In Vitro Effect of Combined Hybrid Molecules from Vitamin E Analogues and Betulinic Acid on Macrophage Activity

A. N. Ivanova, Y. P. Belsky, A. Yu. Spivak*, M. G. Danilets, N. V. Belska, R. R. Chalitova*, A. A. Ligatcheva, and E. G. Uchasova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 6, pp. 638-641, June, 2011
Original article submitted October 29, 2010

Macrophage activity was studied after treatment with hybrid molecules obtained by condensation of terpenic acid residues (betulinic and betulonic acids) and α-tocopherol analogues (α-tocopherol hemisuccinate and Trolox acid). As distinct from betulinic acid and α-tocopherol hemisuccinate, hybrid molecules did not exhibit cytotoxicity in relation to mouse peritoneal macrophages in the MTT test. Test substances inhibited the production of NO by mouse peritoneal macrophages. However, hybrid molecules had no effect on activity of macrophage arginase. Our results indicate that new molecules have anti-inflammatory activity. It can be hypothesized that these substances have immunomodulatory properties.

Key Words: *betulinic acid; α-tocopherol; macrophage; nitric oxide; arginase*

Inflammation-inducing agents polarize macrophages into functionally different types (M1 cells, classically activated macrophages; and M2 cells, alternatively activated macrophages) [9]. M1 macrophages have the anti-inflammatory properties, produce inflammatory mediators (IL-1, IL-6, IL-12, NO, and TNF-α), disintegrate the intercellular matrix, and stimulate apoptosis in infected or transformed cells [4,8,10]. By contract, M2 macrophages possess proinflammatory activity, secrete anti-inflammatory agents (IL-10, IL-1 receptor antagonist, and transformed growth factor-β), and contribute to reparation of damaged tissues (arginase-catalyzed synthesis of angiogenic factors and polyamines) [4,8,10]. A specific feature of activated macrophages is the pathway of arginine utilization. Arginine serves as a substrate of inducible NO synthase in M1 cells, which produces NO and citrulline. In M2 cells, arginine is metabolized by arginase with the formation of urea and ornithine [7,11].

Betulinic acid belongs to lupane triterpenoids. This substance has antitumor activity [12] and *in vitro* inhibits NO production by murine RAW 264.7 cells [5] and TNF-α-induced activation of NF-κB [15]. However, the development of new pharmaceuticals from betulinic acid is not appropriate due to low activity of this compound [5,12]. To preserve or increase biological activity of betulinic acid, many attempts were made to modify chemically the molecule of this substance. These attempts can be successful by the combination of betulinic acid with α-tocopherol or its analogues containing the pharmacophoric chroman fragment of tocopherols. Besides antioxidant activity, tocopherols have anti-inflammatory properties [13]. Previous experiments showed that a course of treatment with α-tocopherol reduces significantly the severity of LPS-induced lung inflammation and septic shock [14].

Institute of Pharmacology, Siberian Division of the Russian Academy of Medical Sciences, Tomsk; *Institute of Petrochemistry and Catalysis, Russian Academy of Sciences, Ufa, Russia. ***Address for correspondence:*** belsky59@mail.ru. Y. P. Belsky

0007-4888/11/1516-0694

There are no data on the synthesis of conjugates from tocopherol and its analogues with lupane pentacyclic triterpenoids. Hybrid compounds can gain new biological properties in the presence of the methylated chroman fragment with high antioxidant activity.

Here we studied anti-inflammatory activity of hybrid molecules from betulinic acid and α-tocopherol hemisuccinate or hydrophilic chroman antioxidant Trolox.

MATERIALS AND METHODS

The experiments were performed on male and female C57Bl/6 mice at the age of 8-12 weeks obtained from the Department of Experimental Biological Models (Institute of Pharmacology, class I strain). The animals were maintained in an incomplete barrier system with the 12:12-h light/dark regimen and had free access to water and food (pelleted feed).

Hybrid molecules were synthesized at the Institute of Petrochemistry and Catalysis. They were obtained by the condensation of betulinic acid with α-tocopherol hemisuccinate or interaction of pre-synthesized betulonic acid chloroanhydrides or betulinic acid chloroanhydrides with the hydrazide derivative of Trolox acid [2].

Macrophages were isolated from the peritoneal fluid. The peritoneal cavity of mice was washed with cold physiological saline (PS, isotonic solution of 0.9% NaCl; Zavod Medsintez). The cells were pelleted and resuspended in the medium of RPMI 1640 (State Research Center Vektor), 10% FBS (HyClone), 20 mM HEPES (Sigma), 0.05 mM 2-mercaptoethanol (Sigma), 50 μg/ml gentamicin (Sigma), and 2 mM L-glutamine (Sigma). They were placed ($1.5\text{-}2.0\times10^6$ cells/ml) in plastic Petri dishes and cultured at 37°C and 5% CO_2 for 2 h. The cells adhering to a plastic surface were collected.

We studied the cytotoxicity and effect of new molecules on functional activity of macrophages. The cells were placed in 96-well flat-bottom plates (2.0×10^5 cells/well), treated with LPS (Sigma) and test substances, and cultured at 37°C and 5% CO_2 for 2 days. Some wells were used to evaluate cytotoxicity in the MTT test [8]. The supernatant was taken from other wells. Nitrite content in the supernatant was measured with Griess reagent on a Titertek spectrophotometer at 540 nm. Cell arginase activity was measured by a modified method [1]. Triton X-100 (0.1%, 0.1 ml) was added to the wells with macrophages. Culturing was conducted at room temperature and constant shaking for 30 min. Tris-HCl (25 mM) and manganese chloride (10 mM, 0.035 ml) were put into tubes to activate arginase. Incubation was performed at 56°C for 10 min. L-Arginine (0.5 M, pH 9.7, 0.05 ml; Sigma) was added to the lysate (0.05 ml). Culturing was conducted at 37°C for 60 min. The reaction was stopped by addition of 0.8 ml mixture of concentrated acids (sulfuric acid and phosphoric acid) and water (1:3:7 v/v). Urea concentration in the solution was measured with Mochevina-450 test system (Bio-LA-Test) according to the instruction manual for a spectrophotometer (540 nm). The amount of arginase catalyzing the formation of 1 mM urea over 1 min was taken as one unit of enzyme activity. MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Serva) in a concentration of 200 μg/ml was added to wells 4 h before the end of culturing to estimate cytotoxicity. The supernatant was removed. The pellet was dissolved with dimethylsulfoxide (dimexide, Tatkhimfarmpreparaty). Absorption of these solutions was measured on a multichannel spectrophotometer at 550 nm.

The results were analyzed by Student's *t* test. The differences were significant at $p<0.05$.

RESULTS

Betulinic acid and α-tocopherol hemisuccinate in concentrations of 25 and 50 μg/ml produced a toxic effect. Hybrid molecules in the same concentrations did not exibit cytotoxicity (Table 1).

The test substances modulated NO production by LPS-stimulated macrophages (Table 2). Betulinic acid (0.1-50 μg/ml) dose-dependently inhibited NO production. α-Tocopherol hemisuccinate and hybrid molecule MR-2009-5 in concentrations of 10, 25, and 50 μg/ml dose-dependently decreased NO production. The hybrid molecule MR-2009-5 was less potent than α-tocopherol hemisuccinate in inhibiting this process. Hybrid molecules MR-2009-2 and MR-2009-4 (25 and 50 μg/ml) dose-dependently decreased the production of NO.

We evaluated the effect of test substances on arginase activity (Table 3). Betulinic acid in concentrations of 10, 25, and 50 μg/ml decreased activity of arginase. α-Tocopherol hemisuccinate in concentrations of 25 and 50 μg/ml also decreased activity of this enzyme. Arginase activity was reduced after treatment with the hybrid molecule MR-2009-4 only in high concentration (50 μg/ml). Other hybrid molecules had no effect on arginase activity.

Our results indicate that parent substances (betulinic and α-tocopherol hemisuccinate) in high concentrations (25 and 50 μg/ml) have a cytotoxic effect. These substances in lower concentrations not only decreased the production of NO (sign M1), but also inhibited arginase activity (sign M2). Hybrid molecules MR-2009-2 and MR-2009-5 produced a selective effect on macrophages. They decreased the production

TABLE 1. Cytotoxic Effect of Betulinic Acid (BA), α-Tocopherol Hemisuccinate (THS), and Hybrid Molecules MR-2009-2, MR-2009-4, and MR-2009-5 on Peritoneal Macrophages (% absorption, *X*±*m*)

Concentration of substances, μg/ml	Substance				
	BA	THS	MR-2009-2	MR-2009-4	MR-2009-5
Control			403±31		
0.01	447±29	463±20	453±26	448±34	411±17
0.1	445±19	403±18	429±24	448±21	357±14
1	452±30	355±52	443±17	459±47	455±47
10	332±49	487±31	452±35	458±45	467±58
25	237±20*	186±43*	427±22	478±28	477±42
50	273±46*	150±20*	462±36	484±44	503±12*

Note. The percentage of absorption in macrophage-containing wells (without LPS) is 425±49. *p<0.05 compared to the control.

TABLE 2. Effect of Betulinic Acid (BA), α-Tocopherol Hemisuccinate (THS), and Hybrid Molecules MR-2009-2, MR-2009-4, and MR-2009-5 on NO Production by Peritoneal Macrophages (*X*±*m*)

Concentration of substances, μg/ml	NO production (nitrite concentration, μM) in the presence of				
	BA	THS	MR-2009-2	MR-2009-4	MR-2009-5
Control			47.8±1.0+		
0.01	45.8±0.3	46.0±1.5	47.9±0.8	46.6±1.0	47.3±1.7
0.1	44.8±0.3*	46.2±1.1	46.4±0.9	45.0±1.4	47.7±0.7
1	41.2±0.9*	45.8±1.1	45.6±0.6	47.7±0.8	47.8±0.4
10	29.2±1.2*	33.1±2.3*	43.8±1.3	44.1±1.7	42.8±1.4*
25	5.9±0.5*	3.4±0.4*	40.5±2.2*	40.6±2.1*	34.4±1.1*
50	1.4±0.8*	1.4±0.9*	33.1±1.2*	19.8±1.2*	13.1±1.3*

Note. The concentration of nitrites in macrophage-containing wells (without LPS) is 7.5±1.8 μM. p<0.05: *compared to the control; +compared to the culture without LPS.

TABLE 3. Effect of Betulinic Acid (BA), α-Tocopherol Hemisuccinate (THS), and Hybrid Molecules MR-2009-2, MR-2009-4, and MR-2009-5 on Arginase Activity in Peritoneal Macrophages (*X*±*m*)

Concentration of substances, μg/ml	Arginase activity (U) in the presence of				
	BA	THS	MR-2009-2	MR-2009-4	MR-2009-5
Control			31.2±1.6		
1	32.0±1.0	30.4±3.3	31.8±0.9	32.1±1.6	31.8±2.7
10	21.7±1.2*	30.4±1.1	28.3±1.4	30.2±1.0	31.3±0.7
25	2.4±0.8*	4.5±1.7*	27.8±1.4	27.9±1.2	31.3±1.1
50	0.3±0.3*	0.6±0.2*	27.1±1.6	13.5±0.7*	29.5±0.7

Note. Arginase activity in macrophage-containing wells (without LPS) is 29.7±1.2. *p<0.05 compared to the control.

of NO, but did not modulate activity of arginase. The exception was the molecule MR-2009-4. This molecule in the highest concentration was shown to inhibit arginase. These data show that hybrid molecules possess anti-inflammatory properties.

M1 cells maintain the Th1-mediated immune response, while anti-inflammatory M2 cells contribute to the Th2-mediated immune response [4,7,8,10]. The destructive potential of classically activated macrophages is a key mechanism for autoimmune diseases [3]. New molecules, which inhibit NO production and do not affect arginase activate, probably have immunomodulatory activity shifting the Th1/Th2 balance towards Th2. This property is of considerable importance for the therapy of autoimmune diseases due to hyperactivation of Th1.

We conclude that modification of betulinic acid and α-tocopherol hemisuccinate molecules is accompanied by a decrease in toxicity of these substances, which results in the formation of conjugates with a selective anti-inflammatory effect.

This study was supported by the Russian Foundation for Basic Research (grant No. 10-03-00105) and Program of the Department for Chemistry and Material Sciences of the Russian Academy of Sciences (Biomolecular and Medical Chemistry).

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