SYNTHESIS OF PEPTIDE ANALOGS OF VV-HEMORPHIN-7 – ANTINOCICEPTIVE AND ANTIHYPERALGESIC EFFECT

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ABSTRACT

Endogenous peptides have various modulating effects on many physiological and pathological processes, including the processing of pain information. Hemorphins are hemoglobin-derived peptides with affinity for both opioid receptors and insulin-regulated aminopeptidase. We recently reported on the antinociceptive effects of hemophins of different lengths and amino acid composition after their supraspinal injection.

The present study aims to elucidate the potential effects of locally (intraplantar) injected hemorphin VV-H7 (Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-NH_2) and its analogs with one or two Arg-Gly-Asp sequence on carrageenan-induced hyperalgesia in rats. We used indomethacin as a reference anti-inflammatory analgesic. Pain thresholds and paw volume were measured before and at 1, 3, and 4 hours after intraplantar carrageenan injection using a paw pressure test and plethysmometry in male Wistar rats.

The precursor peptide VV-H7 showed an antihyperalgesic effect comparable to that of indomethacin, while its peptide analogues one or two Arg-Gly-Asp sequence showed an acceleration of the antinociceptive effect, but shorter duration of antihyperalgesia and lack of efficacy against edema.

Keywords: hemorphins, carrageenan, hyperalgesia, RGD, rats.

INTRODUCTION

Hemorphins are hemoglobin-derived peptides that have an affinity for both opioid receptors and insulin-regulated aminopeptidase [1 - 3]. The synthesis of peptides that contain the Arg-Gly-Asp (RGD) sequence improves their binding to cellular adhesion receptors and is a prerequisite for localizing their effects. We recently reported achievements in the design, synthesis, and physicochemical characterization of two new N- and C-modified analogs of VV-hemorphin-7 containing RGD (Arg-Gly-Asp) residues as potential nociceptive agents after their intracerebral administration [4]. It has been shown that the addition of one or two RGD sequences to natural VV-hemorphin-7 (Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) increases its effect on acute nociception, but reduces it on the inflammatory phase depending on the concentration of the peptide. New data have been acquired about the variation of pharmacological activity of hemorphin analogs containing various natural and/or non-proteinogenic amino acid moieties [5 - 8].

Nociception is a process in which intense thermal, mechanical, or chemical stimuli are detected by peripheral Aδ and C nerve fibers called nociceptors [9, 10]. One subdivision of C fibers more sensitive to chemical stimuli such as capsaicin or histamine is involved in inflammation by altering the release of various substances [11]. The inflammation activates the release of extracellular inflammatory mediators and sensitization both at the site of injury and in the surrounding tissue. Hyperalgesia is an increased sensitivity to pain, which often occurs after an injury,

inflammation, or certain diseases primary in the area of injury, and secondary in a larger, unharmed area around the injury. Intraplantar injection of carrageenan into a rat's hind paw is a well-established model for the induction of hyperalgesia due to pain sensitization [12, 13].

Along with central inhibition of pain signal processing, the peripheral antinociceptive effect of opioids has been demonstrated in visceral pain as well as in the initial phase of carrageenan-induced hyperalgesia [14 - 17].

The present study aims to elucidate the potential effects of locally (intraplantar) injected VV-H7 (Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-NH_2), RGD1, and RGD2 on carrageenan-induced hyperalgesia in rats.

EXPERIMENTAL

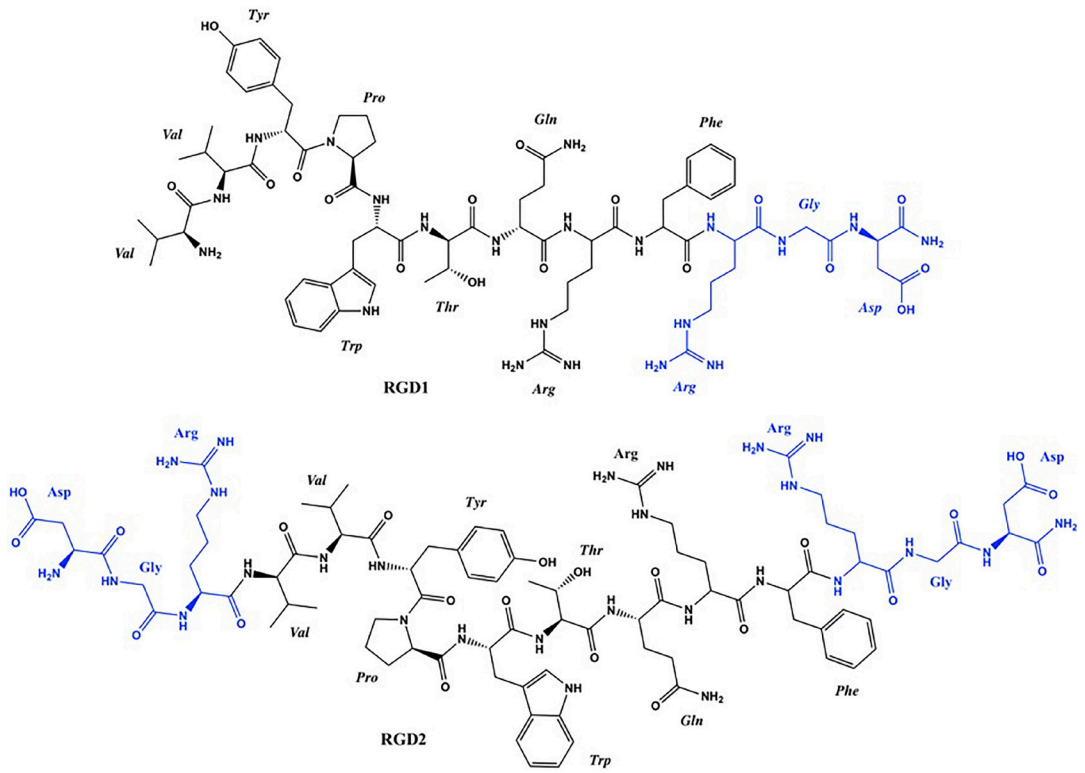
Chemistry

All reagents and solvents were analytical or HPLC grade and were purchased from Merck (Germany). The protected amino acids and Fmoc (9-fluorenylmethoxycarbonyl)-Rink Amide MBHA (4-methylbenzhydrylamine) Resin were purchased from Iris Biotech (Germany). Three-functional amino acids were protected as follows: N^{α}-Fmoc-Arg(Pbf)-OH, N^{α}-Fmoc-Tyr(tBu)-OH, N^{α}-Fmoc-Thr(t-Bu)-OH, N^{α}-Fmoc-Gln(Trt)-OH, and N^{α}-Fmoc-Trp(Boc)-OH.

The peptides were prepared by solid-phase peptide synthesis (SPPS)-Fmoc chemistry. The N- and C-modified analogs of hemorphins (RGD1 and RGD2) containing RGD (arginylglycylaspartic acid) residues following the structures dodecapeptide RGD1 (Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Arg-Gly-Asp-NH_2) and pentadecapeptide RGD2 (Asp-Gly-Arg-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Arg-Gly-Asp-NH_2) were synthesized as described in detail in Georgieva et al., respectively [4]. The chemical structures of the RGD-peptide analogs of VV-hemorphin-7 are presented in Scheme 1.

Animals

Adult male Wistar rats (180 – 200 g) were used. The animals were housed in groups of 8 per cage and kept under a normal 12 h light/dark cycle and 22 ± 2°C temperature. Rats had free access to food and water. All experiments were approved by Bulgarian Food Safety Agency No 218/2021, which is in accordance with EC



Scheme 1. Chemical structures of RGD-analogs of VV-hemorphin-7.

Directive 2010/63/EU for animal experiments.

Carrageenan-induced hyperalgesia and oedema

The rats were injected intraplantar with a solution of VV-H-7, RGD1, or RGD2 at a dose of 5 μg 5μL^{-1} rat, 5 minutes before the administration of the carrageenan as an inflammatory agent. The dose has been selected according to preliminary studies on their effects on brain tissue [4]. λ-Carrageenan (CRG, Sigma Aldrich) was freshly dissolved in saline at a concentration of 1 %, and injected intraplantar into the right hind paw, at an injection volume of 0.1 ml per rat's paw [18].

Measurement of mechanical pain threshold and paw edema

The mechanical pain threshold is determined with an analgesimeter (Ugo Basile, Italy) before, and at the 1st, 3rd, and 4th hour after the carrageenan injection. The required pressure (in grams) that elicits nociceptive reactions such as withdrawal or struggle is set as a threshold [19]. The volume of the animal's paws was measured with a plethysmometer (Ugo Basile, Italy) at the same time intervals as the pain threshold [20].

Data analysis

One and two-way ANOVA were used, followed by Tukey post-hoc test. SigmaStat and SigmaPlot v11.0 were used for calculations as well as the layout of the results. Values are mean ± S.E.M. Values of $P < 0.05$ are considered to show statistical significance.

RESULTS AND DISCUSSION

The present data show significant hyperalgesia in the injected with CRG Controls ($F\ 1, 56 = 42.893$, $p < 0.001$, injected with CRG vs non-injected paw), accelerating during the time ($F\ 1, 56 = 7.247$, $p < 0.001$) (Fig. 1 (A, B)), accompanied by increasing paw volume ($H = 26.079$, $p < 0.001$) (Fig. 2). The precursor peptide VV-H7 did not significantly alter the pain threshold at 5 minutes post-injection but showed a well-defined antihyperalgesic profile both at 1st ($F\ 1, 28 = 15,998$, $p < 0.001$) and at 3rd hour ($F\ 1, 28 = 4,242$, $p < 0,05$) after carrageenan injection (Fig. 1A). VV-H7 decreased the paw edema only during the 1st hour after the irritant ($p = 0.044$) (Fig. 2). RGD1 showed a local antinociceptive effect 5 minutes after its injection in the paw, before the

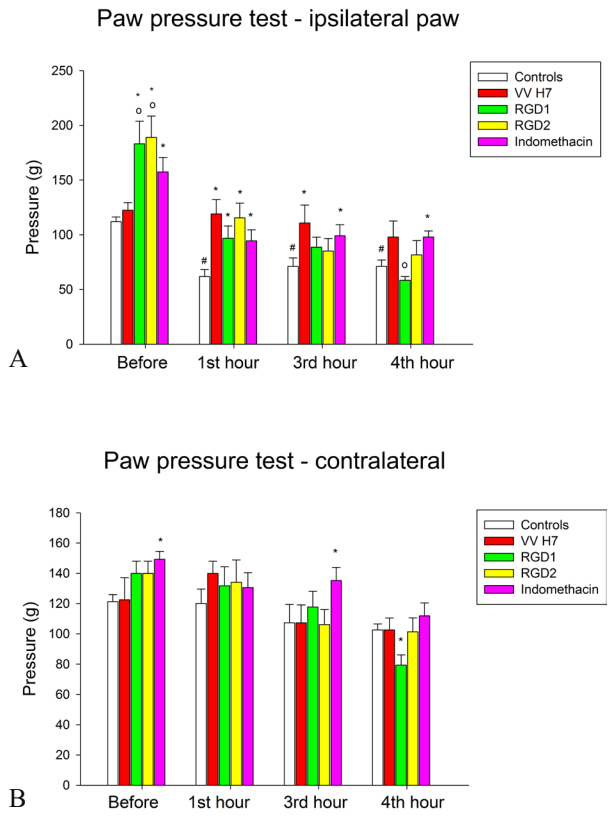


Fig. 1. Pain threshold measured by an analgesimeter A. on the right paw injected with carrageenan (ipsilateral). B. on the left paw not injected with carrageenan (contralateral). Data show the mean ± SEM grams required to induce paw withdrawal before and 1, 3, and 4 hours after the carrageenan injection. *$p < 0.05$ relative to controls in the same time interval; $^{\#}p < 0.05$ compared to controls before carrageenan injection; $^{o}p < 0.05$ relative to the VV-H7 group over the same time interval.

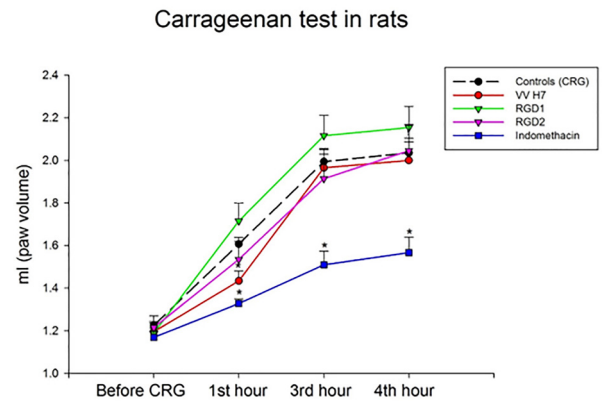


Fig. 2. Carrageenan-induced paw edema in rats. Each value represents the mean ± SEM before and 1, 3, and 4 hours after the carrageenan injection; *$p < 0.05$ compared to the control group.

induction of hyperalgesia ($F\ 1, 28 = 15.175$, $p < 0.001$), as well as an antihyperalgesic effect in the 1st hour after the carrageenan ($F\ 1, 28 = 5.234$, $p = 0.030$) (Fig. 1 (A)) without changes in paw edema (Fig. 2). RGD2 showed a similar local antinociceptive effect before the induction of hyperalgesia ($F\ 1, 28 = 18.648$, $p < 0.001$), as well as an antihyperalgesic effect in the 1st hour after the carrageenan ($F\ 1, 28 = 8.594$, $p = 0.007$) (Fig. 1 A) without changes in paw edema (Fig. 2). The effects of the tested peptides were comparable to the efficacy of the reference drug indomethacin, but it showed a longer antihyperalgesic effect ($F\ 1, 63 = 31.653$, $p < 0.001$) (Fig 1(A)) accompanied by a significant decrease in paw edema ($F\ 1, 63 = 81.8$ $p < 0.001$) (Fig. 2).

The present study provides, for the first time, data on the local antinociceptive and antihyperalgesic effects of peripherally administered hemorphin VV-H7 and its two newly synthesized analogs with RGD modification at one (RGD1) or both chain ends (RGD2). Their effects are as strong as those of the reference anti-inflammatory analgesic indomethacin and are limited to the injected area. The precursor peptide VV-H7 showed a longer-lasting effect than its counterparts, but both RGD analogs had a faster onset of antinociception. These data are informative regarding the molecular structure-efficiency relationship. Apparently, the modification of the ends of the peptide accelerated its local antinociceptive action and preserves its effects on hyperalgesia but decreased activity time and anti-edema effects. Based on the applied low doses and lack of the effect on the contralateral paw, we can assume that the studied peptides have exerted their effect on the level of primary sensory neurons, as they cannot reach a higher level of processing of sensory information. We hypothesize that the chemical modification of peptides alters their half-lives, thereby reducing their duration of efficacy.

In our recent studies, we have already established the antinociceptive effects of supraspinally injected hemorphins of different lengths and amino acid composition in a formalin test, an experimental model of nociceptive and inflammatory pain in mice [5, 21]. Our preliminary data show that hemorphin VV-H7 injected intracerebroventricularly has an antinociceptive effect and the addition of one or two RGD sequences increases its effect on the acute but decreases it on the second, inflammatory phase of the formalin test in a dose-dependent manner [4]. Comparing the current data with the preliminary ones, we can speculate that the lower efficiency in the second phase of the formalin test may be due to the reduced half-life of the tested peptides.

The antihyperalgesic effects of LVV-H7 and ANG IV at the spinal level have been established after their intrathecal injection in a model of thermal hyperalgesia [22]. The authors hypothesize at least two putative mechanisms of action of this group of hemorphins: the first is their action as atypical agonists of opioid receptors, and the second is their activity as insulin-related aminopeptidase inhibitors (IRAPs). The authors hypothesize at least two putative mechanisms of action of this group of hemorphins: the first is their action as atypical agonists of opioid receptors, and the second is their activity as insulin-related aminopeptidase inhibitors (IRAPs). Thus, the authors speculated that the effect should be not solely a result of the activation of opioid receptors. Moreover, their suggestion is in line with the findings of a similar binding affinity of LVV-H7 and ANG IV to IRAP [23]. In addition, both LVV-H7 and VV-H7 have been confirmed as receptor ligands for the human MAS-bound G protein-bound receptor X1 (MRGPRX1), which plays a role in somatosensory neurons involved in the perception of pain and itching, as well as in mast cells, where this receptor is responsible for Ig E-independent degranulation and stimulation of chemokines release [24 - 28].

Although RGDs containing peptides are often less effective than parental proteins, they are used as tools to promote cell adhesion, increase biocompatibility, prevent apoptosis, and facilitate the delivery of drugs for cancer therapy [29, 30]. The encouraging results for the antinociceptive and antihyperalgesic effects of the studied peptides indicate the need for further detailed study of their receptor-mediated cellular mechanism of action.

CONCLUSIONS

Our results showed that local administration of hemorphin VV-H7 and its analogs RGD1 and RGD2 containing RGD (Arg-Gly-Asp) moieties produced significant antinociceptive and anti-hyperalgesic effects in an experimental model of edema and hyperalgesia in rats. Modification of VV-H7 with one or two RGD sequences accelerates the antinociceptive effect but shortens the duration of antihyperalgesia and abolished anti-edema activity.

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