

ANTI-INFLAMMATORY POTENTIAL OF SOME 4-AMINOPYRIDINE'S PEPTIDE DERIVATIVES – IN VIVO AND IN VITRO EVALUATION

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Abstract

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system, characterized by inflammatory demyelination and neurodegeneration. Fampyridine (4-aminopyridine, 4-AP) is a drug used for MS treatment as it readily passes through the blood-brain barrier exhibiting anti-inflammatory effects. Its high toxicity, however, limits its wider application. To reduce 4-AP high toxicity, we have designed new peptides-containing 4-AP derivatives showing about 150 times lower toxicity than 4-AP. This work aims to investigate the cytotoxicity and in vivo anti-inflammatory effects of three newly synthesized peptide derivatives of 4-AP, defined as substances A, B, and C. In addition, their inhibitory potential against MMP-2 and -9 proteolytic activity was evaluated as well. To study the anti-inflammatory effect of the 4-AP-derivatives we used a rat model for immune complex-induced inflammation. In vitro cytotoxicity test was performed on WEHI-164 cells. Inhibition of matrix metalloproteinase (MMP-9 and -2) proteolytic activity was assessed by gelatinase zymography and densitometric analysis. Our results showed that treatment with the 4-AP derivatives kept cells viable and metabolically active showing no cytotoxicity. Substances A and C inhibited the proteolytic activity of both isoforms of MMP-2 and -9 even when applied in the lowest concentration, whereas substance B had no inhibitory effect on the MMP-9 activity and inhibited MMP-2 activity

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only applied at higher concentrations. Along with its inhibitory effect on the MMP-2 and -9 proteolytic activity, substance A demonstrated a clear anti-inflammatory effect either preventing or inhibiting the production of antibodies in the blood circulation of rats. Substance A might be very promising as a drug molecule.

Key words: MS, 4-AP derivatives, ant-inflammatory effect, cytotoxicity, MMP-2 and -9

Introduction. Multiple sclerosis (MS) is an autoimmune neurodegenerative disease of the central nervous system (CNS) characterized by inflammation, demyelination, loss of oligodendrocytes, and varying degrees of axonal degeneration [1]. In MS, the immune system attacks and destroys its own tissue such as myelin coating the axons and thus, the signals cannot be conducted effectively [2]. Currently accumulating data showed that B- and T-lymphocyte infiltration is found to be responsible for the formation of lesions at particular locations of the CNS [3]. Although the exact pathogenesis remains unknown several studies have shown a beneficial effect of anti-inflammatory and immunomodulatory drugs [4]. Various antibodies targeting autoantigens in the CNS have been identified in the serum of MS patients, signifying the involvement of humoral immunity in the process of inflammatory demyelination [5]. Very recently in *Frontiers of Immunology* 2023 IGLESIAS et al. [6] published an interesting study about the dual role of peripheral B cells in MS as emerging remote players in demyelination. The authors identified autoreactive myelin antibodies in extracellular vesicles (EV) released by peripheral blood B cells, able to cross BBB due to their small size. This finding “triggers a call for a reconsideration of peripheral immune response contributions to the pathogenesis of MS” [6]. 4-aminopyridine (4-AP) is one of the drugs used for the treatment of MS [7]. It was introduced in the 70s as Pimadine in the pharmaceutical industry of Bulgaria for neurodegenerative disease treatment [8]. In 2010 it was approved as Ampyra by the US Food and Drug Administration (FDA) and as Fampyra by the European Medicines Agency to treat patients with MS-related walking difficulties. 4-AP facilitates neural conduction in different neurologic diseases [9, 10]. In addition, 4-AP penetrates the blood-brain barrier and blocks potassium channels, leading to an increase in the acetylcholine’s level in brain providing an anti-inflammatory effect [11]. A significant disadvantage limiting the wider application of 4-AP is its high toxicity and higher doses of 4-AP used for the treatment of MS show a risk of epileptic seizures and impaired consciousness [12]. Thus, reduction of 4-AP toxicity is highly desirable. Our previous studies of 4-AP derivatives modified with peptide fragments revealed 8–18 times reduced toxicity [13]. They were tested on the cuprizone model of demyelination of corpus callosum in the mouse brain and on scopolamine-induced memory impairment, revealing a promising effect of two of them [14]. Recently, we designed three new peptides-containing 4-AP derivatives showing 150 times lower toxicity than 4-AP [15]. This study aimed to evaluate their cytotoxicity

and anti-inflammatory effects in vivo and in vitro.

Materials and methods. Chemicals. Three derivatives of 4-AP were designed and synthesized as follows: 1) Substance **A** (Boc-**Tle**-Leu-Asn-Leu-Ala-4-AP), 2) substance **B** (Boc-**Val**-Asn-Leu-Ala-4-AP), and 3) substance **C** (Boc-**Nva**-Asn-Leu-Ala-4-AP) [15]. The molecular structures of the compounds differ slightly, by only one amino acid at the N-side of the peptide chain as follows: compound **A** contains Tle, compound **B** contains Val and compound **C** contains Nva.

Animals and experimental model of mild immune complex-induced inflammation (ICII). Ten-week-old female Wistar rats weighing 200–250 g were housed in an Animal facility at IBIR-BAS and provided with standard food and water ad libitum. Rats were allowed at least one week to acclimate to the facility before use in any experiments to eliminate the effects of stress. Rat mild ICII model was performed according to [16] with minor modifications. In this model by an immunization with BSA-CFA (bovine serum albumin complete Freund's adjuvant), a production of anti-BSA antibodies was generated in the circulation of the rats without deposition of immune complexes in the rats' tissues. Briefly, BSA was dissolved in sterile water at a concentration of 2 mg/ml and before injection was emulsified with an equal volume of 1 mg/ml CFA (Sigma Aldrich). Rats were subjected to two subcutaneous applications (100 μ l of emulsion) in the base of the tail on day 0 and day 15 (booster dose BSA only). Four immunized experimental groups were defined for each 4-AP derivate, as follows: two groups treated with the compound at doses 80 and 160 μ g per animal per day seven days before immunization (BI-80 and BI-160) and two groups treated with the compound at doses 80 and 160 μ g per animal per day seven days after immunization (AI-80 and AI-160). The doses were based on a range at or below the mass-adjusted values for humans [17]. The compound was administered to the intraperitoneal (ip) compartment. The animals were anesthetized and sacrificed 24–72 hours following administration of the compound for AI groups and of booster dose for BI groups. Rats that were immunized and treated with PBS before or after immunization were included as control groups. All experiments were performed with a minimum of three animals per group. The animals were reared and handled following Bulgarian Veterinary Law (25/01/2011) regarding the life conditions and welfare of experimental animals, which is adapted to the European Union regulation 86/609 and Directive 2010/63/EU. The experimental protocols were approved by the National Ethics Commission for Animals ("Permission for using animals in the experiments", 84/04.10.2013).

Clinical and histological examination. The rats were clinically examined before killing. After killing tissue material from rat paws and knees was proceeded for embedding in paraffin for histological evaluation to confirm the lack of local inflammatory reaction due to the deposition of the immune complexes. The paws and knees of the animals were clinically assessed at a score of 1 to 4 as follows: 0

– no visible inflammation, 1 – swelling and/or redness, 2 – involvement of 2 joints, 3 – involvement of more than 2 joints, 4 – severe inflammation of entire paw and fingers. For the histological examination collected histological material from the paws and knees of the rats was fixed in 10% formalin, decalcified, and embedded in paraffin. The 5 μ m sections were obtained and stained with hematoxylin-eosin (X/E). Ligaments and muscle damage was assessed on the base of inflammatory cell infiltration and both cartilage and subchondral bone destruction. The scoring was carried out in a blinded manner.

Detection and quantification of anti-BSA antibodies. After rats killing individual blood samples were taken by intracardiac puncture and obtained sera were frozen at -20°C until use. Sera samples were subjected to ELISA for detection and quantification of anti-BSA antibodies (rat IgG ELISA, Online Antibodies). The ELISA test was performed according to the instructions of the manufacturer. The OD was read at 570 nm using a microplate reader Microplate reader LKB 5060-006.

Cell culture and MTT assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay has become the gold standard for determination of cell viability since its development [18]. In general, the MTT-based colourimetric assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple colour. Formazan crystals are then dissolved using a solubilizing solution and absorbance is measured at 500–600 nanometers using a plate-reader. Darker solution means a greater number of viable and metabolically active cells.

Mouse fibrosarcoma cell line WEHI-164 was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and penicillin-streptomycin at 100 units/ml and 100 $\mu\text{g}/\text{ml}$, respectively, (all from Sigma Aldrich) defined as complete medium. Exponential-phased WEHI-164 cells were harvested and seeded in 96-well plates at density 4×10^4 cells/well in 100 μl medium per well. Following a 24-hour incubation at 37°C and 5.2% CO_2 , the cells were treated for 48 h with two-fold serial dilutions of the investigated substances in the concentration range from 0.1 to 0.8 $\mu\text{g}/\text{ml}$ in triplicate. Non-treated cells (NT) were used as controls. After the incubation the conditioned media (CM) were collected and screened on zymography. To each well of the culture plate 50 μl sterile MTT substrate solution (5 mg/ml in PBS) was added. After 4-hour incubation purple insoluble precipitates of the formazan were completely dissolved in dimethyl sulphoxide (DMSO, 100 $\mu\text{l}/\text{well}$). The solution was stirred for 30 min and the absorbance was measured (wavelength: 570 nm; reference: 630 nm) using a microplate spectrophotometer. Collected absorbance values were blanked against MTT and solvent solution and normalized to the mean value of untreated control (100% cell viability). Absorbance readings from test samples were divided

by those of the control and multiplied by 100 to give the percentage of cell viability or proliferation. Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation. The MTT assay is a quantitative and sensitive detection of cell proliferation as it measures the growth rate of cells by a linear relationship between cell activity and absorbance.

Zymography and densitometry analysis. Matrix metalloproteinase (MMP-9 and -2) activity was detected by gelatinase zymography. Twenty microliters from each CM were loaded to each line for zymography. The sample was electrophoresed under non-reducing conditions in a 10% SDS-PAGE containing 0.1% gelatin as substrate (Hoefer miniVe system, Amersham Biosciences, USA). The gel was run at 120 V for approximately 3.5 h at 4 °C. SDS in the gel activates the latent forms of MMPs and dissociates the active forms from their inhibitors permitting their detection. After electrophoresis, the gel was washed in renaturing buffer (2.5% Triton X-100 in dH₂O) with gentle agitation 2×30 min at room temperature and incubated for 20 h at 37 °C in zymography digestion buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl₂, 1 μM ZnCl₂, 0.02% NaN₃, pH 7.6). The gel was stained with 0.5% Coomassie blue R250 in 40% methanol, and 10% glacial acetic acid solution for 2 h and de-stained in the same solution without Coomassie blue R250 for 4 h. The enzymatic (proteolytic) activity was visualized as clear bands in a blue (undigested gelatin) background. The gel was scanned and finally dried and archived. Pre-stained SDS-PAGE molecular weight standards were used to estimate the average sizes of MMPs. The approximate molecular weights of the proteolytic bands were calculated using CurveExpert software. The relative intensity of the bands from zymography was calculated by comparison with parallel background readings of equal area by densitometry analysis. Comparisons were made only between samples run on a single gel.

Statistical analysis. All data were expressed as mean ± SE (ELISA) or means (± SD) (zymography). Statistical analysis was performed using GraphPad Prism 5 software. In each experiment, samples were run as triplicates with the average of the three values. The comparisons were made using Student's *t*-test (ELISA) or one-way ANOVA (zymography). *P* values less than 0.05 were considered significant.

Results and discussion. In this study, we tested the cytotoxicity and the anti-inflammatory effect of three newly designed 4-AP derivatives, containing peptide fragments attached to the amino group of 4-AP [16]. The tested 4-AP derivatives were defined as substance **A** (Boc-Tle-Asn-Leu-Ala-4-AP), substance **B** (Boc-Val-Asn-Leu-Ala-4-AP), and substance **C** (Boc-Nva-Asn-Leu-Ala-4-AP).

In vitro cytotoxicity test was performed on WEHI-164 cells as three experiments were conducted (Fig. 1). The response of the cells to the investigated substances applied at different doses (0.1, 0.2, 0.4, and 0.8 ug/ml) showed no cytotoxic effect of any substance at any dose. Thus, all tested 4-AP derivatives

provided viable and metabolically active cells. Moreover, all tested substances increased mitochondrial respiration of the cells and had a positive effect on cell proliferation.

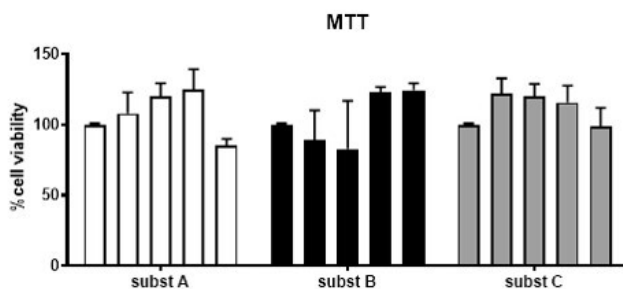


Fig. 1. Viability of WEHI-164 cells after treatment with the investigated compounds in different concentrations. Each bar represents mean $\pm$ SEM, from the first to last bar as follows: control (untreated cells) and cells, treated with 0.1, 0.2, 0.4, or 0.8 ug/ml of each compound. Three experiments were performed

Mild ICII of rats immunized with BSA-CFA was used as a model for the production of anti-BSA antibodies into circulation without deposition of immune complexes and inflammation in the tissues. Clinical and pathohistological evaluation of the paws of immunized treated and non-treated rats confirmed the lack of clinical signs of local inflammation and thus, severe inflammation was excluded. Clinically, the rats did not show peri-articular erythema and edema of their paws. The histological evaluation of the paws did not reveal any signs of acute and severe inflammation along with inflammatory cell infiltration, tissue edema, joint destruction, and bone erosion (Fig. 2, upper panel). It is important to note that the model used in this study resembles mild chronic inflammation, which is typical for autoimmune diseases such as MS. In this model, the presence of the immune complexes is restricted to the blood circulation and there was no triggering of inflammatory reactions in the tissues. Such a rat model can be used to assess the anti-inflammatory potential of different compounds. To check how this model is working before starting treatment with the tested substances we set up two groups with three animals per group – an immunized group and an unimmunized group. The levels of anti-BSA antibodies in both groups were measured by ELISA and compared. We found a significantly higher amount of anti-BSA antibodies in the sera of the immunized compared to unimmunized rats (18.29 ± 1.3 mg/ml vs. 12.02 ± 0.42 mg/ml, $P < 0.01$). We hypothesized that 4-AP derivatives could have anti-inflammatory effects via prevention/inhibition of antibody production. To check this possibility, we treated the rats with certain 4-AP derivate before and after immunization to evaluate its effect on the prevention and inhibition of antibody production, respectively.

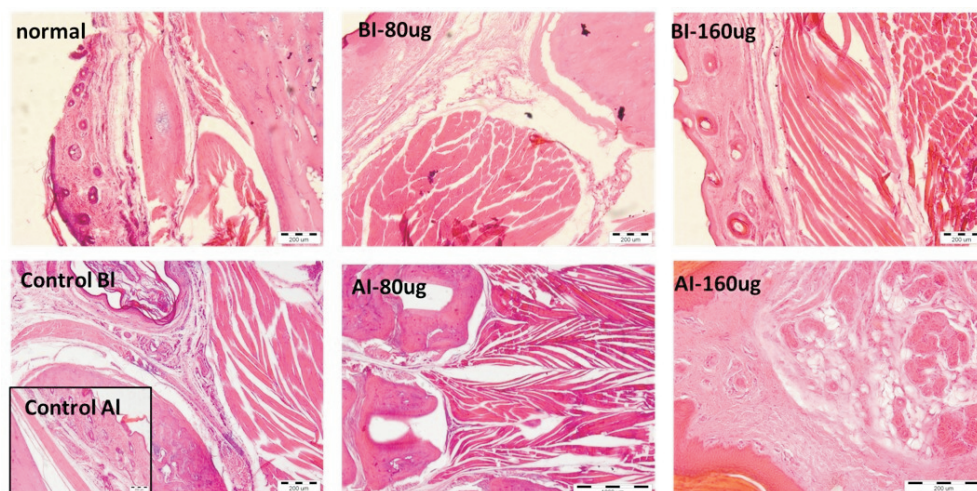
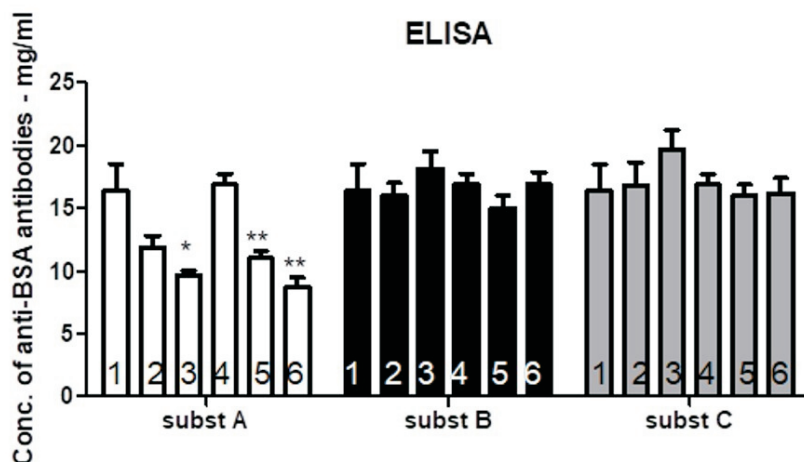


Fig. 2. Histology of rat paws and anti-BSA antibody levels in rat sera. Upper panel – paraffin sections of paws stained with X/E obtained from the normal (non-immunized/non-treated), control (immunized/non-treated), and immunized rats treated with substance **A** – representative pictures. Histology of rat paws showing no signs of acute and severe inflammation such as inflammatory cells infiltration, tissue edema, joint and muscle destruction, or bone erosion in any of the rats, treated with substance **A** before (BI) or after immunization (AI) as compared to control and normal animals. Down panel – Comparison of anti-BSA antibody levels in the circulation between rats treated with substances **A**, **B**, or **C** compared to the control group. 1 and 4 – control rat groups BI and AI, respectively; 2 and 3 – rats treated before immunization with 80 ug/rat/d and 160 ug/rat/d, respectively; 5 and 6 – rats treated after immunization with 80 ug/rat/d and 160 ug/rat/d, respectively. Each bar represents mean $\pm$ SEM, * denotes $P < 0.05$, ** $P < 0.01$ of comparison between experimental (treated) vs. control groups

Our results showed that the treatment with 160 ug/rat/d of substance **A** significantly reduced the levels of anti-BSA antibodies in comparison to immunized non-treated rats when applied either before ($P < 0.05$) or after immunization ($P < 0.01$, Fig. 2, down panel). However, at the lower dose of 80 ug substance **A** decreased the antibodies amount only when it was applied after immunization of the rats ($P < 0.01$, Fig. 2, down panel). Substances **B** and **C** did not influence the levels of anti-BSA antibodies neither at 80 ug/rat/d nor at 160 ug/rat/d regardless of the time of administration.

Thus, substance **A** demonstrates a clear anti-inflammatory effect either preventing or inhibiting the production of antibodies in the rat blood circulation. However, no effect was observed upon treatment with substances **B** and **C**.

The main enzymes in ECM synthesis and degradation include matrix metalloproteinases (MMPs), zinc-dependent proteases that play a crucial role in ECM remodelling via proteolytic degradation of its components during inflammation [19]. MMP-9 and MMP-2 (also known as gelatinases A and B) particularly degrade gelatin, elastin, as well as various types of collagens [20]. By using a gelatin zymography we found proteolytic bands in the range of 100–55 kDa in the CM derived from WEHI-164 cell culture, non-treated and treated with different concentrations of **A**, **B**, and **C** substances (Fig. 3A). Densitometry analysis of the proteolytic bands, corresponding to the enzymatic activity of proMMP-9 (inactive form) and MMP-9 showed the following trend. Substances **A** and **C** had an inhibitory effect on the proteolytic activity of both MMP-9 isoforms at a concentration of 0.1 ug/ml only and the higher concentrations eliminated this effect (Fig. 3B, left upper and down). On the contrary, substance **B** had an inhibitory effect on both MMP-9 isoforms only when applied at higher concentrations of 0.2–0.8 ug/ml (Fig. 3B, middle left). Regarding the inhibitory effect of the tested substances on the proteolytic activity of MMP-2 zymogen and active form, we found a similar trend: 1) substances **A** and **C** had an inhibitory effect on the activity of both MMP-2 isoforms at a concentration of 0.1 ug/ml only (Fig. 3B, right upper and down), and 2) there was no inhibition of MMP-2 activity upon any treatment with substance **B** (Fig. 3B, middle right).

Conclusions. All tested 4-AP derivatives (substances **A**, **B**, and **C**) provided viable and metabolically active cells. The substances **A** and **C** inhibit the proteolytic activity of both metalloproteinases MMP-2 and -9 even when applied in the lowest concentration, whereas substance **B** had no inhibitory effect on the MMP-9 activity and inhibits MMP-2 action only applied at higher concentrations. Our results distinguished compound **A** as very promising. Along with its inhibitory effect on the MMP-2 and -9 proteolytic activity, it demonstrates also a clear anti-inflammatory effect either preventing or inhibiting the production of antibodies in the blood circulation of rats.

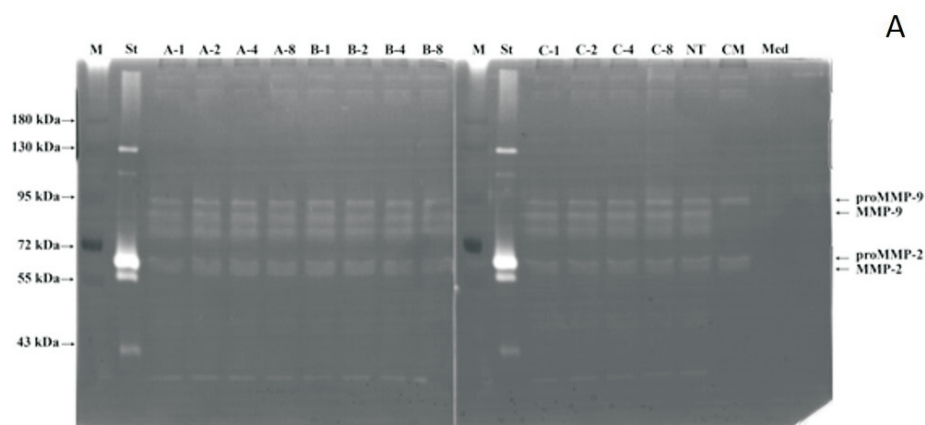
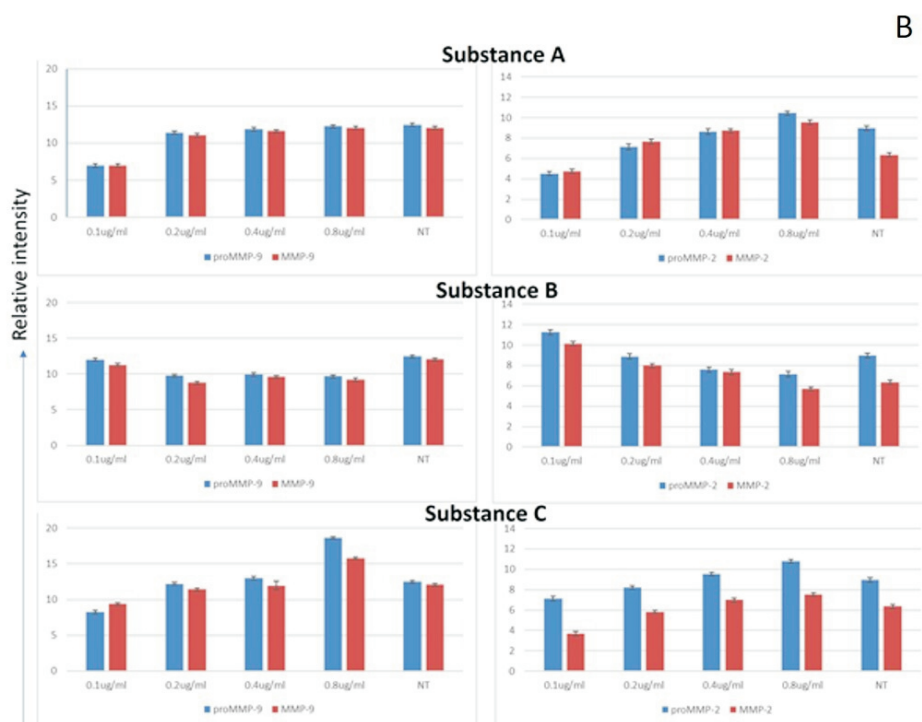


Fig. 3. Inhibitory effect of the substances **A**, **B** and **C** on the proteolytic activity of MMP-9 and MMP-2 isoforms (pro- and active). **A** – representative zymogram gel loaded with CM showing gelatinolytic bands with an approximate molecular weight of 93 and 86 kDa corresponding to the inactive (pro, zymogen) and active forms of MMP-9, respectively, and bands with an approximate molecular weight of 70 and 62 kDa, corresponding to the inactive and active forms of MMP-2. M – a marker for molecular weight, St – recombinant MMP-2, A-1, A-2, A-4, A-8 – CM from cell culture treated with substance **A** at doses of 0.1, 0.2, 0.4, and 0.8 ug/ml; the same for substances **B** and **C**; NT – CM from not treated cells, CM – complete medium, Med – serum-free medium. **B** – Densitometric analysis of gelatin zymography ($n = 3$) showing the inhibitory effect of the substances **A**, **B**, and **C** on the proteolytic activity of MMP-9 (left graphs) and MMP-2 (right graphs) isoforms

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