Effect of Cycloalkyl Glycosides of Muramyl Dipeptide on Antibacterial Resistance of Mice and Cytokine Production by Human Mononuclear Cells

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β-Cyclohexylmethyl-, β-cyclohexylethyl-, and β-4-*tert*-butyl-cyclohexyl glycosides of muramyl dipeptide were shown to increase the resistance of mice to intraperitoneal infection with cultures of *Staphylococcus aureus* and *Escherichia coli*. These compounds increased the production of cytokines by mononuclear cells from healthy donors. β-Cyclohexylethyl glycoside of muramyl dipeptide was more potent than muramyl dipeptide and other derivatives in increasing *in vivo* antibacterial resistance and *in vitro* production of interleukin-1β, interleukin-6, tumor necrosis factor-α, and interferon-γ. This glycopeptide had a strong stimulatory effect on the production of interleukin-4 and tended to stimulate the synthesis of interferon-α. β-Cyclohexylmethyl glycoside of muramyl dipeptide was most potent in stimulating the production of interleukin-4. Biological activity of β-4-*tert*-butyl-cyclohexyl glycoside of muramyl dipeptide was lower than that of other glycosides of muramyl dipeptide.

Key Words: *muramyl dipeptide; cycloalkyl glycosides; resistance; cytokines; mononuclear cells*

N-acetylmuramyl-*L*-alanyl-*D*-isoglutamine (muramyl dipeptide, MDP) was extensively studied by immunopharmacologists for more than 35 years. Chemical modification of this molecule resulted in the development of some immunotropic substances and large group of pharmacological drugs to increase the antiinfectious and antitumor immunity [2,3,8]. The molecular mechanisms for action of MDP and its derivatives include an agonistic relationship between pattern-recognizing receptors NOD-2 [9]. These data provide the basis for the synthesis of efficient and safe analogues of MDP. We previously attempted to attach a cyclic structure to the glycoside site of MDP. Biological activity of glycopeptide (β-cyclohexyl glycoside of MDP and β-adamantyl glycoside of MDP) increased [5,6,7], remained unchanged, or even decreased under these conditions (α- and β-cyclododecyl glycosides of MDP) [4].

Here we evaluated the capacity of new cycloalkyl glycosides of MDP to increase the resistance of mice to infections with gram-positive and gram-negative bacteria and to stimulate the production of cytokines by human mononuclear cells.

MATERIALS AND METHODS

β-Cyclohexylmethyl-, β-cyclohexylethyl-, and β-4-*tert*-butyl-cyclohexyl glycosides of MDP were synthesized as described elsewhere (Fig. 1) [1]. Unmodified MDP was synthesized [10] and served as the reference agent in all biological tests.

For *in vivo* and *in vitro* studies, MDP glycosides were dissolved in 10% dimethylsulfoxide (10 mg/ml,

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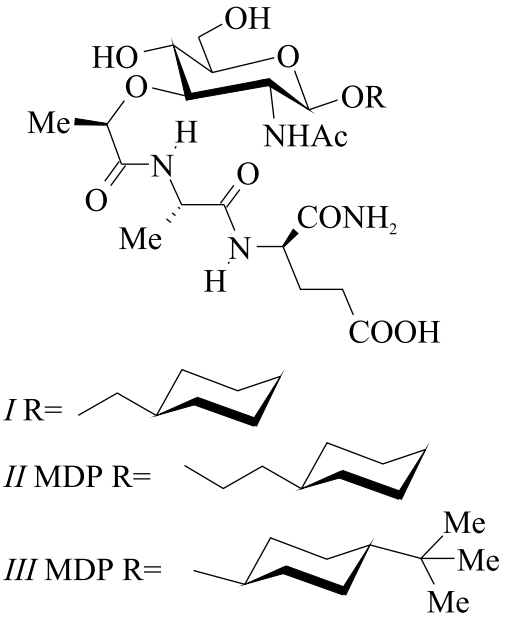

Fig. 1. Structure of β-cycloalkyl glycosides of MDP. β-Cyclohexylmethyl glycoside of MDP (*I*), β-cyclohexylethyl glycoside of MDP (*II*), and β-4-*tert*-butyl-cyclohexyl of MDP (*III*).

Tatkhimfarmpreparaty). This solution was brought to the desired concentration by adding 0.9% NaCl or culture medium.

The effect of glycopeptides on the resistance to bacterial infection was studied on outbred albino mice aging 20-25 days and weighing 12-14 g (Stolbovaya nursery) [4]. MDP glycosides in a final volume of 0.5 ml were injected intraperitoneally to animals with sepsis induced by *S. aureus* strain Wood 46 (doses 0.15, 1.5, and 15 mg/kg) or *Escherichia coli* strain 264 (doses 3.75, 75 μg/kg, and 1.5 mg/kg). Control mice received 0.5 ml 0.9% NaCl. The animals were infected with an 18-h culture of infectious agents 24 h after treatment. The amount of microbial bodies was estimated in preliminary experiments (10^9 and 2×10^7 for *S. aureus* and *E. coli*, respectively) and served as the minimum infectious dose (death of 100% animals over the first 3 days). The animals were examined for 10 days. The efficacy of MDP glycosides was estimated from animal survival.

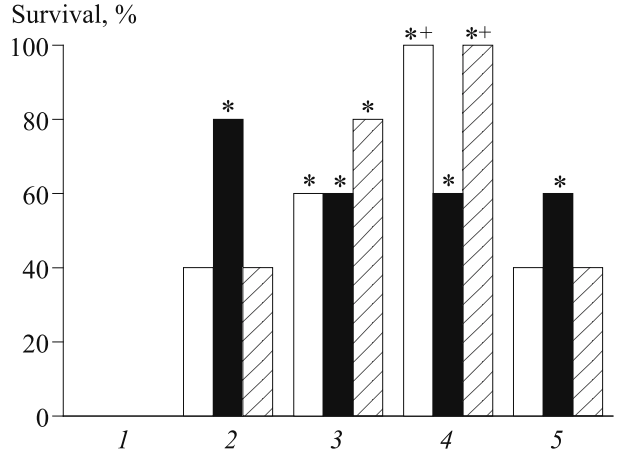

Fig. 2. Effect of β-cycloalkyl glycosides of MDP on the resistance of animals to intraperitoneal infection with *S. aureus*. Light bars, 0.15 mg/kg; dark bars, 1.5 mg/kg; shaded bars, 15 mg/kg. Results of one of three independent experiments. Here and in Fig. 3: control (*1*); MDP (reference control, *2*); β-cyclohexylmethyl glycoside of MDP (*3*); β-cyclohexylethyl glycoside of MDP (*4*); β-4-*tert*-butyl-cyclohexyl of MDP (*5*). $p<0.05$: *compared to the control; +compared to the reference control (MDP).

Mononuclear cells were isolated from the venous blood of healthy donors by the standard method using a Ficoll-Paque density gradient (Pharmacia). The cells (final concentration 2×10^6 cells/ml) were cultured in RPMI 1640 medium containing 10% inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES buffer (Flow Lab), and 50 μg/ml gentamicin (KRKA). Culturing was performed in a humid atmosphere at 37°C and 5% CO_2 for 24 h. The test substances in a final concentration of 0.2, 2, or 20 μg/ml were added to the medium before incubation. Phytohemagglutinin (PHA, Difco) in a concentration of 1 μg/ml was added to the cell culture, which served as a positive control. The concentrations of interferon-α (IFN-α), IFN-γ, interleukin-1β (IL-1β), IL-4, IL-6, and tumor necrosis factor-α (TNF-α) were measured by solid-phase enzyme immunoassay with Vector-Best kits according to the manufacturer's recommendations.

The results were analyzed by Statistica 7.0 software.

RESULTS

Pretreatment with cycloalkyl glycosides of MDP increased survival of mice infected with *S. aureus* (Fig. 2). β-Cyclohexylethyl glycoside of MDP had the strongest protective effect. This substance in high (15 mg/kg) and low doses (0.15 mg/kg) prevented death of 100% animals (two of three experiments). β-Cyclohexylmethyl glycoside of MDP had a wide range of effective doses. However, the survival rate of infected animals after injection of this substance was lower compared to that observed in experiments with β-cyclohexylethyl glycoside of MDP. Activity of β-4-*tert*-butyl-cyclohexyl glycoside of MDP was similar to that of unmodified MDP. This substance significantly decreased mortality rate (dose 1.5 mg/kg) or tended to increase survival of animals (other doses).

β-Cyclohexylethyl glycoside of MDP was most potent in increasing the antibacterial resistance during experimental sepsis induced by intraperitoneal injection of *E. coli* culture (Fig. 3). This glycopeptide in a dose of 1.5 mg/kg prevented death of 100% animals (much

more significant than MDP). β-Cyclohexylmethyl glycoside of MDP in the same dose increased survival of infected animals to 60%. MDP and β-4-*tert*-butylcyclohexyl glycoside of MDP tended to decrease the mortality rate of infected mice.

β-Cyclohexylethyl glycoside of MDP was more potent than other derivatives of MDP in stimulating *in vitro* production of IL-1β, IL-6, TNF-α, and IFN-γ by human mononuclear cells (Table 1). As differentiated from other glycosides of MDP, this glycopeptide increased production of IL-4 and tended to stimulate the synthesis of IFN-α. β-Cyclohexylethyl glycoside of MDP in a dose of 20 μg/ml significantly increased the production of this cytokine. β-Cyclohexylmethyl glycoside of MDP was most effective activator of IL-4 production and its effect in a dose of 2 μg/ml far surpassed the effect of the reference preparation. It should be emphasized that both glycopeptides in concentrations of 2 and 20 μg/ml increased the production of IL-6 (similarly to PHA in a dose of 1 μg/ml). Similarly to *in vivo* experiments, biological activity of β-4-*tert*-butyl-cyclohexyl glycoside of MDP was lower than that of MDP and other derivatives. This glycoside of MDP only in a concentration of 20 μg/ml stimulated the production of IL-4 and IL-6.

The increase in biological activity of cycloalkyl glycosides of MDP in *in vivo* and *in vitro* test systems was shown to correlate with mobility of the cyclic aglycone (relative to the glycoside site of MDP). β-Cyclohexylethyl glycoside of MDP was most potent in stimulating antibacterial resistance of mice and cytokine production by human mononuclear cells. Its structure includes a two-carbon spacer, which provides greater conformational mobility of the lipophilic aglycone. These features contribute to

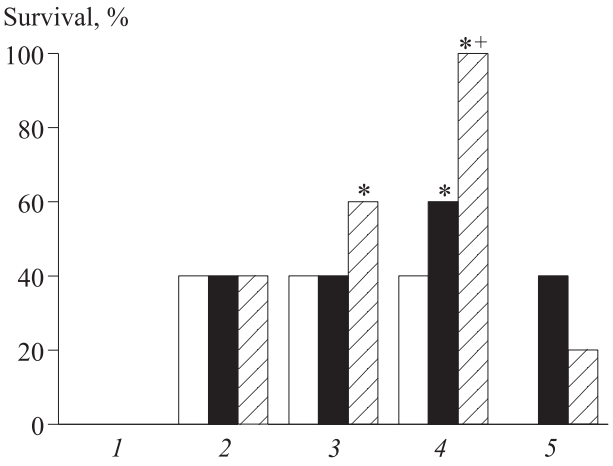

Fig. 3. Effect of β-cycloalkyl glycosides of MDP on the resistance of animals to intraperitoneal infection with *E. coli*. Light bars, 3.75 μg/kg; dark bars, 75 μg/kg; shaded bars, 1.5 mg/kg.

TABLE 1. Effect of Cycloalkyl Glycosides of MDP on Cytokine Production by Human Mononuclear Cells (pg/ml, *M*±*m*)

Test substances	Concentration of substances, μg/ml	IFN-α	IFN-γ	TNF-α	IL-1β	IL-4	IL-6
Spontaneous production (control)	—	18±6	15±4	92±21	30±9	6.1±3.2	81±19
PHA	1	25±4	189±42*	737±95*	1020±110*	41±19*	1250±210*
MDP (reference control)	0.2	19±5	11±5	116±29	37±10	11±7	72±16
	2	14±6	27±5*	154±21*	40±12	20±4*	144±22*
	20	15±7	26±4*	161±25*	68±11*	29±6*	163±29*
β-Cyclohexylmethyl glycoside of MDP	0.2	15±4	14±3	128±20	44±6	21±5*	237±51*+
	2	19±7	31±5*	171±22*	51±6*	42±7*+	975±120*
	20	20±6	25±6	159±26*	59±7*	41±5*	1090±140*+
β-Cyclohexylethyl glycoside of MDP	0.2	22±5	22±6	160±19*	60±7*+	19±5*	365±62*+
	2	21±6	35±6*	203±25*	69±6*+	21±3*	1330±150*+
	20	31±4*+	34±5*	211±24*	111±15*+	40±5*	1290±110*+
β-4-*tert*-Butyl-cyclohexyl of MDP	0.2	14±5	17±5	84±16	23±6	5.4±2.9	85±13
	2	12±7	12±4+	98±11+	24±7	11±3	101±14+
	20	21±4	14±3+	110±19+	39±8+	16±4*+	179±21*

Note. Results of 3 independent experiments. $p<0.05$:*compared to the control; +compared to the reference control (MDP).

biological advantages of β-cyclohexylethyl glycoside of MDP over other derivatives of MDP. By contrast, cyclic aglycone of β-4-*tert*-butyl-cyclohexyl glycoside is firmly attached to MDP. This glycoprotein had minimum immunotropic effect and was less potent than MDP (in several test systems). Further studies should be performed to evaluate the relationship between the structure and mobility of aglycones (relative to the glycoside site in cycloalkyl glycosides of MDP) and interaction of these glycopeptides with intracellular receptors NOD-2.

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