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*Short Communication*

# RM-11, a New Izoxasole Derivative, Is a Potent Stimulator of the Humoral and Cellular Immune Responses in Mice

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**Abstract.** In this report we describe immunostimulatory properties of RM-11 in several *in vivo* and *in vitro* tests in the murine model. We found that RM-11 significantly stimulated the humoral immune response to sheep erythrocytes (SRBC) when given intraperitoneally (i.p.) at doses of 10 and 100 μg or *per os* (doses of 20 and 200 μg) 3 h before immunization. The compound was also stimulatory with regard to generation of delayed type hypersensitivity (DTH) to SRBC when given i.p. or *per os* (doses of 10, 100 and 500 μg/mouse). The described immunostimulatory activities of RM-11 were higher compared to that of the reference drug, levamisole. RM-11 stimulated, in addition, concanavalin A (ConA)-induced splenocyte proliferation. Lastly, we showed that RM-11 was not toxic when given to mice *per os* at doses 250 mg/kg body weight. Taken together, RM-11 appeared to be a universal stimulator of the immune response in mice. Lack of toxicity and the ability to stimulate the immune response, when administered *per os*, predispose the compound for further preclinical studies.

**Key words**: stimulation; humoral; cellular immune response; izoxazole.

## Introduction

Despite enormous progress in medicine and pharmacy, we witness a significant increase in bacterial and viral infection rates. The aggravating problem of infectious diseases results from the acquirement of resistance of phatogens to antibiotics[2, 3, 4, 9, 11]. In the opinion of many experts a "dark age" of the pre-antibiotic era is approaching[9]. Other dangerous illnesses include neoplastic diseases and those caused by innate or acquired immunological deficiencies. The search for and application of immunostimulating drugs is crucial in combating diseases resulting from the malfunctioning of the immune system. In recent years, 5 new immunoregulatory drugs have been introduced: Carfenil (1986) – disodium salt of 4-chloro-2, 2'-iminodibenzoic acid, Rimatil (1987) – N-(2-mercapto-2-methyl-propionylo)--L-cysteine, Polimod (1993) – 3-L-piroglutamylo-L-thiazolidyne carboxyl acid, Orel (1994) – 4-N-acetyl-4--aminophenylacetic acid, and Cellcept (1995) – 1-hydroxy-3-methoxy-4-methylbenzopyrrolidine-2-hex-2--ene-carboxylic acid N-mor-pholineethyl ester, exhibiting differential antiinflammatory, immunomodulatory, antiviral and antiarthritic activity. All these preparations, besides their immunomodulatory activity, possess high toxicity and have numerous side-effects.

Because of the increased significance of new immunomodulatory drugs, the search for new compounds is the subject of studies by leading pharmaceutical companies. In the course of our previous investigations we

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obtained new izoxazole derivatives exhibiting immunomodulatory activity higher than levamisole. We have undertaken the synthesis of amides of 5-amino-3-methyl--4-isoxazolecarboxyl acid. These compounds demonstrated particularly interesting immunostimulatory activity which was equal to or even higher than that of levamisole, but being nontoxic at the same time. We also found a rarely observed correlation between structure and immunological activity[14]. As an effect of our search for new immunostimulatory drugs, we have synthesized a preparation whose immunological activities are presented in this report.

## Materials and Methods

*Animals*. 8–10-week-old CBA and 129 mice of both sexes were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy, Wrocław, Poland.

*Preparation of the compound for in vitro experiments.* Five mg of a compound was suspended in 0.3 ml of a mixture of cremophor (Sigma) and ethanol at a ratio of 0.36 : 0.64, followed by a 20 min incubation in an ultrasonic bath. Then the compound was further diluted in 0.9% NaCl or culture medium. As a reference drug levamisole was used, dissolved in PBS and applied at the same doses as RM-11.

*Humoral immune response in vivo*. CBA mice (5 animals per group) were treated with RM-11 3 h before immunization with SRBC. The doses of RM-11 for i.p. treatment were 10 and 100 μg per mouse and for *per os* administration 20 and 200 μg. Levamisole was used at the same dosage. Control mice were untreated or given solvent 3 h before immunization. Four days later the number of plaque-forming-cells (PFC) in the spleens was determined by the method described by MISHELL and DUTTON (10). The results are presented as mean values of PFC per $10^6$ cells ± standard error (SE).

*Delayed type hypersensitivity response in vivo*. CBA mice (7 per group) were treated with the compound 3 h after sensitization of mice i.v. with SRBC ($10^6$/mouse) at 10, 100 and 500 μg i.p. or *per os* doses. Four days later the DTH reaction was elicited by subcutaneous (s.c.) injection of $10^8$ SRBC into the hind foot pads as described by LAGRANGE et al.[8] and 24 h later the foot pad thickness was measured using a caliper with 0.05 mm accuracy. The results are presented in DTH units (1 U = 0.1 mm).

*Proliferative response of splenocytes to concavalin A (ConA).* Spleens from CBA mice were pressed through a plastic screen into a precooled Hanks' medium. Erythrocytes were lyzed using 0.84% $NH_4Cl$, the lymphocytes washed 3 times with Hanks' medium and resuspended in a culture medium supplemented with 5 mM L-glutamine sodium pyruvate, 10% fetal calf serum and antibiotics. The cells were then distributed into 100 μl aliquots ($2 \times 10^5$ cells) in 96-flat-bottom well plates. ConA was used at a concentration of 5 μg/ml and the preparation at concentrations to 0.1, 1 and 5 μg/ml. Control cultures contained solvent at appropriate concentrations. After 3-day culture, cell proliferation was determined using the MTT colorimetric method[5]. Cell proliferation was expressed as mean optical density (OD) values from quadriplicate wells ±SE.

*Statistical evaluation*. The results were subjected to statistical analysis using Student's *t*-test. Data were considered to be statistically significant when $p \leq 0.05$. The effects of the compounds were compared to appropriate solvent control.

## Results

### *Effects of RM-11 on generation of humoral immune response to SRBC*

Table 1 presents the effects of i.p. and *per os* administration of RM-11, 3 h before immunization on the magnitude of the humoral immune response as measured by PFC numbers. In the case of i.p. administration, RM-11 was strongly stimulatory (4-fold enhancement at 100 μg/mouse). Levamisole was less active in this test. Stimulation of the humoral response with *per os* administration of RM-11 was slightly weaker but still very significant.

### *Effect of RM-11 on development of delayed type hypersensitivity to SRBC*

The effects of i.p. and *per os* administrations of RM-11 were also investigated with regard to generation of DTH (shown in Table 2). Also in this case the stimulatory activity of RM-11 was strong, particularly when the compound was given i.p., and was higher than that of levamisole.

### *Effect of RM-11 on ConA-induced proliferative response of mouse splenocytes*

Pilot experiments with human PBMC showed that RM-11 exhibited some stimulatory effect on PHA-induced cell proliferation. Table 3 presents results of an

**Table 1**. Effect of RM-11 administration on magnitude of the humoral immune response to SRBC in CBA mice

| Preparation | Dose (μg/mouse) | PFC/$10^6$cells | ±SE | p |
|---|---|---|---|---|
| Control | | 1624 | 134 | |
| Solvent i.p. | | 1656 | 47 | |
| Levamisole i.p. | 10 | 1704 | 27 | NS |
| | 100 | 3504 | 293 | <0.01 |
| RM-11 i.p. | 10 | 2400 | 89 | <0.01 |
| | 100 | 6848 | 605 | <0.001 |
| Solvent *per os* | | 1584 | 47 | |
| Levamisole *per os* | 20 | 2376 | 77 | <0.01 |
| | 200 | 3192 | 105 | <0.001 |
| RM-11 *per os* | 20 | 3088 | 258 | <0.01 |
| | 200 | 4600 | 308 | <0.001 |

NS – not significant.

**Table 2**. Effect of RM-11 administration on generation of delayed type hypersensitivity to SRBC in CBA mice

| Preparation | Dose (μg/mouse) | DTH units | ±SE | p |
|---|---|---|---|---|
| Control | | 5.28 | 0.81 | |
| Solvent control | | 5.43 | 0.76 | |
| Levamisole i.p. | 10 | 4.70 | 0.36 | NS |
| | 100 | 7.14 | 0.51 | NS |
| | 500 | 8.66 | 0.72 | <0.05 |
| RM-11 i.p. | 10 | 9.57 | 1.09 | <0.05 |
| | 100 | 9.28 | 0.61 | <0.01 |
| | 500 | 10.40 | 0.75 | <0.01 |
| Levamisole *per os* | 10 | 5.57 | 0.86 | NS |
| | 100 | 7.71 | 0.99 | NS |
| | 500 | 8.57 | 0.48 | <0.05 |
| RM-11 *per os* | 10 | 4.71 | 0.66 | NS |
| | 100 | 7.57 | 0.72 | NS |
| | 500 | 9.28 | 0.68 | <0.01 |

NS – not significant.

**Table 3**. Effect of RM-11 on concanavalin A (ConA)-induced proliferation of CBA mouse splenocytes

| Preparation | Dose (μg/ml) | Xmean OD550 nm | ±SE | p |
|---|---|---|---|---|
| Control (medium only) | | 0.210 | 0.005 | |
| ConA only | | 0.677 | 0.009 | |
| ConA (1 μg/ml) | | | | |
| Solvent | 0.1 | 0.623 | 0.016 | |
| | 1 | 0.600 | 0.012 | |
| | 5 | 0.596 | 0.006 | |
| RM-11 | 0.1 | 0.696 | 0.010 | NS |
| | 1 | 0.602 | 0.008 | NS |
| | 5 | 0.669 | 0.006 | <0.01 |
| ConA only | | 0.889 | 0.010 | |
| ConA (5 μg/ml) | | | | |
| Solvent | 0.1 | 0.890 | 0.012 | |
| | 1 | 0.864 | 0.010 | |
| | 5 | 0.860 | 0.006 | |
| RM-11 | 0.1 | 0.981 | 0.003 | <0.01 |
| | 1 | 0.931 | 0.008 | <0.01 |
| | 5 | 0.996 | 0.010 | <0.01 |

NS – not significant.

experiment testing the stimulatory activity of RM-11 in ConA-induced splenocyte proliferation. The stimulatory effect of RM-11 was statistically significant in all used doses when the optimal concentration of ConA (5 μg/ml) was applied. At lower mitogen concentration (1 μg/ml) the stimulatory effect of RM-11 was observed only at a 5 μg/ml dose of the compound. RM-11 did not significantly change the spontaneous proliferation of cells (not shown). Levamisole, at the doses tested, is not active, demonstration of its immunostimulatory action *in vitro* requires 10 times higher doses (not shown).

## Discussion

This preliminary report reveals strong, immunostimulatory properties of RM-11 in the mouse system. The stimulatory effects of the compound are not selective with regard to a particular type of immune response (humoral or cellular), suggesting that RM-11 does not preferentially affect specific types of antigen presenting cells or induce specific activation pathways or sets of cytokines. One of the possible mechanisms of action is a strong upregulation of TNF-α production by RM-11 as observed in the response of human PBMC to LPS (unpublished data). TNF-α is a cytokine clearly involved in the induction phase of the immune response[6]. Other interesting unpublished data on mice showed that pretreatment of animals with RM-11, followed by LPS injection, significantly lowered LPS-induced TNF-release into the circulation. This protective type of action suggests an interaction of RM-11 with cells of the reticuloendothelial system. It is interesting that RM-11 is also active when administered *per os*. Such a feature is desirable and expected for potential drugs. It also suggests that the compound is efficiently absorbed by the gut and affects gut-associated lymphoid tissue. The stimulatory property of RM-11 is less pronounced at suboptimal dose of mitogen (Table 3). Such a finding indicates that RM-11 acts rather by amplifying the signals leading to generation of the immune response than by providing costimulatory signals during that process, a feature characteristic of adjuvants.

The activity of RM-11 in this study was compared with levamisole, an immunoregulatory drug with a long, fascinating history[1], now again appreciated due to its regulatory properties. It appears that levamisole affects immunodeficient but not normal individuals[1, 16]. Although applied in the treatment of many diseases[15], its major use was associated with cancer therapy[13] and enhancement of vaccination effectiveness[7]. In our study, RM-11 was more effective compared with levamisole in the mouse model. In addition, RM-11 was devoid of toxicity when mice were administered the compound *per os* at a dose of 250 mg/kg body weight and observed for 7 days. Moreover, (data not shown) RM-11 upregulated to various degrees PHA-induced proliferation of human peripheral blood lymphocytes. Levamisole, at the same range of dosage (0.2–5.0 μg/ml) was inactive. We, therefore, suppose that RM-11 can potentially replace levamisole. Although data presented in this report are prelimiary, we envisage application of this compound in several categories of patients, for example as an immunopotentiating agent following chemotherapy.

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