



Immunosuppressory activity of an isoxazolo[5,4-e]triazepine-compound RM-33

II. Effects on the carrageenan-induced inflammation

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Abstract:

The aim of this investigation was to evaluate effectiveness of RM-33, a new isoxazolotriazepine, in the model of carrageenan-induced inflammation in rats. Wistar rats were pretreated with intraperitoneal (*ip*) or oral (*po*) doses of RM-33, at daily doses ranging from 250 to 1000 µg, administered 1–3 days before elicitation of the carrageenan reaction. We showed that both routes of RM-33 administration were effective in significantly diminishing the footpad edema. The effects were dose-dependent and better pronounced at the *ip* administration of the compound. We found a lower production of tumor necrosis factor alpha (TNF-α) by mitogen-stimulated splenocytes isolated from rats pretreated with RM-33 and injected with carrageenan, as well as lower serum TNF-α levels in these rats, as compared to the respective control. Histological analysis of the skin reaction site revealed that in the rats pretreated with RM-33, the carrageenan-induced inflammation was reduced, as reflected by a lesser damage of mast cells, smaller infiltration by macrophages and a diminished edema of the connective tissue. Together with our previous data, indicating the antagonistic action of RM-33 in the adjuvant-induced footpad inflammation in mice, the present results confirm the anti-inflammatory activity of RM-33 compound.

Key words:

isoxazolotriazepines, carrageenan, rats, TNF-α

Introduction

Compounds with potential anti-inflammatory activity have been a subject of extensive research among chemists and pharmacologists. The 1,2,4-triazepine heterocyclic system has been recently investigated for its interesting chemical and pharmacological proper-

ties [4, 12, 14, 17]. In the previous article, we presented immunosuppressory activities of a new isoxazolotriazepine, compound RM-33 [13]. The compound exhibited potent immunosuppressory actions both in the humoral and cellular immune response in mice. A strong diminution of the adjuvant-elicited skin inflammation by RM-33 suggested that the immunosuppressory activity of the compound may be

associated with anti-inflammatory properties in other, conventional, pharmacological tests.

The model of carrageenan-induced inflammation in rats is useful for pharmacologists to evaluate potential anti-inflammatory activities of new compounds [1, 7, 9]. That inflammatory reaction consists of a non-phagocytic inflammation, followed by a phagocytic inflammatory response in dermis and an epidermal nonphagocytic inflammatory response [18]. The reaction is initiated by an injury of mast cells resulting in cell degranulation. The intermediates of arachidonic acid and serotonin mobilize neutrophils, producing an edema and hyperalgesia. Later, the inflammatory site is infiltrated by monocytes which contribute to diminution of the reaction intensity by phagocytosis of neutrophils and cellular debris. Several studies have indicated that isoxazole derivatives are potent inhibitors of the carrageenan-induced inflammation in rats [6, 10, 11]. The isoxazole derivatives exhibited higher activity in that test as compared with quinazolines [11]; a combination of the two structures was also tested [10]. Derivatives of leflunomide were particularly anti-inflammatory and it was postulated that their activities were more pronounced in compounds possessing substituents of an electron donor or acceptor in character [6].

The aim of this study was to evaluate efficacy of RM-33 to inhibit carrageenan-induced inflammation in rats after intraperitoneal and oral administration of the compound. We evaluated an increase in footpad edema, production of TNF- α and performed the histological analysis of the inflamed tissue.

Materials and Methods

Animals

Female and male Wistar rats weighing 175 g, on average, were supplied by the Animal Facility of the Wrocław Medical University. The animals were fed a granulated, commercial food and water *ad libitum*. The local Ethics Committee approved the study.

Reagents

RM-33 was synthesized in the Department of Organic Chemistry, Faculty of Pharmacy, Wrocław Medical

University, as described previously [13]. The compound was initially dissolved in DMSO, then in 0.9% saline. Carrageenan, DMSO, concanavalin A and lipopolysaccharide (LPS) from *Escherichia coli* serotype O111:B4, were from Sigma, USA.

Treatment of rats with RM-33 and elicitation of the carrageenan reaction

Rats were given RM-33 dissolved initially in DMSO and then in 0.9% NaCl, at 250 or 500 μg doses, in a volume of 0.2 ml, intraperitoneally, at 48 and 24 or 72, 48 and 24 h before carrageenan injection, as indicated in figure legends. Alternatively, rats were given RM-33 *per os* using a stomach tube, at a dose at 500 or 1000 μg , 48 and 24 h before carrageenan injection. Control rats were given DMSO, diluted in 0.9% NaCl. The final concentration of DMSO was 4% (v/v) in volume of 0.2 ml. To elicit the inflammatory reaction, the rats were given 0.05 ml of carrageenan (1% solution in 0.9% NaCl), into hind footpads, intradermally. The footpad swelling was determined using a caliper before carrageenan administration and after 1, 2 and 3 h. The results are presented as the footpad thickness measured after 3 h (a peak of the reaction) and expressed in millimeters. Each group comprised 5 rats.

Preparations of splenocytes for cell cultures and induction of TNF- α production

Spleens were removed aseptically 3 h after elicitation of the carrageenan reaction. A single cell suspension (10^7 cells/ml) was prepared in a culture medium consisting of RPMI 1640, supplemented with glutamine and 10% FCS (fetal calf serum, Gibco). The cells were cultured in 24-well culture plates (10^7 /ml/well) with an addition of 2 $\mu\text{g}/\text{ml}$ of LPS and 2 $\mu\text{g}/\text{ml}$ of concanavalin A (mix). After 24 h of incubation in a cell culture incubator, the supernatants were harvested, frozen at -80°C until cytokine determination.

Preparation of serum for determination of TNF- α

For determination of TNF- α in serum, rats were exsanguinated by a heart puncture under general anesthesia 3 h after elicitation of the carrageenan inflammation. After blood clotting, serum was separated by centrifugation at $800 \times g$, aliquoted and kept frozen at -80°C until TNF- α determination.

Determination of TNF- α activity

TNF- α activity was determined by a bioassay [3]. In brief, WEHI-164.13 cells were cultured in 96-well plates at a density 2×10^4 cells/well with serial dilutions of either tested supernatants or serum. Cell kill was evaluated after 20 h using MTT colorimetric method [5]. Optical density was measured at 550 nm and reference wavelength of 630 nm using a colorimetric reader (Dynatech 5000). In that bioassay, one unit of TNF- α (corresponding to 10 pg/ml) is defined as an inverse dilution of the studied sample, at which 50% cells are killed. The method sensitivity was 2.5 pg/ml.

Preparation of histological sections

Feet were fixed with 4% formalin solution for 24 h, then were dehydrated and embedded in paraffin. Fragments of the preparations were subsequently serially cut into 6–7 μ m sections and stained with hematoxylin according to Delanfield and eosin and with metylene blue according to van Gieson. The preparations were microscopically evaluated at 400 magnification by a histologist.

Statistical evaluation

The results are presented as the mean values \pm standard error (SE). The W Shapiro-Wilk's test was used to verify the normal distribution of the data. The Levene's test was used to determine the homogeneity of variance between groups. When the variance was homogeneous, analysis of variance (ANOVA) was applied, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of the difference between groups. Nonparametric data were evaluated by the Mann-Whitney's U test or the Kruskal-Wallis analysis of variance on ranks (to compare multiple groups). Significance was defined as $p < 0.05$.

Results

Effect of RM-33 treatment on the carrageenan reaction

Preliminary experiments (not shown) indicated that administration of RM-33 twice before elicitation of the inflammatory reaction, was more suppressory than

its single dose in reduction of the inflammation. In addition, application of drug doses lower than 250 μ g, like 125 μ g or 60 μ g did not lead to a statistically significant inhibition of the carrageenan-induced reaction. Figure 1A shows that RM-33, given *ip* to rats

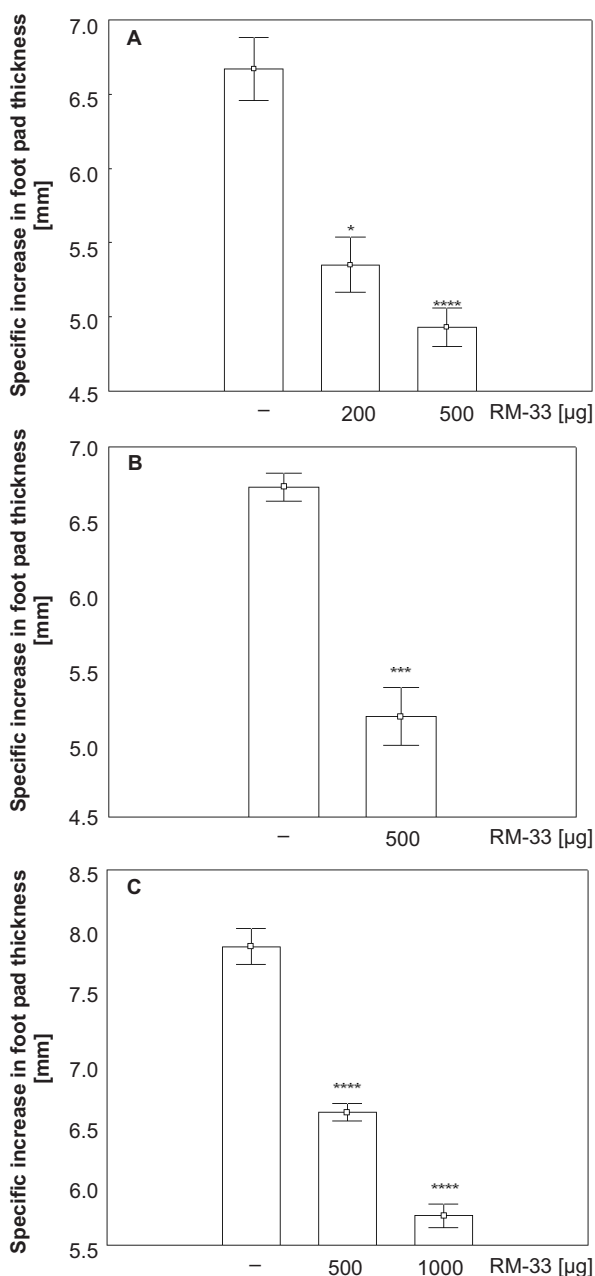


Fig. 1. Effect of the intraperitoneal (A, B) or oral (C) pretreatment of rats with RM-33 on the magnitude of carrageenan-induced edema. Rats were given RM-33 at 250 or 500 μ g doses, intraperitoneally, at 72, 48 and 24 h (B) or 48 and 24 h (A) before carrageenan injection. Alternatively, rats were given RM-33 using a stomach tube, at a dose of 1000 or 500 μ g, 48 and 24 h before carrageenan injection (C). The data are shown as the mean \pm SE. (A) * $p < 0.05$, **** $p < 0.0001$ vs. control (Kruskal-Wallis test). (B) *** $p < 0.001$ vs. control (Mann-Whitney U-test). (C) **** $p < 0.0001$ vs. control (ANOVA)

48 h and 24 h before elicitation of the reaction, led to a significant reduction of the footpad edema, and the effect was clearly dose-dependent. Application of three doses of RM-33 (72, 48 and 24 h before elicitation of the reaction) did not further enhance the inhibitory effect of the compound (Fig. 1B). Figure 1C, shows that oral treatment of rats with RM-33 is also effective in inhibition of the reaction, that effect was also dose-dependent and better pronounced at a dose of 1000 $\mu\text{g}/\text{dose}$.

Effect of RM-33 treatment on TNF- α production during carrageenan-induced inflammation

We also tested the ability of splenocytes, isolated from rats at the time of maximal carrageenan reaction (3 h), to produce TNF- α upon stimulation with LPS

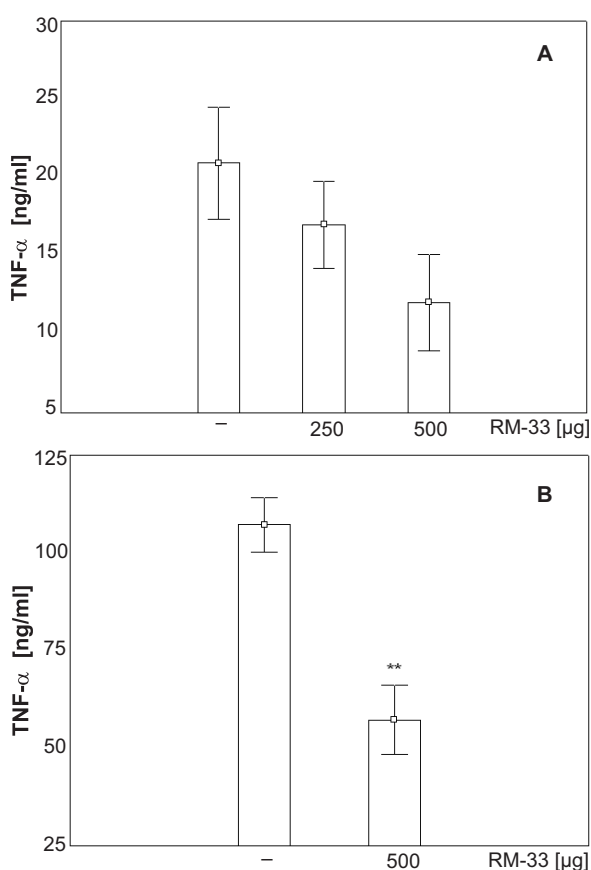


Fig. 2. Effects of the pretreatment of rats with RM-33 on the TNF- α production *in vitro* and *in vivo* at the maximum carrageenan-induced reaction. The ability of splenocytes, isolated from rats at the time of maximal carrageenan reaction (3 h), to produce TNF- α upon stimulation with LPS and ConA (A). The serum TNF- α level in RM-33-treated rats at 3 h after carrageenan-induced reaction (B). The data are shown as the mean \pm SE. (A) (NS) vs. control (ANOVA). (B) ** $p < 0.01$ vs. control (ANOVA)

and ConA. The results (Fig. 2A) show that treatment of rats with RM-33 resulted in a dose-dependent inhibition of TNF- α production (the effect not statistically significant). No suppression was observed when LPS alone was used as a stimulant (data not shown). On the other hand (Fig. 2B), the serum TNF- α level in RM-33-treated rats was significantly reduced at 3 h after carrageenan-induced reaction.

Histological evaluation of the footpads in rats treated with RM-33 and carrageenan

In the footpads of rats given carrageenan, a distinct decrease in the mastocyte number in the connective, subepidermal tissue of the dermis was observed (Fig. 3B). Most of the mast cells has undergone degranulation. Macrophages and neutrophils appeared at a high number. In addition, single lymphocytes were noticed, particularly around blood vessels. Reduction in density of the dermal fibres was also noted. Generally, the histological picture presented an edema of the connective tissue. In the rats pretreated with RM-33, the subepidermal zone of the connective tissue was inflamed to a lesser degree (Fig. 3C). Single mastocytes appeared around blood vessels. In the adjacent zone only few macrophages were present. Neutrophils were observed at similar numbers as in the former group of rats. Figure 3A shows the histological picture of a footpad from control, naive rats.

Discussion

The results of this investigation demonstrated that a new isoxazolotriazepine, compound RM-33, exhibits a potent anti-inflammatory activity in the carrageenan-elicited skin reaction in rats. These data add more information to the results described in the preceding report [13] with regard to a plausible mechanism of action of the compound. The hitherto accumulated data have indicated that RM-33 may inhibit inflammatory reactions elicited both by bacterial antigens (Freund's complete adjuvant) or a plant compound (carrageenan). RM-33 can also inhibit antigen-specific immune responses, both cellular and humoral, possibly by a mechanism associated with suppression of the nonspecific accessory signals during initiation of the immune response, delivered by

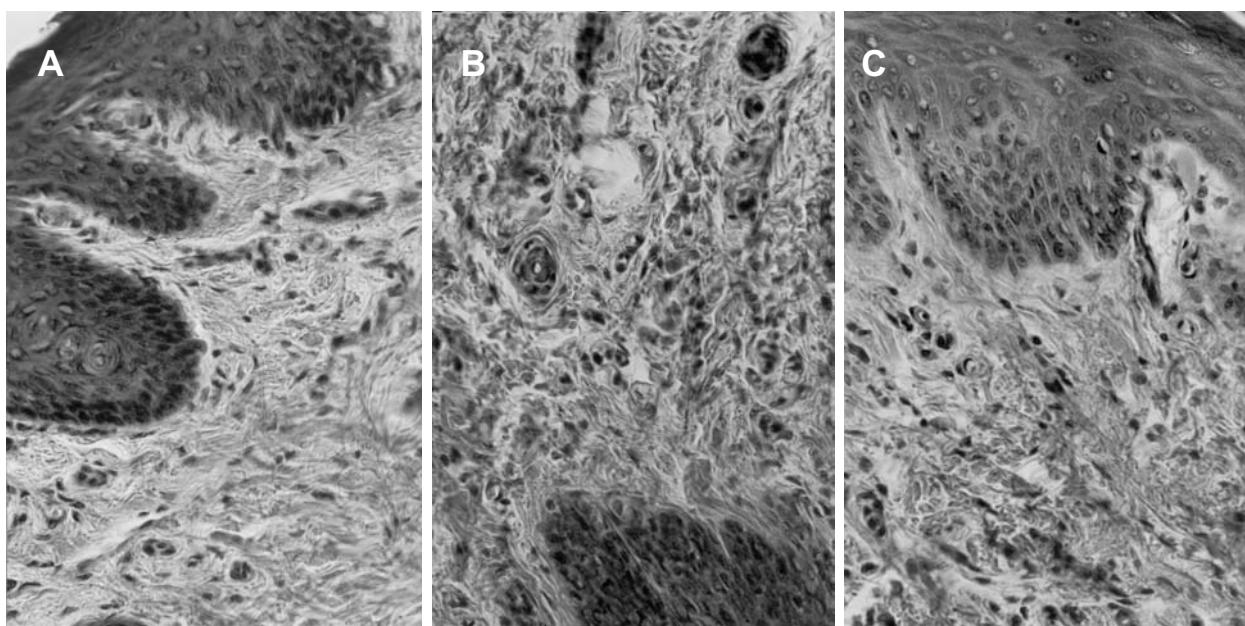


Fig. 3. Effects of the pretreatment of rats with RM-33 on the histological picture of footpads injected with carrageenan. (A) A footpad from a control, naive rat. (B) A footpad from a rat injected with carrageenan. (C) A footpad from a rat pretreated with RM-33 and injected with carrageenan. See the Results section for details

the adjuvant. In addition, our unpublished results showed that the compound significantly reduced the severity of experimental encephalomyelitis in Lewis rats, probably as a result of a significant diminution of the initial footpad inflammation, caused by injection of the sensitizing dose of antigen, admixed with *Mycobacterium tuberculosis*, as an adjuvant.

Carrageenan-induced inflammation is correlated with appearance of proinflammatory cytokines, such as interleukin (IL)-1 and IL-6 [16], and TNF- α plays also a role in mediation of that inflammatory response [15]. Rat peripheral mononuclear cells, incubated with carrageenan, produce in culture, cytokines such as IL-1, 2, 6, 8 and TNF- α , and that supernatant causes hyperalgesia and edema when injected into rat footpads [2]. We have previously found that pretreatment of rats with lactoferrin [19] led to a significant reduction of carrageenan-induced footpad edema, associated with decreased production of IL-6 and TNF- α in the splenocytes isolated 24 h after the peak of the carrageenan-induced inflammation. The results presented in this investigation support our previous findings [19] that the anti-inflammatory actions in the carrageenan model are associated with a decrease in mitogen-induced TNF- α production (Fig. 2A). Moreover, we found that TNF- α serum level at the peak of the carrageenan reaction was significantly de-

creased in the rats pretreated with RM-33 (Fig. 2B). Other unpublished data showed that RM-33 inhibited IL-6 and TNF- α production in the culture of peritoneal rat cells. Taken together, the inhibition of TNF- α production was correlated in our studies with diminution of the carrageenan-induced edema. The exact mechanism of this phenomenon, in the case of RM-33, remains to be elucidated. It is most likely that the compound causes some kind of nonspecific hyporeactivity of the target cells like macrophages and mastocytes which become less responsive to proinflammatory stimuli. A hypothesis to be also tested assumes a direct inactivation of some proinflammatory cytokines as in the case of migration inhibition factor, whose tautomerase enzymatic activity was blocked by another isoxazole derivative [8]. It was also postulated that isoxazole derivatives of leflunomide were particularly effective in the carrageenan assay if they contained groups of an electron donor or acceptor in character [6]. Although RM-33, unlike leflunomide, does not bear electrodonor groups, it possesses NH-group in position 7, which is a strong electron acceptor, thus it may fulfill such a criterion. The present data are in agreement with other reports on the anti-inflammatory activities of isoxazole derivatives in the carrageenan assay [6, 10, 11]. Histological examination confirmed the results of the macroscopic evalua-

tion of the inflamed footpads and revealed a lesser damage of mast cells correlated with diminished infiltration of macrophages whose role consist in phagocytizing cell debris. In addition, there was no passage of lymphocytes through the blood vessels in RM-33-pretreated rats. Interestingly, the infiltration of the connective tissue by neutrophils was comparable in both groups of animals. That type of cells, however, did not apparently contribute to footpad edema since in RM-33-pretreated rats, the footpad thickness was significantly lower as compared to respective control.

In conclusion, the anti-inflammatory properties of RM-33 were shown in several models (ref. 13 and this report). The compound is low toxic and bioaccessible when administered also *per os*, therefore, further studies are justified to evaluate its potential therapeutic application.

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