Determination of the anti-inflammatory properties and analgesic activity of the AA3052 chimeric peptide against CFA-induced inflammatory pain*

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In this study, we determined the antihyperalgesic and anti-inflammatory activity of AA3052, a novel opioid-substance P hybrid peptide in a rat Complete Freund's adjuvant (CFA) inflammatory

pain model. Central antinociceptive activity of AA3052 was assessed after intracerebroventricular injection using plantar and Randall-Selitto assays. The antinociceptive potency of the hybrid peptide was then compared with its opioid pharmacophore DALDA. In addition, intraplantar injection of AA3052 was performed to analyze its peripheral activity. Serum levels of IL-1β, IL-6, IL-10, and TNF-α accompanied with COX-2 activity were determined by ELISA. Histopathological analysis of the inflamed paws was also conducted to determine the possible modulatory effect of AA3052 on cytokine release. Intracerebroventricular AA3052 was found to act as an inhibitor of inflammatory pain as it effectively attenuated both thermal and mechanical nociceptive behaviors in CFA-treated rats. However, it had no effect on peripheral inflammation as no changes were observed in serum levels of pro- and anti-inflammatory cytokines regardless of the route of administration. Furthermore, a significant increase in paw edema in intraplantarly AA3052-treated rats compared with control was observed, possibly indicating tissue remodeling. Histopathological analysis revealed extensive necrotic damage in the paws of DALDA-treated rats, which correlated with profound hyperalgesia; and this was in contrast to the AA3052-treated group.
Our findings indicate that our newly designed hybrid peptide can modify the progression of the CFA-induced inflammatory phenomena.

KEY WORDS: cytokines / DALDA / hybrid peptide / immunomodulator / inflammation

Abbreviations

CFA – complete Freund's adjuvant; DALDA – H-Tyr-(D)Arg-Phe-Lys-NH$_2$; i.c.v – intracerebroventricular; IGF – insulin-like growth factor; i.pl – intraplantar; MOR – mu opioid receptor; MPE – maximal possible effect; NK1 – neurokinin 1 receptor; PDGF – platelet-derived growth factor; SP – substance P; VEGF-A – vascular endothelial growth factor.

Inflammation is strictly related to several events from which pain is one of the most important. Induction of inflammation either by chemical agents (e.g. carrageenan, complete Freund's adjuvant, zymosan), mechanical injury, or microbial infection (e.g. *Staphylococcus aureus, Pseudomonas aeruginosa*, fungi) leads to painful sensations. Pain can be further intensified by thermal or mechanical stimulation. During the inflammatory process, numerous pro-inflammatory mediators are produced and released rapidly into the inflammatory milieu. These include interleukin 1β (IL-1β), interleukin 6 (IL-6), cyclooxygenase-2 (COX-2), and tumor necrosis factor (TNF-α). Furthermore, both the production and inhibition of such pro-inflammatory cytokines is mediated by several endogenous neuropeptides. Opioids and substance P (SP) have long been implicated in immune system modulation and in the genesis and propagation of inflammatory processes [O'Connor *et al.* 2004, Pintér *et al.* 2014, Rittner *et al.* 2009, Stein 2013, Walker 2003]. Although both the aforementioned endogenous peptides participate strongly in the inflammatory response, they exert opposite actions. Indeed, numerous reports declare opioids and opioid receptor agonists act as potent inhibitors of inflammation and associated pain [Green and Levine 1992, Stein 2013, Stein and Küchler 2012, Yaksh 1988]. In contrast, SP expresses pro-inflammatory effects [Bost 2004, Douglas and Leeman 2011] and neurokinin 1 (NK1) receptor-related antagonists are reported to be potent anti-inflammatory drugs [Binder *et al.* 1999, Landau *et al.*

2007, Makino *et al.* 2012, Uematsu *et al.* 2011]. Considering aforementioned, we have synthesized a chimeric compound (codenamed AA3052) that encompasses in one entity both NK1 receptor- and opioid receptor–related fragments. Furthermore, we have compare its activity to the tetrapeptie DALDA ([D-Arg2, Lys4]-dermorphin-(1-4)-amide), a selective MOR agonist.

Recently, this opioid-substance P hybrid peptide (AA3052) was found to produce modest analgesic activity in an animal model of acute pain after intracerebroventricular (i.c.v) administration. This effect was not related to opioid receptor binding, as AA3052 stimulated G protein activation, both with affinity and intrinsic activity, typical of partial agonists that are not reversed by naloxone. This phenomenon may be a consequence of DALDA pharmacophore inactivation. However, the SP-related fragment of the chimera remains intact and is able to interact with NK1 receptors, as shown by attenuation of AA3052 binding by spantide I, an SP antagonist [Kowalczyk *et al.* 2016]. Despite producing weak central analgesic activity, AA3052 does not induce motor impairment or trigger tolerance development at the dose examined [Kowalczyk *et al.* 2016]. Therefore, AA3052 may be useful in long-term treatment of persistent inflammatory pain. Thus, in this study we aimed to investigate the central (intracerebroventricular) and local (intraplantar) antihyperalgesic activity of AA3052 in the CFA rat model of inflammatory pain. Such route of administrations was based on the following criteria: 1) DALDA possesses limited permeability across the blood–brain barrier [Samii *et al.* 1994]; 2) a central site of action of NK1 receptor-related ligands appears to be important for the antinociceptive effects [Campbell *et al.* 1998, Patel *et al.* 1996]. On the other hand, considering clinical utility where peripherally active drugs are highly requested, we have tried to determine the hybrid-induced effect after its intraplantar injection.

Additionally, AA3052 influence on inflammatory mediators release was also evaluated.

Material and methods

Drugs and reagents

AA3052 (H-Tyr-*D*-Arg-Phe-Lys-*D*-Phe-Phe-*D*-Phe-Leu-Leu-NH$_2$) and DALDA (Tyr-*D*-Arg-Phe-Lys-NH$_2$) were synthesized at the Department of Neuropeptides at Mossakowski Medical Research Centre PAS (Poland) using a Solid Phase Peptide Synthesis procedure. Complete Freund's adjuvant (CFA; 1 mg/ml of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate) was purchased from DIAG-Med (Poland).

Animals

Male WAG (Wistar Albino Glaxo; Warsaw) rats weighing 200 - 220 g were used in the study. Animals (n=40) were randomly divided into four experimental groups. Group size was determined based on the power analysis. The optimal number of animals

in each group was calculated to be 6. Rats showing no signs of inflammation or those that either removed their cannulas or showed signs of paralysis were not included in the study. Treatment groups were as follows: i) CFA-injected group that received test drugs, AA3052 (n = 7) and DALDA (n = 7) i.c.v; ii) CFA-injected control group treated i.c.v with vehicle (control; n = 7); iii) sham (non-CFA) animals receiving i.c.v vehicle (sham; n = 7); iv) CFA-injected group given AA3052 intraplantarly (i.pl) (n = 6). All drugs were dissolved in sterile distilled water and administered in a volume of 3 μl. The experimenters were all blinded to treatments.

The experimental protocols for animal use were approved by the I and II Local Commissions for the Care and Use of Laboratory Animals for Experimental Procedures in Warsaw (Permit numbers: 35/2011, 15/2015 and 134/2016) and followed the compliance guidelines published in the European directive 2010/63/EU on the protection of animals used for scientific purposes.

Behavioral studies

Intracerebroventricular implantation of cannula. Rats were cannulated according to the method of Noble and Wurtman [1967] with slight modifications as described by Robinson *et al.* [1969] and Strada *et al.* [1970]. Briefly, before the surgery, animals were anaesthetized with a combination of ketamine (70 mg/kg, i.p) and xylazine (6 mg/kg, i.p). An incision was made to expose the skull. Cannulas (Intramedic™ Polyethylene Tubing, Clay-Adams Company, New York) (ID = 0.28 mm, OD= 0.61 mm and 13 cm length) were implanted into the right lateral ventricle. Each guide cannula was fixed with a special cement substance to the bone of the skull. After the surgery, the animals were placed in separate cages and allowed a five-day recovery period.

CFA-induction of inflammation and drug administration. Complete Freund's adjuvant (CFA) was used to induce inflammatory pain. For this purpose, animals were injected i.pl with 50 μl of CFA into a right hind paw (group i, ii and iv). Sham animals (group iii) were injected with water (i.c.v) instead of CFA. The induction of inflammation was confirmed by paw edema formation as well as the assessment of nociceptive behavior (paw withdrawal or paw licking) upon thermal stimulation. The initial experiment was performed 7 days post CFA-injection in order to induce a well-evolved inflammatory process. AA3052 and DALDA were administered to rats by an i.c.v route for 6 consecutive days.

From the clinical point of view, it is very important for a drug to be active peripherally. Therefore, in order to examine the local action of AA3052 the compound was delivered i.pl into the CFA-injected paw within the same time period as stated above. In each case AA3052 was dissolved in water and injected at a dose of 100 μg/ rat (this dose was chosen based on our previous report and following dose screening studies [Kowalczyk *et al.* 2016]). DALDA was injected at a dose of 10 μg/rat. Both i.c.v and i.pl administration of drugs was performed using a Hamilton syringe.

Measurement of behavioral pain responses after mechanical or thermal painful stimuli. *Thermal hyperalgesia:* The pain-relieving effect of tested drugs as a response to thermal noxious heat was assessed using the plantar test (Ugo Basile, Italy)

according to the method described by Hargreaves *et al.* [1988]. Before testing, each rat was placed in a plexiglas chamber for 15 min in order to habituate to the environment. Next, radiant heat with the IR adjusted to 80 was applied to the plantar surface of the rat hind paw. Paw lifting or licking were scored as nocifensive behavior and recorded as paw withdrawal latency (PWL). PWL was measured every day in triplicate. The cut-off time was set to 20 s in order to prevent tissue damage.

Mechanical hyperalgesia: To quantify mechanical sensitivity the Randall-Selitto test was performed using the analgesymeter (Ugo Basile, Comerio, Italy). Briefly, the threshold was measured by application of incremental pressure to the dorsal surface of the hind paw. The measurement was terminated when the animal showed a clear sign of discomfort or escape. Cut-off time was set at 250 g to prevent tissue damage. In both tests, drug-induced antinociception was expressed as the percent of maximal possible effect, calculated according to the following equation: % MPE = 100 × [(test score – baseline score) / (cut-off score – baseline score)], where the baseline and test scores indicate the score before and after drug administration, respectively.

Blood collection and ELISA assays

Immediately after behavioral testing, rats were deeply anaesthetized with isoflurane and arterial blood samples were collected by cardiac puncture (with a 21-gauge needle; 0.8 x 40 mm; Becton Dickinson S.A., Spain). Blood was allowed to clot for 30 min at 37°C, and serum was separated by centrifugation (MPW Med. Instruments, Poland) at 1,800 rpm for 10 min. Serum was stored at -80 °C. Serum levels of IL-1β, IL-6, IL-10, TNF-α (Thermofisher, Poland), and COX-2 (Wuhan EIAab Science, China) were measured using ELISA kits according to the manufacturers' specifications. Absorbance at 450 nm was measured in each well with a spectrophotometer (BIO-RAD Microplate Reader, Warsaw, Poland).

Measurement of paw weight

The degree of edema as a marker of CFA-induced inflammation, both in control and drug-injected groups, was determined by paw weighing on an analytical balance (Ohaus, USA).

Paw punch biopsy and histopathological studies

After decapitation, rat paw was cut by sterile scissors at the level of hock joint. Then, a small sterilized skin punch (5 mm in diameter and with tip length of approx. 8 mm) was introduced perpendicularly to the surface of pad skin (central part on rat paw), rotated and advanced until reaching approx. 3–4 mm depth. Skin sample was immediately fixed in 10% (v/v) buffered formalin for 24 h. Preparations were embedded in paraffin, sectioned (5 μm thickness), and stained with either hematoxylin and eosin (H&E) or Masson-Trichrome.

Hematoxylin and Eosin staining. Briefly, 5 μm skin sections were de-waxed in a series of xylene solutions for 30 min and rehydrated in graded ethanol solutions. Sections were washed with tap water and stained for 20 min with hematoxylin to visualize nuclei

and again washed with tap water. Tissues were differentiated in 0.5% (v/v) acid-alcohol solution, washed and counter-stained with Eosin Y for 10 min and washed again. Later, sections were dehydrated in graded ethanol, acetone, cleared with xylene, and mounted on slides. All reagents were bought from Sigma-Aldrich (Germany).

Masson-Trichrome staining. To observe collagen deposition, the Masson-Trichrome staining kit (DiaPath S.p.A, Martinengo Italy; code 010210) was used. After de-waxing, skin samples were stained according to a standard protocol by DiaPath. Sections were examined under the Eclipse Ni-U Nikon light microscope (Nikon, Japan) equipped with a CCD camera and a PC-based image analysis system.

Immunohistochemistry

Briefly, de-waxing was performed as described for H&E staining. Skin biopsies were washed three times (3 x 5 min) with PBS and blocked in 10% (v/v) goat serum (in PBS with 0.25% (v/v) Triton X-100) for 60 min at room temperature. Next, skin biopsies were incubated for 60 min with a Polyclonal Rabbit Anti-CD68 antibody (Rockland - cat no. 600-401-R10; 1:100) dissolved in PBS. After three washes (3 x 5 min) in PBS, biopsies were incubated for 60 min with Fluor™ 488 Goat Anti-Rabbit IgG (H+L) Antibody (Applied BioProbes - cat. no. L110B; 1:200). Hoechst 33342 dye was used to stain the nuclei. Immunofluorescence was analyzed under a fluorescent microscope (Nikon, Japan) equipped with a CCD camera and a PC-based image analysis system. Specificity of the staining was tested by running the same procedure on the respective sister sections with the primary antibody absent in the incubation mixture. Control sections revealed no immunosignal.

Data analysis

Mechanical and thermal hind paw withdrawal thresholds were converted to percentage of maximum possible effect (%MPE). All data are presented as the mean ± SEM of 6-7 rats. Behavioral data were analyzed using two-way ANOVA, followed by the Newman-Keuls post-hoc test, unless otherwise stated. The degree of inflammation together with the results obtained from ELISA experiments were analyzed by one-way ANOVA with Tukey post test. Differences were considered significant at $P < 0.05$. All comparisons were calculated using GraphPad Prism 5.0 software for Macintosh (GraphPad software, San Diego, CA).

Results and discussion

The impact of AA3052 on CFA-induced mechanical and thermal hypersensitivity

When given i.c.v for 6 days, AA3052 produced modest, but long-lasting anti-nociceptive effect to mechanical and thermal stimuli following CFA-induced inflammation (Fig. 1 and 2, respectively). On the other hand, DALDA showed hardly any effect, except for the first day of treatment. Interestingly, it seems that the effectiveness of the chimera depends on the stimulus modality used. Namely,

repeated administration of AA3052 did not result in dampening of the analgesic effect in response to mechanical stimuli within the entire period of treatment (% MPE ranging from 11 to 16%; Fig. 1A). Conversely, a gradual decline in thermal threshold was observed in the AA3052-treated groups (Fig. 2A, C). Moreover, unlike central administration, intraplantar administration increased both mechanical and thermal hyperalgesia when measured either after a single injection (Fig. 1B and Fig. 2B) or following daily administration (Fig. 1C and Fig. 2C).
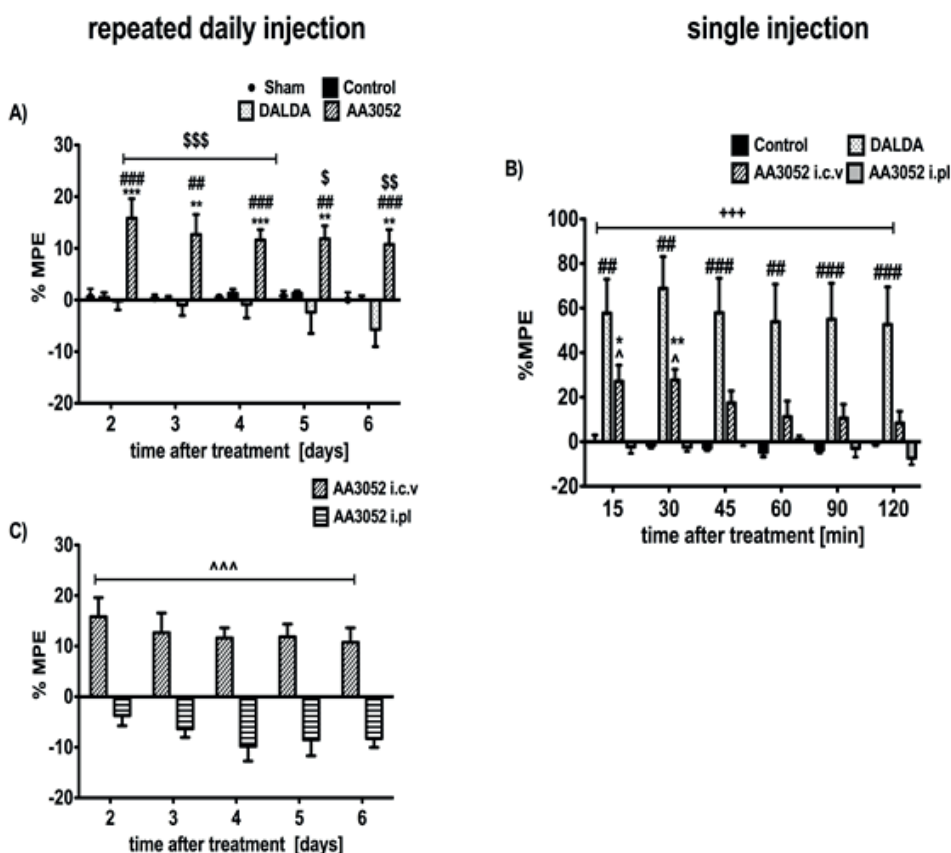

Fig. 1. AA3052-induced time-dependent effect on mechanical hypersensitivity. A) Antinociceptive effect of repeated daily i.c.v administration of AA3052 (100 µg/rat) and DALDA (10 µg/rat); B) Acute antinociceptive effect of i.c.v DALDA (10 µg/rat) and i.pl and i.c.v AA3052 (100 µg/rat); C) Comparison of % MPE of i.c.v and i.pl AA3052. Two-way ANOVA followed by Newman-Keuls post test revealed significant differences between DALDA and control (+++P<0.001; panel B), DALDA and AA3052 i.c.v (##P < 0.01 and ###P < 0.001; panel A and B), AA3052 i.c.v and control (**P<0.01 and ***P<0.001 for panel A, and *P<0.05 and **P<0.01 for panel B, respectively), AA3052 i.c.v and sham ($P<0.05, $$P <0.01 and $$$P<0.001; panel A), and AA3052 i.c.v and AA3052 i.pl (^P < 0.05 for panel B and ^^^P < 0.001 for panel C, respectively). There were no statistical changes observed for AA3052 i.pl and control (panel B). % MPE = percentage of maximal possible effect; n = 6-7 rats/group.
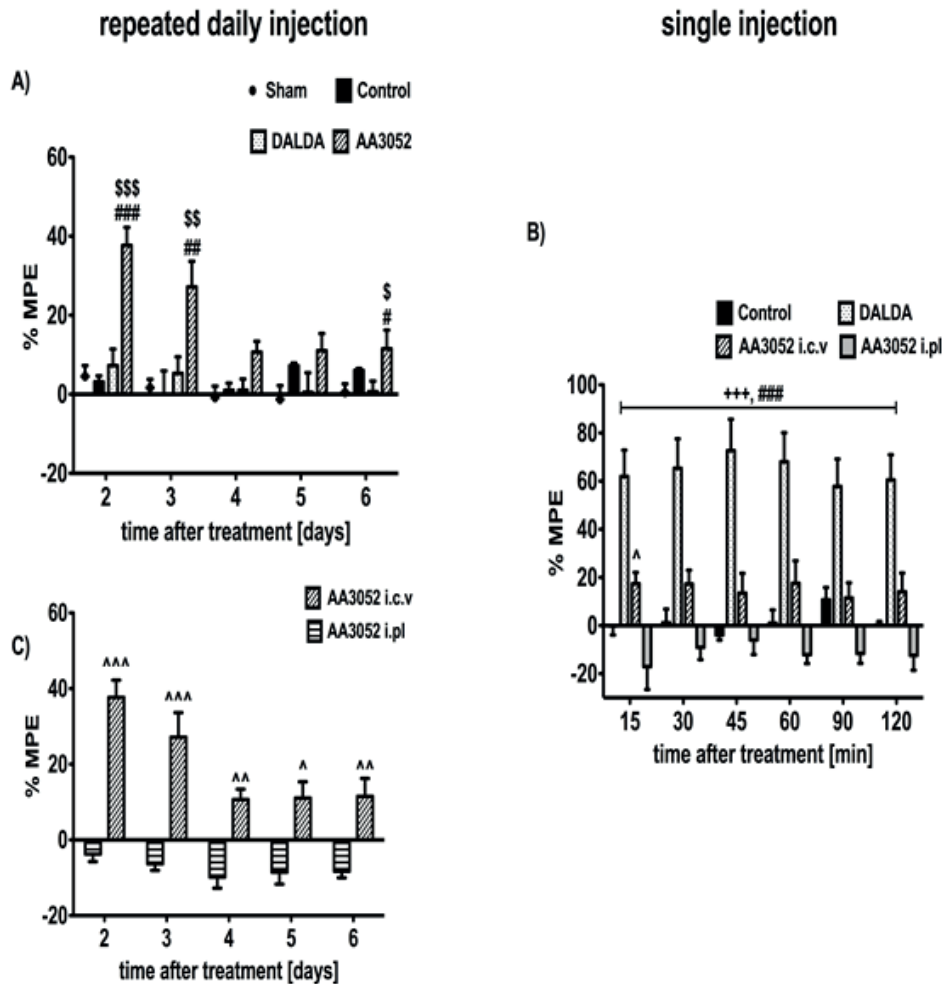
Fig. 2. AA3052-induced time-dependent effect on thermal hypersensitivity. A) Thermal pain sensitivity after subchronic i.c.v administration of AA3052 (100 µg/rat) in comparison to i.c.v DALDA (10 µg/rat) and water-treated CFA-groups; B) Acute anti-nociceptive effect of i.c.v DALDA (10 µg/rat) and i.pl and i.c.v AA3052 (100 µg/rat) in comparison to control; C) Comparison of % MPE of i.c.v and i.pl AA3052. Two-way ANOVA followed by Newman-Keuls post test revealed significant differences between DALDA and control (+++$P<0.001$; panel B), DALDA and AA3052 i.c.v (#$P<0.05$, ##$P<0.01$ and ###$P<0.001$ for panel A and ###$P < 0.001$ for panel B), AA3052 i.c.v and control (**$P<0.01$ and ***$P<0.001$; panel A), AA3052 i.c.v and sham ($\$P<0.05$, $\$\$P<0.01$ and $\$\$\$P<0.001$; panel A), and AA3052 i.c.v and AA3052 i.pl (^$P<0.05$ for panel B and ^$P<0.05$, ^^$P<0.01$ and ^^^$P<0.001$ for panel C, respectively). No statistical differences were observed for DALDA vs. sham ($P>0.05$; panel A) and AA3052 i.c.v vs. control ($P>0.05$; panel B). In addition, intraplantar injection of AA3052 did not exert a significant change when compared with control (panel B). % MPE = percentage of maximal possible effect; n = 6-7 rats/group.

Paw weight changes after AA3052 administration in CFA rats

Rats injected with CFA (control) demonstrated a marked increase in paw volume and paw weight when compared with sham animals (Fig. 3). In addition, CFA-induced edema was not affected by i.c.v injection of AA3052 ($P>0.05$). However, when administered intraplantarly, AA3052 caused a slight, but significantly larger swelling in comparison with control animals (1.73 ± 0.07 g vs. 2.11 ± 0.16 g, $P<0.05$).
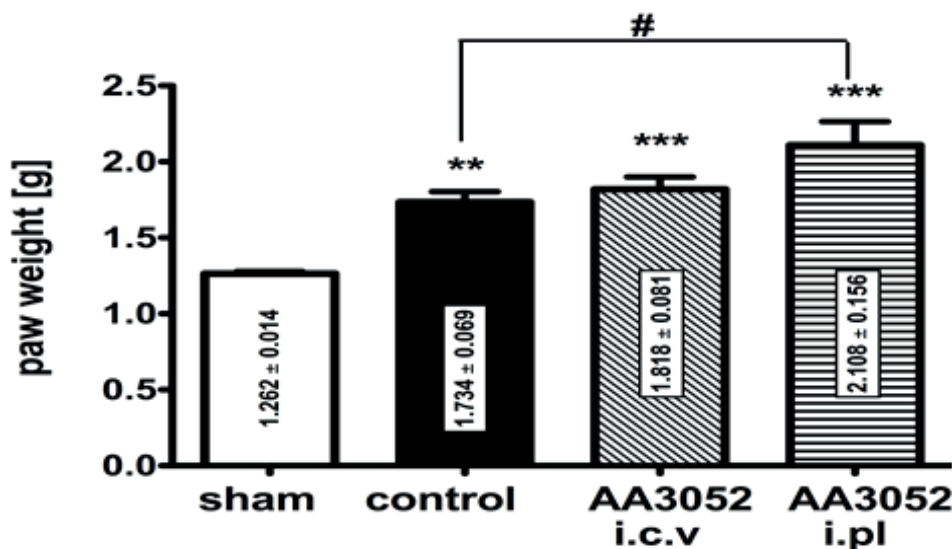

Fig. 3. Intracerebroventricular and intraplantar administration of AA3052 affected CFA-induced paw edema in rats to a similar extent. Visible changes in paw weight of rats treated with AA3052; One-way ANOVA followed by Tukey post test revealed no significant differences between i.c.v and i.pl AA3052. However, in comparison to controls, i.pl AA3052 caused a significant increase in CFA-induced edema (#$P<0.05$). Furthermore, a significant difference was observed between both i.pl and i.c.v AA3052 in comparison to sham group (***$P<0.001$), and between the control and sham group (**$P<0.01$), respectively. No differences were noted between i.c.v and control.

Effect of AA3052 on serum levels of inflammatory mediators in CFA rats

AA3052 given either i.c.v or i.pl to rats with CFA-induced inflammation did not affect serum levels of selected cytokines after 6 days of repeated administration (Fig. 4A and 4B) when compared to CFA alone. Although we saw a slight downward trend in IL-6 levels they did not reach statistical significance. Additionally, there was no difference in cytokine levels between control and sham groups. However, a significant reduction in IL-1β was observed in DALDA-treated rats when compared with control and sham group rats.
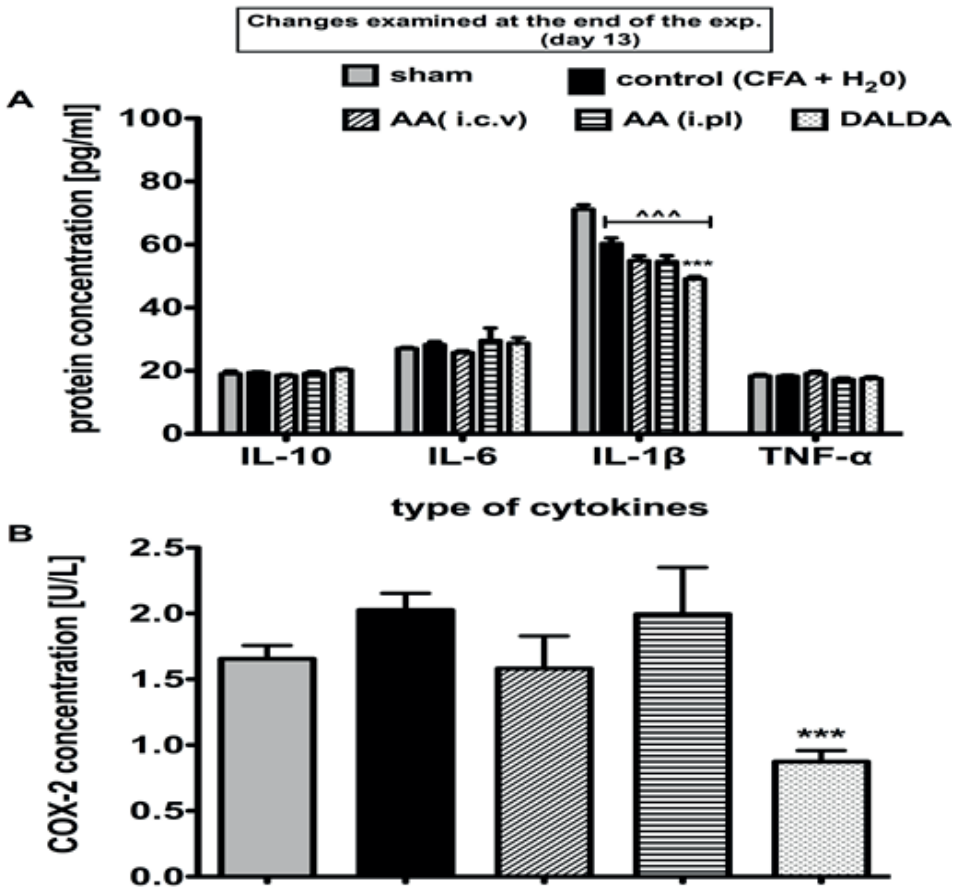
Fig. 4. Effect of repeated AA3052 intracerebroventricular administration (100 µg/rat) on serum levels of IL-1β, IL-6, COX-2, and TNF-α. Drugs were delivered to animals for 6 days beginning from the last day of CFA-injection (day 7). Measurements were taken on day 13 starting from the beginning of the experiment (panel A and B). One-way ANOVA followed by Tukey post-hoc test was used to show significant differences as compared with control (water-treated animals with CFA-induced inflammation). Differences were considered significant when $P<0.05$; * – in comparison to control and ^ – in comparison to sham groups.

**The effect of subchronic AA3052 treatment on histopathological changes
in paw tissue of CFA rats**

H&E staining revealed that i.c.v administration of AA3052 resulted in several changes in the CFA-injected paw tissue. A mixed histiocytic and lymphocytic dermal and subdermal infiltrate with different quantity of lymphocytes was noted (Fig. 5-A7 and 5-A8). In some rats, the inflammatory response also revealed blood vessel

pathology, such as fibrinoid wall necrosis, thrombin in the lumen, and extravasated erythrocytes (Fig. 5-A7 and 5-A8). Additionally, the presence of neutrophils, especially around the paraffin droplets, was noticed. In DALDA-treated rats (Fig. 5-A9 and 5-A10) the main histopathological observation from this group included a strong, nodular, compact mixed cell infiltrate with significant fibrosis. Moreover, a histiocytic, granulomatous response was dominated by lymphocytes and neutrophils forming intradermal microabscesses in the inflammatory infiltrate. Additionally, dermal necrosis was clearly visible. In this group, erosions and superficial ulcers caused by skin necrosis were observed. The missing epidermis was substituted by fibrin and a neutrophilic crust.

The tissues in the control group (i.pl CFA and i.c.v water; Fig. 5-A3 and 5-A4) were characterized by a superficial and deep dense lymphohistiocytic infiltrate, with the formation of granulomas with accompanying lymphocytes in response to CFA. In contrast, histology from sham animals revealed typical morphology of the paw (Fig. 5-A1 and 5-A2), which included an epidermis covered with a compact layer, wavy epidermal-dermal junction, dermis with blood vessels, and nerves with scattered lymphocytes and mast cells between vessels and collagen bundles.

Surprisingly, AA3052 injected into the paw (i.pl; Fig. 5-A5 and 5-A6) resulted in a typical granulomatous response with epithelioid histiocytes coalescing together to form small granulomas with accompanying lymphocytes without caseation of necrosis corresponding to tuberculoid granulomas. Such tuberculoid granulomas were additionally mixed with paraffinomas belonging to foreign body granulomas and with interstitial granulomatous dermatitis. In addition, no neutrophils were found. All presented features were absent in the contralateral paw of each group tested.

Masson-Trichrome staining showed that in the control group (Fig. 5-B2) medium and deep dermal collagen was pushed aside by oval tuberculoid granulomas and paraffinomas. A similar picture was obtained with i.c.v administration of AA3052 (Fig. 5-B4). Whereas, when AA3052 was administered i.pl (Fig. 5-B3), Masson-Trichrome staining revealed more dense and tightly packed collagen fibers. The more fibrotic arrangement of collagenous stromas presented with less slit like spaces between collagen bundles. Finally in DALDA-treated rats (Fig. 5-B5), Masson-Trichrome staining showed densely packed, thick collagen bundles throughout the dermis (almost to the homogenous layer). Spaces between granulomas appeared dark blue, indicating marked fibrosis.
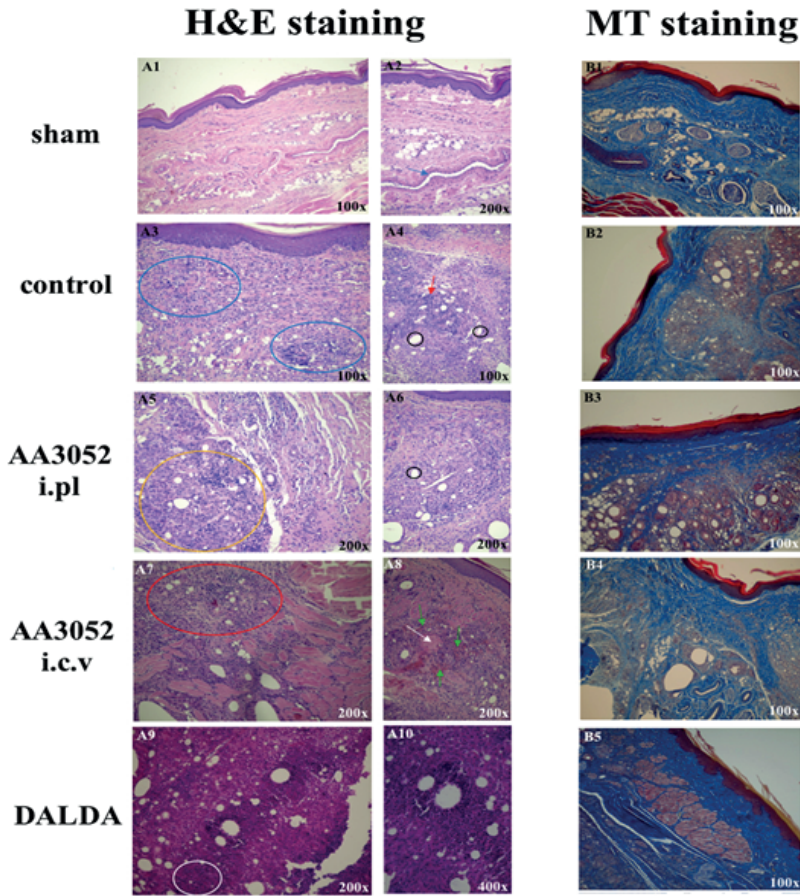

Fig. 5. Histopathological changes in rats with CFA-induced inflammation treated with AA3052 (i.c.v or i.pl) and DALDA (i.c.v) as revealed by H&E staining (A) and Masson-Trichrome staining (B). Blue circle – lymphohistiocytic infiltrate, blue arrow – blood vessel, red circle and arrows – lymphocytes, black circles – paraffin droplets, yellow circle – tuberculoid granulomas, white arrows – thrombi in the lumen, green arrow – erythrocytes, white circle – necrosis.

In animals injected i.c.v with AA0352, anti-CD68 staining revealed that cells from the macrophage lineage including monocytes, histiocytes, and giant cells (white arrows) predominated (Fig. 6). Similar results were observed in i.pl AA0352 and control groups, however, the signal was weaker compared with animals injected with i.c.v AA0352. In tissue sections taken from the DALDA group, a very low density of CD68-positive cells was observed compared with both i.c.v and i.pl AA3052. Also, a very weak anti-CD68 staining was demonstrated for the sham group.
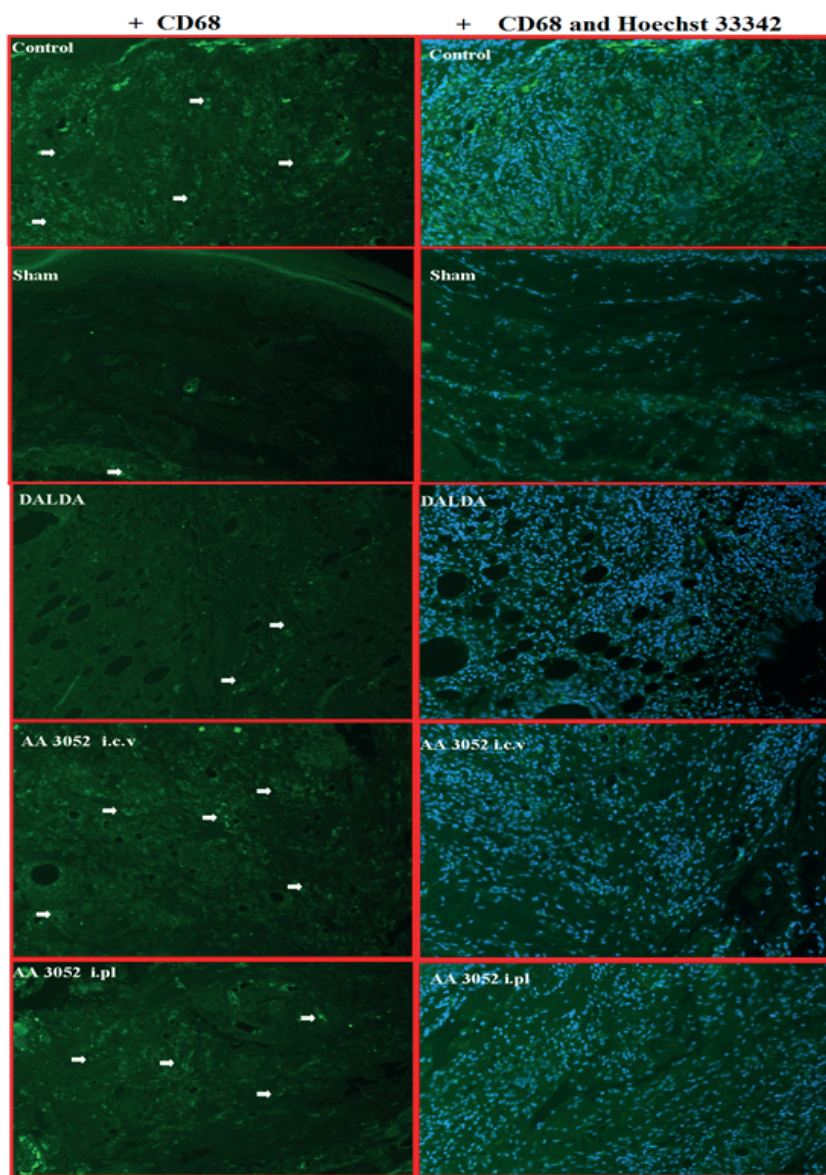
Fig. 6. CD68 immunostaining of paw skin biopsies following AA3052 administration in comparison to DALDA and control. The tissues were immunostained for the macrophage marker CD68 (light green marked with white arrows; left panel). Hoechst 33342 (blue fluorescent) was used to visualize nuclear staining (right panel). Original magnification, 100×.

Opioids are highly involved in the attenuation of inflammation [Chicre-Alcântara *et al.* 2012, Iwaszkiewicz *et al.* 2013, Walker 2003], while SP is a well-known pro-inflammatory agent [Massaad *et al.* 2004, Rameshwar 1997]. Following the definition of hybrid compounds as structures possessing multiple functions, we designed a novel opioid-NK1 peptide tailored to target inflammation and related pain. Here, the antinociceptive properties of AA3052 were only observed after central administration. Similarly to previously reported results, in an acute pain model [Kowalczyk *et al.* 2016], one-day treatment with DALDA administered at a much lower dose (10 µg/kg) than its hybrid analog (100 µg/kg), led to a significantly stronger anti-nociceptive effect (Fig. 1B and 2B). In contrast, after subchronic i.c.v administration, AA3052 produced a much more potent and long-lasting effect than its structural pharmacophore DALDA in response to thermal and mechanical stimulation (Fig. 1A and 2A). Importantly, some degree of opioid-induced mechanical hyperalgesia was noted in the DALDA-treated group.

Subsequently, we attempted to investigate whether locally administered AA3052 was able to elevate pain thresholds as well as exert anti-inflammatory activity. In this aspect, AA3052 seems to not possess the desired properties, as significant hyperalgesia in both mechanical and thermal pain tests (Fig. 1C and Fig. 2C) occurred. It is possible that the action of i.pl AA3052 depends heavily on either the route of administration or could have been induced by local tissue irritation. It is also possible that AA3052 could undergo systemic absorption and produce hyperalgesia as a result of conformational changes. Nonetheless, from the clinical point of view, these results imply that AA3052 might not be useful for further clinical use. However, considering that several new enhanced transport techniques are available (e.g. carriers transporting through a lipid bilayer such as liposomes or nanoparticles, oil-in-water nanoemulsion formulation), we cannot dismiss any of the potentially effective drugs which activities are only hampered by poor blood-brain barrier permeability. Such drugs should be specifically optimized to have the desired pharmacological profile to enable their clinical application.

Furthermore, apart from the results shown in Fig. 1A and 1C, data representing central delivery of drugs are consistent with several papers well documenting the effectiveness of chronic use of opioid agonists in various inflammatory pain models. In fact, several lines of evidence suggest that chronic use of opioid agonists caused desensitization of µ opioid receptors in several brain areas [Aoki *et al.* 2014, Hurley and Hammond 2001], thus leading to tolerance. This finding seems true for DALDA, however, the rapid loss of its antinociceptive activity is puzzling. Based on recent findings indicating that mainly long-term activation of peripheral opioid receptors in the presence of several inflammatory agents is responsible for the absence of tolerance to opioid-induced antinociception [Börzsei *et al.* 2008, Tokuyama *et al.* 1998], DALDA may not contribute to their activation. However, this could depend solely on the route of administration, as subcutaneous injection of DALDA compound revealed opposite results in rats with spinal nerve ligation (SNL)-induced neuropathic

pain [Tiwari *et al.* 2016]. On the contrary, the analgesic activity of AA3052 remains a matter of discussion. Subchronic central administration of the opioid-NK1 chimera did not result in tolerance development to mechanical pain thresholds in inflamed paws (Fig. 1C). Therefore, we can hypothesize that this compound did not lead to dysregulation of μ opioid receptor function. This in turn, provides additional evidence for the inactivation of the hybrid's opioid pharmacophore. Our recent findings showed that AA3052 did not readily compete for the same site with the potent μ-selective agonist DAMGO [Kowalczyk *et al.* 2016]. Therefore, the analgesic effect induced by this compound in an inflammatory pain model is obviously mediated either by activation or inhibition of an unknown receptor system, likely through the modulation of the NK1 receptor. There is also little probability of the involvement of other opioid receptors, i.e. δ and κ opioid receptors, as ligands activating these receptors revealed potent analgesic properties [Abdallah and Gendron 2017, Cahill *et al.* 2007 and 2014, Chavkin 2011]. For example, if we assume that the DALDA moiety constituting the AA3052 chimera adopts a conformation corresponding to the binding site of the receptors mentioned above and acting as an agonist, this would confirm the pain-relieving response exerted by the whole molecule. Additionally, this would reflect findings indicating that opioid ligands, including those targeting both types of receptors, modulate inflammation at different stages [Stein and Küchler 2012]. In fact, opioid receptor agonists, rather than antagonists, are constantly demonstrated to produce strong peripherally mediated anti-inflammatory effects [Binder *et al.* 2001, Romero *et al.* 2005]. However, considering our previous findings, which showed no reversal of AA3052 activity by naloxone [Kowalczyk *et al.* 2016], there is no doubt that this effect is not due to interactions between the compound and any of the three common opioid receptors. Hence, the pain-relieving actions of AA3052 in both acute and inflammatory pain models are apparently mediated through the activation of other unknown receptor systems and pathways. However, this hypothesis demands further investigation.

This concept seems quite plausible as hybrids represent a completely different group of compounds with highly diverse biological activity when compared to each pharmacophore administered alone [Kleczkowska *et al.* 2016]. Also, the behavior of the NK1 receptor-related fragment of AA3052 may be altered, particularly when we assume that the other chemically linked pharmacophore could influence its activity. In line with this, it was previously demonstrated that AA3052 bears features of an NK1 receptor partial agonist [Kowalczyk *et al.* 2016]. Therefore, assuming that effects elicited by the peptide likely result from 'switching on' its antagonist over agonist activity, this hypothesis would explain the results obtained. To strengthen this explanation it was previously reported that: i) peripheral inflammation results in transcriptional activation of several genes in the spinal cord and brain [Ceccarelli *et al.* 1999, Takeda *et al.* 2012], including NK1 receptor genes [Duric and McCarson 2007, Hamity *et al.* 2014]; ii) NK1 receptor antagonists are well known anti-inflammatory and analgesic agents [Makino *et al*. 2012, Stucchi *et al*. 2003], and iii) a number of pro-inflammatory mediators (e.g., IL-6, IL-8, TNF-α) are up-regulated as a result

of SP activity [Azzolina *et al.* 2003]. Additionally, NK1 receptor antagonists were shown to decrease the expression of some cytokines, particularly IL-6 [Martinez *et al.* 2015]. In fact we did observe a tendency of IL-6 to decrease in the AA3052-treated group, however, the results did not reach statistical significance (Fig. 4 A).

It is known that CFA-induced inflammation covers two phases: the acute phase lasting 24 h and the chronic phase characterized by the development of secondary hyperalgesia [Fraser *et al.* 2000]. Within each phase, the level of pro- and anti-inflammatory cytokines changes and many are phase-specific [Schaible *et al.* 2002]. Their release may not be obvious as it is often dependent on the type of drug used [Teng *et al.* 2005, Yang *et al.* 2016]. Also in our study, the expression of cytokines seems to be extensively modulated by examined compounds, particularly DALDA. Biochemical results obtained for DALDA do not correspond with the behavioral response. In fact, this selective μ opioid agonist was found to reduce serum levels of both COX-2 and IL-1β on day 13 after CFA (Fig. 4A and 4B), which is in parallel with the study by Philippe *et al.* [2003] showing DALDAs anti-inflammatory effect in colon inflammation. Nevertheless, the animals still remained hyperalgesic to both thermal and mechanical stimulation (Fig. 2A and 3A). Although COX-2 produces prostaglandins that enhance pain sensation [Funk 2001], they are obviously not the sole factor responsible for it. Additionally, COX-2 levels increase in inflammation, but the inflammatory process is suggested not to be the primary stimulus for COX-2 expression [Abdalla *et al.* 2005]. Furthermore, considering that IL-1β release is responsible for hyperalgesia [Ferreira *et al.* 2002], the results are controversial. On the other hand, inhibition of COX-2 corresponds to increased apoptosis [Peng *et al.* 2008]. Histologically, we did observe some signs of damaged tissue, but in the form of dermal necrosis with pronounced fibrosis. These two processes were thought not to bear any similarities as they are triggered by different factors (natural process *vs.* external factors). However, recent findings clearly indicate the existence of crosstalk between both models of cell death [Nikoletopoulou *et al.* 2013]. In fact, regarding *in vivo* conditions, a specific programmed form of necrosis, termed necroptosis, is very common [Jouan-Lanhouet *et al.* 2012]. In line with this, we cannot exclude the possibility that robust apoptosis, resulting cell debris, triggers neutrophil activation and induces tissue necrosis and significant fibrosis.

With regards to AA3052 and its ability to interfere with the inflammatory cascade, it was revealed that when injected i.c.v it caused neutrophil infiltration, albeit to a lesser extent and at a much slower rate than for DALDA (Fig. 5 and 6). Neutrophil activity is observed in the primary innate immune response [Salthouse 1984] and is intended to destroy infected or damaged tissue with subsequent reconstruction by monocytes and macrophages. This process leads to necrosis. However, it is difficult to say whether in our study neutrophil-mediated processes leading to tissue remodeling are favorable or deleterious. In fact, necrosis promotes the removal of damaged tissue and enhances healing, as the presence of macrophages, particularly the M2 type, is essential for activation of collagen synthesis [Mosser and Edwards 2008, Salegio *et*

al. 2011]. Macrophages are also crucial for the restoration of tissue homeostasis; they remove cellular debris and synthesize several various cytokines and growth factors (i.e. PDGF, VEGF-A, IGF-1) [Okabe and Medzhitov 2016]. In contrast, the necrotically altered tissue will be deprived of full properties, as some of them will not be restored (incomplete healing and repair, i.e. scarring or fibrosis). Therefore, it is obvious that a new tissue, although made up of the same elements (proteins) as the undamaged skin, consequently doesn't look the same; and this is because of the way of its arrangement.

Taken together, additional studies need to be performed in order to determine for instance, the character/phenotype of macrophages (pro- vs. anti-inflammatory) that are present in the studied tissue, levels of all inflammatory cytokines along with M(IFN-γ):M(IL-4) macrophages ratio (where M(IL-4) macrophages are considered as tissue repair macrophages in contrast to the first one). Additionally, the information about the accumulation level of γδT cell fraction at injury sites, which are widely reported as being pro-regenerative [Jameson *et al.* 2002], could be significant.

As seen in histological slices, AA3052 induced bleeding (Fig. 5), which could be mistaken for a highly unfavorable sign of tissue damage. However, several papers revealed that SP plays an important role in regulation of hematopoiesis and blood cell function [Goto *et al.* 1998, Imai *et al.* 1994]. Therefore, we can assume that this effect is possibly related to AA3052 shifting to an agonist role towards the NK1 receptor, rather than acting as an antagonist. Moreover, its impact on hematopoietic cells is more likely direct, as indirect interactions are mediated through stromal cells that are further stimulated to produce cytokines, particularly IL-6 and TNF-α [Lotz *et al.* 1987]; and this is not in parallel with our biochemical results (Fig. 4).

Conclusions

Although the results obtained in this study were difficult to interpret, there is evidence supporting the notion that AA3052, an opioid receptor-NK1 receptor related chimera, modulates the innate immune response. Additionally, the ability of AA3052 to produce antinociception insensitive to the development of tolerance upon subchronic treatment in acute and inflammatory pain models, speaks in favor for further assessment of its effectiveness in other pain states. The mechanism of action of AA3052 is highly complex, and we can only speculate that this novel compound acquires either NK1 receptor agonist or antagonist properties depending on the ongoing pathological condition.

Conflicts of interest: none

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