3052 may be related not only with the activity of a substance P pharmacophore, but may be a consequence of the modulation of  $\alpha$ 2AR by DALDA (i.c.v). Of note,  $\alpha$ 2-adrenergic receptor agonists given intracerebroventricularly were also found to exert pain-relieving effect (Hayes et al., 1986; Pertovaara et al., 1991).

Futhermore, it is well documented that activation of noradrenergic system may bring several benefits such as facilitation of cognitive functions (Coull 1994; Mair and McEntee, 1983), relieve in neuropathic pain (Di Cesare Mannelli et al., 2016; Leiphart et al., 2004) or alleviation of drug-induced withdrawal symptoms (Linnoila et al., 1987; Redmond 1987). In line with this, it is possible that AA3052 may occure to be useful in management of several various diseases. Moreover, although its administration did not result in tolerance developing

(Fig. 6), we in fact did not studied animal behavioral effects after the abruption of discontinuation or decrease in intake of this chimera. However, considering that AA3052 acts as  $\alpha 2$ -adrenergic receptor agonist with the ability to reduce withdrawal signs and symptom, we can believe that cessation of AA3052 would be harmless for the patient.

## 5. Conclusions

In summary, AA3052 seems to be a promising drug candidate since it may enhance pain directed therapies, while lacking the side effects typically caused by conventional opioid therapies (i.e. tolerance). However, further studies need to be performed in order to determine the source of its analgesic properties and a possible role of DALDA pharmacophore in the activity of hybrid's molecule.

## Abbreviations

DALDA, H-Tyr-(D)Arg-Phe-Lys-NH<sub>2</sub>; EGTA, ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; GDP, guanosine 5'-diphosphate; GTP; i.c.v, intracerebroventricular; i.p, intraperitoneal; MOR, mu opioid receptor; NK1, neurokinin 1 receptor; SOM, somatostatin; SP, substance P; SPPS, solid phase peptide synthesis; VP, vasopressin

## Acknowledgments

The research of AE and SB was partially supported by an OTKA-108518 grant provided by National Research, Development and Innovation Office (NKFIH), Budapest, Hungary. Research of AB has been supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. CM and SB thank the Research Foundation Flanders (FWO Vlaanderen) for financial support.

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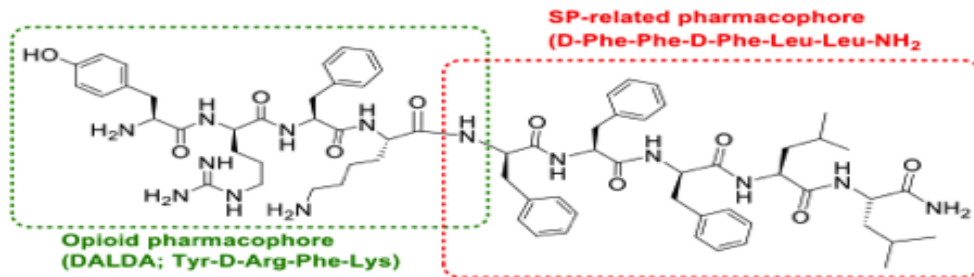
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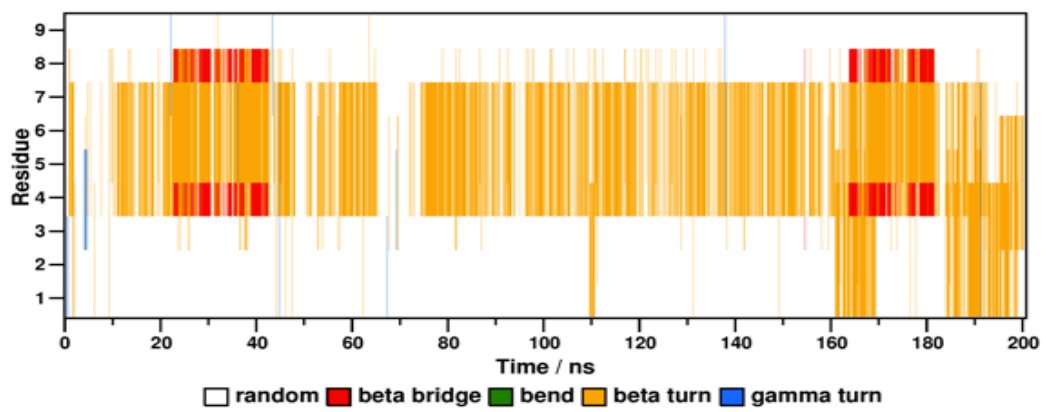
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## Figures

### Figure 1



### Figure 2



### Figure 3

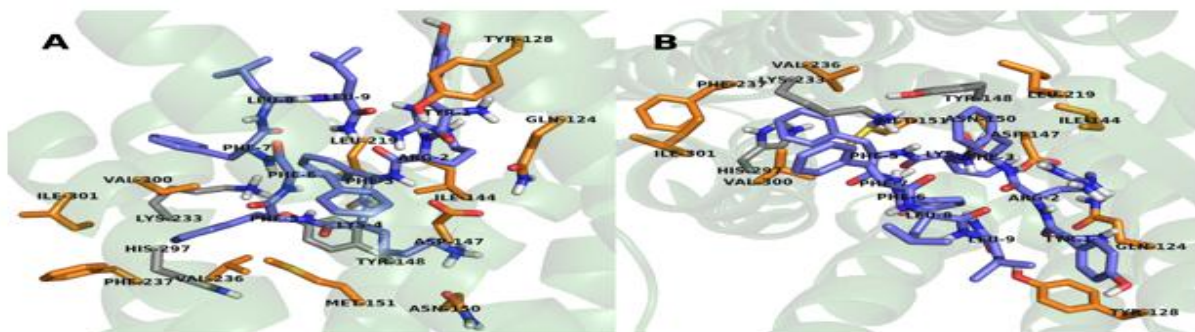


Figure 4

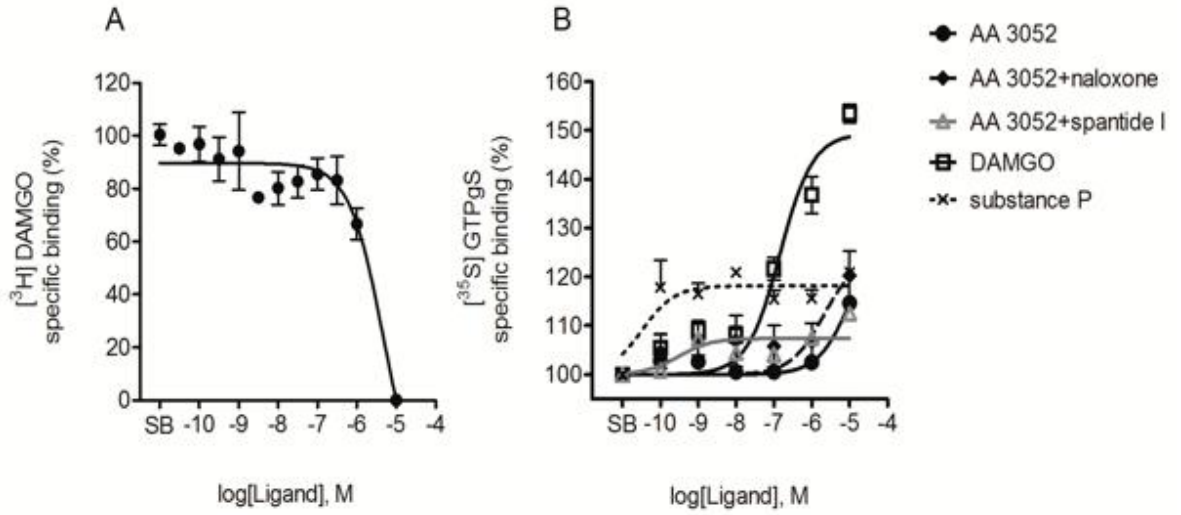


Figure 5

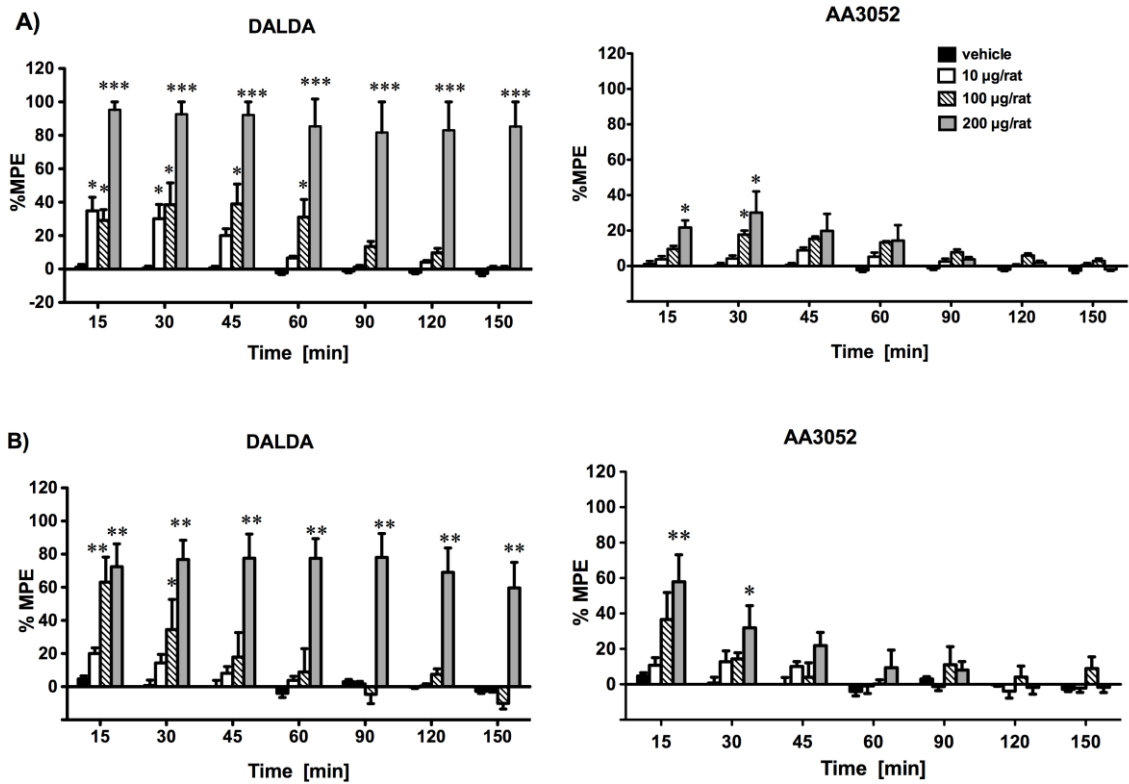


Figure 6

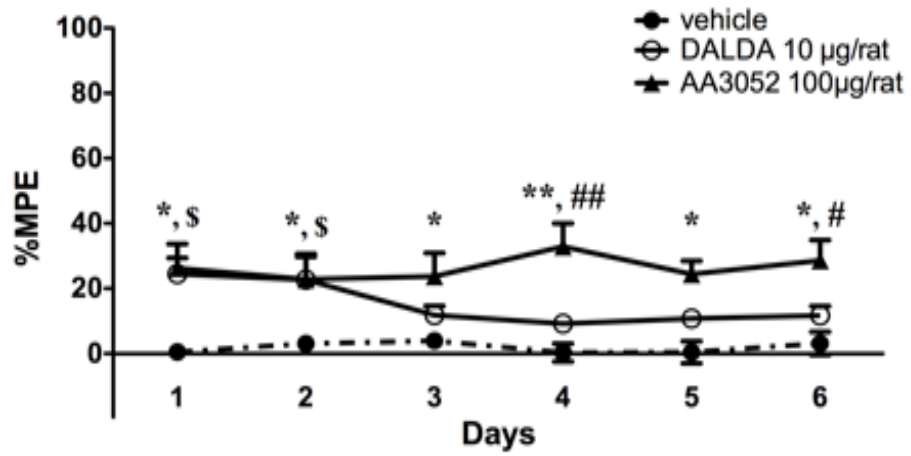
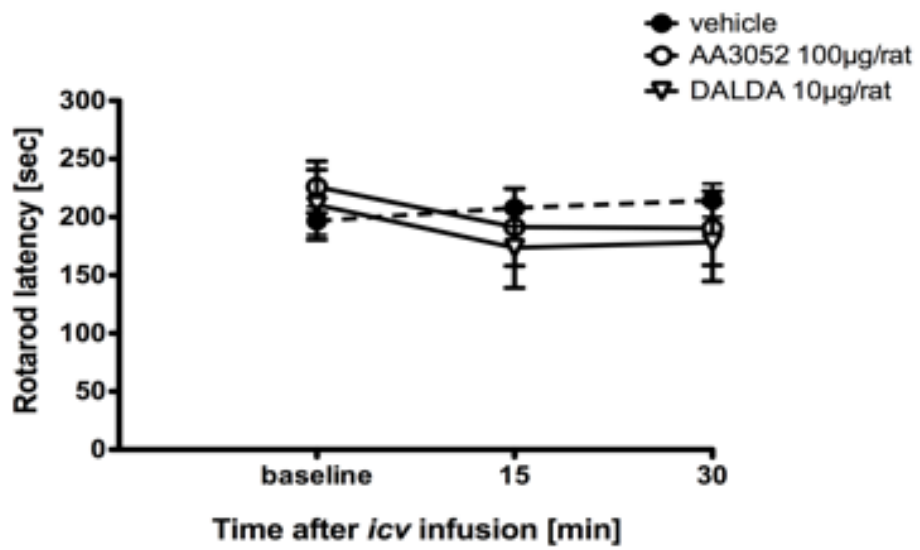


Figure 7



## Figure legends

**Figure 1.** Chemical structure of AA3052, a novel hybrid peptide comprising an opioid and a substance P-related pharmacophore.

**Figure 2.** Occurrence of different secondary structural element observed via STRIDE analysis of the MD trajectory of AA3052 (H-Tyr<sup>1</sup>-D-Arg<sup>2</sup>-Phe<sup>3</sup>-Lys<sup>4</sup>-D-Phe<sup>5</sup>-Phe<sup>6</sup>-D-Phe<sup>7</sup>-Leu<sup>8</sup>-Leu<sup>9</sup>-NH<sub>2</sub>).

**Figure 3.** The lowest binding free energy docked complex of AA3052 and the mu opioid receptor from side (A) and extracellular (B) view. Binding pocket side chains in close contact with the ligand are shown in orange.

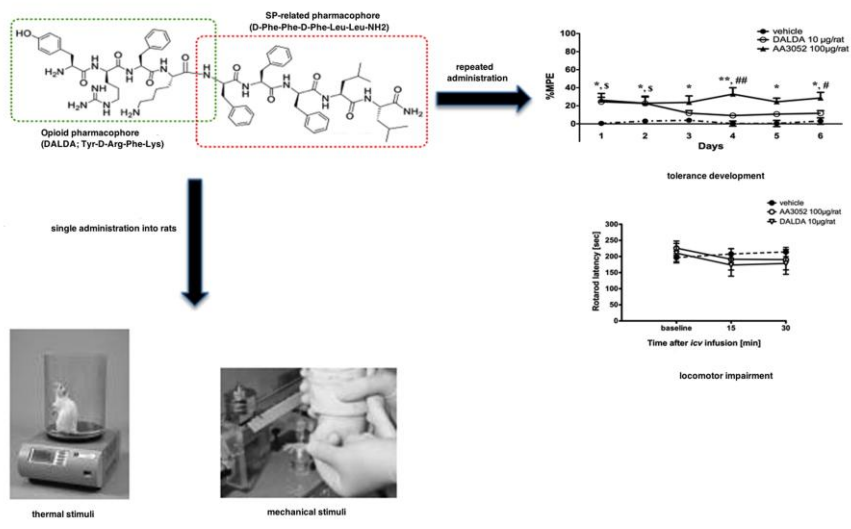
**Figure 4.** Competitive displacement of [<sup>3</sup>H]DAMGO (A) and specific stimulation of [<sup>35</sup>S]GTPγS (B) binding of (●) AA3052, (◆)AA3052 + naloxone and (Δ) AA3052 + spantide I. DAMGO (□) and SP (x) were used as reference compounds. Selective antagonists spantide I and naloxone were used in a 10 μM concentration in the [<sup>35</sup>S]GTPγS assay. Points represent means ± S.E.M. for at least three independent experiments, each performed in triplicate.

**Figure 5.** The effect of intracerebroventricular DALDA (10-200 μg/rat) and AA3052 (10-200 μg/rat) injection on the mechanical (A) and thermal (B) thresholds assessed in the Randall-Selitto and hot plate tests, respectively. \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001 vs. vehicle treated naïve rats at corresponding time; *n* = 6. %MPE = percentage of maximal possible effect.

**Figure 6.** Changes in mechanical pain threshold following repeated daily intracerebroventricular administration of AA3052 (100 μg/rat) and DALDA (10 μg/rat), assessed in the Randall-Selitto test. Analgesic responses were recorded every day, 30 min. after administration of tested compounds. \$ *p* < 0.05 indicated significant differences between DALDA and vehicle-treated naïve rats on a corresponding day. Similarly; \**p* < 0.05 and \*\**p* < 0.01 depicts differences between AA3052 and vehicle; # *p* < 0.05; ## *p* < 0.01 is a

comparison between AA3052 and DALDA at the same time point;  $n = 7-9$ . %MPE = percentage of maximal possible effect.

**Figure 7.** Total time spent by rats walking on a rotating rod after intracerebroventricular administration of AA3052 (100  $\mu\text{g}/\text{rat}$ ) and DALDA (10  $\mu\text{g}/\text{rat}$ ). The cut-off time was set at 300 s. Data are shown as mean latency  $\pm$  S.E.M. \*\*\* $p < 0.001$  vs. vehicle treated naïve rats at corresponding time;  $n = 7-9$ .



Graphical abstract