Coll. Antropol. **35** (2011) 4: 1219–1223
Original scientific paper

A Tetrahydrophthalazine Derivative »Sodium Nucleinate« Exerts a Potent Suppressive Effect upon LPS-Stimulated Mononuclear Cells *in vitro* and *in vivo*

Tomislav Jukić[1], Musa Abidov[2] and Alojz Ihan[3]

[1] »J. J. Strossmayer« University, School of Medicine, Department of Internal Medicine, History of Medicine and Ethics, Osijek, Croatia
[2] Russian Academy of Natural Sciences, Institute of Immunopathology, Moscow, Russia
[3] University of Ljubljana, Medical Faculty, Institute for Microbiology and Immunology, Ljubljana, Slovenia

A B S T R A C T

We described the use of a new chemical substance Sodium nucleinate (SN) as an immunomodulatory substance exhibiting antiinflammatory properties. Sodium nucleinate (SN) registrated in Russian Federation as Tamerit®, is 2-amino-1,2,3,4-tetrahydrophthalazine-1,4-dione sodium salt dihydrate, derivative of well known chemical substance luminol. To comprehend the mechanisms of SN immunomodulatory activity, we examined the SN modulation of the innate inflammatory cytokine response of human PBMC stimulated with LPS in vitro. Furthermore, we studied the immunomodulatory effects of SN in mice challenged with E. coli LPS in vivo to investigate a possible novel approach to therapy of excessive inflammation that interfere with the response to endotoxin and inflammatory mediators. Our results demonstrated that SN is an efficient inhibitor of sepsis development in mice model of LPS-induced sepsis. The changes induced by SN include decreased mice plasma inflammatory cytokine production. Simmilary we demonstrated a decreased TNF-α, IFN-γ and IL-6 response in human LPS-stimulated PBMNCs. SN was therefore shown to be a promising inhibitor of multiple inflammatory cytokine secretion.

Key words: *sepsis, lipopolysaccharide, tumor necrosis factor alpha, gamma interferon, interleukin 6, interleukin 12*

Introduction

Sodium nucleinate (SN) registrated in Russian Federation as Tamerit®, is 2-amino-1,2,3,4-tetrahydrophthalazine-1,4-dione sodium salt dihydrate, derivative of well known chemical substance luminol. SN is described in Russian pharmacopeia as immunomodulatory substance exhibiting antiinflammatory and antioxidative properties. Another derivative of luminol, a refined monosodium 5-amino-2-3-dihydro-1-4-phthalazine dione (galavit) has been also extensively studied by Russian scientists due to its antiinflammatory properties. Both compound were not available in EU or United States until recently. Only in 2002 a first article desribing clinical immunomodulatory properties of galavit appeared[1].

In 2006 Jiang Y et al. described galavit as a poverfull protector of neurodegeneration in mice infected with the cytotoxic retrovirus *ts*1, a mutant of Moloney murine leukemia virus[2]. Since the protection of infected mice was not associated with suppressed viral replication, an antiinflammatory/antioxidant properties of galavit was supposed to cause the protection mechanism. The idea was also supported from studies showing that antioxidant defense mechanisms are activated in cultured astrocytes in response to their infection by ts1 and that as many as half of these cells survive infection by successfully mobilizing these pathways[3].

The immunomodulatory properties of second luminol derivative SN has been not described in western medical literature yet. SN consists of 2 highly active aminophthalhydrazides (Figure 1). To comprehend the mechanisms of SN immunomodulatory activity, we examined

Received for publication October 30, 2011

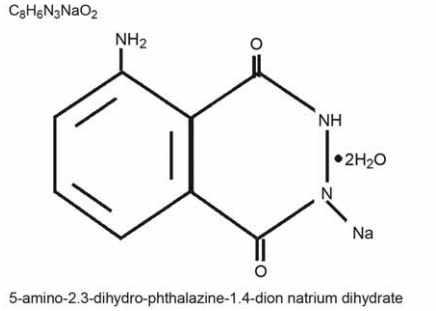

Fig. 1. Structural and Chemical Tamerit® Formula.

the SN modulation of the innate inflammatory cytokine response of human PBMC stimulated with LPS *in vitro*. Furthermore, we studied the immunomodulatory effects of SN in mice challenged with *E. coli LPS in vivo* to investigate a possible novel approach to therapy of excessive inflammation that interfere with the response to endotoxin and inflammatory mediators[4].

Materials and Methods

Reagents and preparation of LPS

Water, RPMI-1640 medium supplemented with 25 mM Hepes buffer, (all sterile, cell culture-tested and endotoxin-tested; endotoxin level ≤ 1EU/mL), and LPS from *Escherichia coli* (strain 0111:B4) were commercially obtained from Sigma-Aldrich Corp., St. Louis, USA. LPS was dissolved as 1 mg/mL stock solution in water, further diluted in a cell culture medium and titrated in a preliminary experiment[5].

Isolation and stimulation of human PBMC

Eight healthy volunteers (age range from 20 to 55 years) with no symptoms of allergy, autoimmune diseases or acute infections were included in the study. PBMC from venous blood with EDTA were isolated by density gradient centrifugation with Ficoll-Paque™. The cells were counted by the trypan blue dye exclusion method and cultured in the RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 10% heat-inactivated human serum. The 1×10^6 cells (final culture volume of 1.5 mL) were plated in 24-well culture plates with medium alone, with SN alone, or together SN with LPS at 37°C in a humidified atmosphere of 5% CO_2 in the air. To establish the TNF-α response to the LPS challenge, PBMCs were incubated for 18 hours with LPS (from E. coli, 0111:B4; Sigma, St. Louis, Missouri, USA), with a final culture concentration of 10 ng/mL. Evaluation of the IFN-γ response was performed by simultaneous polyclonal activation by phorbol 12-myristat 13-acetate (PMA), with a final concentration of 3.33 ng/mL, and ionomycin (IONO), with a final concentration of 500 nM. Plates were then incubated for 40 hours. Plates with LPS and plates with IONO&PMA were incubated at 37°C, in a 5% CO2 atmosphere and 95% humidity. The cell-free supernatants were collected at 18 and 40 hours, respectively. Supernatant samples as well as serum samples were stored at -20°C before being evaluated for cytokines using commercially available ELISA kits for measuring human TNF-α, IFN-γ (Pierce – Endogen, Rockford, IL, USA), IL-6 and IL12 (R&D Systems, Minneapolis, MN, USA).

Mouse experiments

Female BALB/c mice were obtained at eight weeks of age. Each mouse was assessed daily for weight gain, feed intake, clinical status and behavioral changes. SN in doses of 2, 20, 200, and 2000 ug/ mouse was injected intraperitoneally 2 h before injection with LPS (from E. coli, 0111:B4; Sigma, St. Louis, Missouri, USA) to study the modulation of cytokine response to LPS. Serum samples were stored at -20°C before being evaluated for three cytokines using commercially available ELISA. TNF-α and IL-6 were were quantified by using the Pharmingen protocol (Pharmingen, San Diego, USA). The concentrations of IFN-γ were measured using commercially available ELISA kits purchased from Thermo Scientific, USA.

Statistical analysis

Descriptive statistics were calculated for all the observed parameters. Independent sample t-tests were performed to asses the difference in means between the two groups A $p\leq0.05$ value was considered statistically significant. All statistical analyses were performed using SPSS for Windows software (version 15.0.1.1., Chicago, IL, 2007).

Results

Effect of SN on *in vitro* cytokine production induced by LPS in human PBMC.

The culture conditions were established to assess the ability of SN to modulate the LPS-induced secretion of cytokines in human PBMC. PBMC from 8 healthy volunteers were stimulated with a – LPS (10 ng/mL); b – LPS (10 ng/mL) + SN (0.16–100 μg/mL); Cytokine levels in cell culture supernatants were measured, and a modulatory effect of each SN concentration on the LPS-induced cytokine production was evaluated. As shown in Figure 2, a SN addition to LPS had a significant inhibitory effect on the LPS-induced TNF-α (SN concentrations 10–100 μg/mL), IFN-γ (SN concentrations 4–100 μg/mL), and IL-6 (SN concentrations 10–100 μg/mL).

SN treated mice ameliorate the *in vivo* inflammatory response to LPS.

In animal study we investigated whether SN can reduce the cytokine response and thereby improve survival in mouse endotoxin shock. To induce lethal shock, we injected 1 mg i.p. LPS/mouse corresponding to a dose of 40 mg/kg b.wt. This dose produced 60% mortality within 72 hr, which is in agreement with numerous studies in mice using LPS at doses of 30 to 60 mg/kg i.p., which usually result in mortality rates of >50% (Redmond et al., 1991; Novogrodsky et al., 1994). As seen in Table 1, a single in-

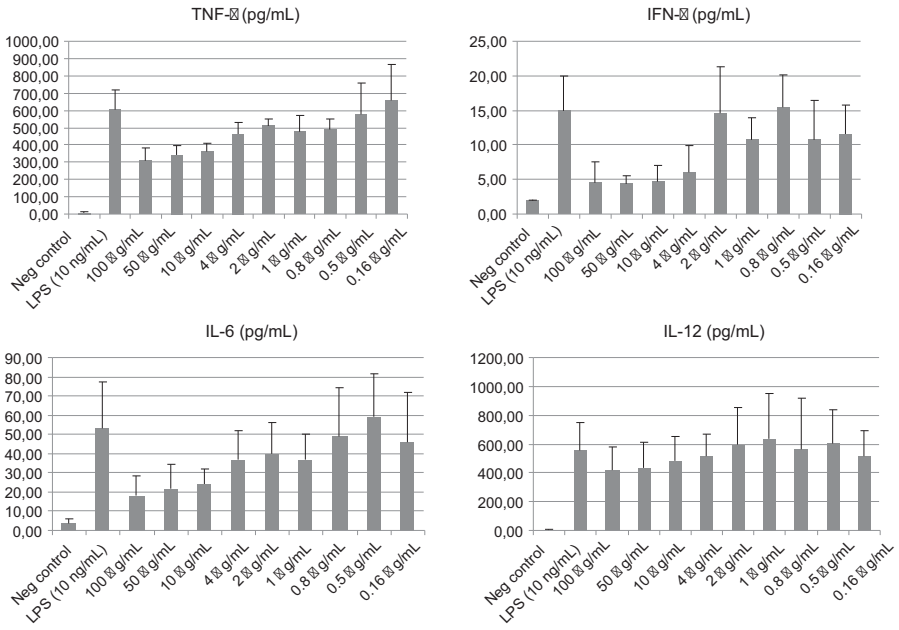

Fig. 2. The effect of SN on the LPS-induced secretion of TNF-α, IFN-γ, IL-6 and IL-12 in human PBMC. PBMC (1×10^6 cells/mL) were isolated from venous blood of a healthy volunteer and stimulated for 4 h with with a – LPS (10 ng/mL); b – LPS (10 ng/mL) + SN (0.16–100 μg/mL). Each point represents the mean cytokine concentration with SEM of 8 cultures.

jection of SN 2 hr before LPS significantly reduces the mortality of endotoxin shock from 60% to 40%.

To determine the release of inflammatory cytokines after LPS challenge, blood (≈1 mL) was collected by cardiac puncture after sternotomy in pyrogen-free, nonheparinized microtubes. To minimize distress, mice were kept under deep anesthesia breathing an enflurane/oxygen mix delivered from a vaporizer over a nose cone during these procedures and then killed. Blood in microtubes was allowed to clot at room temperature and centrifuged in a microfuge (12,000 rpm for 2 min). Sera were transferred into Eppendorf cups and stored at -20°C until assayed. Serum concentrations of TNF-α and IL-12 after LPS-injection were significantly lower in animals pretreated with Tamerit compared with controls (Table 1).

Discussion

In the current study, we first demonstrated that SN is an efficient inhibitor of sepsis development in mice model of LPS-induced sepsis. The changes induced by SN include decreased mice plasma inflammatory cytokine production. Similarly we demonstrated a decreased TNF-α, IFN-γ and IL-6 response in human LPS-stimulated PBMNCs. SN was therefore shown to be a promising inhibitor of multiple inflammatory cytokine secretion. It was shown many times before that multiple cytokine secretion may trigger or prolong a vast number of pathological conditions, the inhibition of cytokine release may therefore serve as a tool for a disease control[6-8]. In the current study we demonstrated that SN is an effective cytokine inhibitor in severe sepsis that often results with organ dysfunction and is associated with significant morbidity and mortality[9]. Sepsis results in a cascade of events, including the production of pro- and anti-inflammatory cytokines and a shift from a Th1 to a Th2 immune phenotype, as well as the induction of apoptosis of immune effector cells[5,10]. The early phase of sepsis is dominated by a hyperinflammatory state mediated by systemic production of inflammatory cytokines, including interleukin 1 (IL-1), interleukin 6 (IL-6), tumor ne-

TABLE 1
EFFECTS OF TAMERIT ON SURVIVAL AND BLOOD CYTOKINES IN LPS CHALLENGED MICE. EACH CYTOKINE POINT REPRESENTS THE MEAN CYTOKINE CONCENTRATION WITH SEM OF 10 SAMPLES

Parameter	Control	LPS	LPS + SN, mg/mouse			
			2	20	200	2000
Mouse survival (72hr)	100 %	40 %	40 %	40 %	60 %	60 %
IFN-γ (pg/mL)	3±2.1	21±15.3	16±12.4	14±8.6	6±4.3	4±2.1
TNF-α (pg/mL)	12±8.2	222±136.7	154±97	156±112	84±54	35±26
IL-6 (pg/mL)	40±32.4	13600±8234	11561±543	6512±3411	1874±876	1811±911

crosis factor alpha (TNF-α), and gamma interferon (IFN-γ)[3,7]. Althought anticytokine therapies have been introduced in Gram-negative sepsis as a means of preventing excessive immune reactions, the usefulness of antiinflammatory strategies in sepsis in order to block the activity of a single mediator in the cascade of immune reactions is under discussion. Despite promising experimental data, monoclonal antibodies and receptor antagonists neutralizing a particular inflammatory cytokine, such as TNF-α and IL-1β, have failed so far to improve the clinical outcome of patients with sepsis[9]. Therefore, novel approaches to therapy of sepsis are being investigated that interfere with the intracellular signal transduction to modulate the entire cellular response to endotoxin and inflammatory mediators.

In this study, we investigated whether SN can reduce the cytokine response and thereby improve survival in mouse endotoxin shock. To induce lethal shock, we injected 1 mg i.p. LPS/mouse corresponding to a dose of 40 mg/kg b.wt. This dose produced 60% mortality within 72 hr, which is in agreement with other studies in mice using LPS at doses of 30 to 60 mg/kg i.p., which usually result in mortality rates of >50%[10]. We found that a single injection of SN 15 min before LPS significantly reduces the mortality of endotoxin shock from 60% to 40%. The mechanisms responsible for a better survival in SN treated animals, however, have not been clarified. Because LPS is a major trigger for inflammatory cytokines, which accounts for most of its toxic and lethal effects *in vitro* and *in vivo*, we examined the effect of SN on the release of TNF-α, IL-6 and IL-12 during endotoxin shock[11]. Our findings demonstrate that SN usage in mouse endotoxin shock has inhibitory effect on the release of all cytokines measured after LPS-challenge. These are the first data demonstrating an inhibitory effect of SN on the LPS--stimulated cytokine release. Because IL-6 in particular is known to cause lethal shock in various animal models synergistically with TNF-α the suppression of cytokines by SN in this endotoxin model most likely is a major factor for the protection from endotoxin lethality observed in SN-treated mice[12]. The reduced IL-12 release in SN--treated mice may further ameliorate the inflammatory response to LPS (*e.g.*, induction of acute phase proteins), although IL-12 *per se* does not seem to be a major pathogenic factor for endotoxin lethality[13].

In conclusion, our findings imply SN as potentially important drug inhibiting the pathogenicity of LPS by modulating cytokine response. Further studies are warranted to evaluate whether SN may serve as a novel approach to therapy of sepsis.

Abbrevations

LPS lipopolysaccharide
TNF-α tumor necrosis factor alpha
IFN-γ gamma interferon
IL-6 interleukin 6
IL-12 interleukin 12

REFERENCES

1. NELYUBOV MV, Bull Exp Biol Med, 134 (2002) 165. — 2. YUHONG J, VIRGINIA L, MINGSHAN Y, WENAN Q, AMY J, WILLIAM S, PAUL KY, Journal of Virology, 80 (2006) 4557. — 3. QIANG W, KUANG JL, LIU VL, SCOFIELD AJ, REID YJ, STOICA WSL, WONG PK, J Virol, 80 (2006) 3273. — 4. DINARELLO CA, GELFAND JA, WOLFF SM, JAMA, 269 (1993) 1829. — 5. SHALABY MR, HALGUNSET J, HAUGEN OA, AARSET H, AARDEN L, WAAGE A, MATSUSHIMA K, KVITHYLL H, BORASCHI D, LAMVIK J, Clin Immunol Immunopathol, 61 (1991) 69. — 6. BARIŠIĆ-DRUŠKOV, RUČEVIĆ I, Coll Antropol, 28 (2004) 277. — 7. KATALINIĆ V, SALAMUNIĆ I, PAŽANIN S, MULIĆ R, MILIŠIĆ M, ROPAC D, Coll Antropol, 31 (2007) 165. — 8. GLASNOVIĆ M, BOŠNJAK I, VČEV A, SOLDO I, KOŠUTA M, LENZ B, GLASNOVIĆ-HORVATIĆ E, SOLDO-BUTKOVIĆ S, MIĆUNOVIĆ N, Coll Antropol, 31 (2007) 173. — 9 NOVOGRODSKY A, VANICHKIN A, PATYA M, GAZIT A, OSHEROV N, LEVITZKI A, Science, 264 (1994) 1319. — 10. TATEDA K, MATSUMOTO T, MIYAZAKI S AND YAMAGUCHI K, Infect Immunol, 64 (1996) 769. — 11. PRASHANT RM, SHIRSHENDU DG, SNEHA R, RAJANI G, MANGE RY, Exp Opin Therap Pat, 20 (2010) 31. — 12. ESPOSITO E, CUZZOCREA S, Curr Med Chem, 16 (2009) 3152. — 13. MATERA G, MUTO V, VINCI M, ZICCA E, ABDOLLAHI-ROODSAZ S, VAN DE VEERDONK FL, KULLBERG BJ, LIBERTO MC, Clin Vaccine Immunol, 16 (2009) 1804. — 14. LAMOTHE B, CAMPOS AD, WEBSTER WK, GOPINATHAN A, HUR L, DARNAY BG, J Biol Chem, 283 (2008) 24871.

A. Ihan

University of Ljubljana, Medical Faculty, Institute for Microbiology and Immunology, Zaloška 4, 1000 Ljubljana, Slovenia
e-mail: alojz.ihan@mf.uni-lj.si

DERIVAT TETRAHIDROFTALAZINA »NATRIJ NUKLEINAT« IMA SNAŽNI SUPRESIVNI UČINAK NA LPS-OM STIMULIRANE MONONUKLEARNE STANICE IN VITRO I IN VIVO

S A Ž E T A K

Opisali smo upotrebu nove kemijske tvari Natrij nukleinat(SN) kao imunomodulatorne tvari koja pokazuje anti-upalna svojstva. Natrij nukleinat(SN) registriran u Ruskoj Federaciji kao Tamerit® je 2-amino-1,2,3,4-tetrahidroftalazin-1,4 diion, dihidrat natrijeve soli, derivat dobro poznate tvari luminol. Da bi shvatili mehanizam SN imunomodulacije, ispitali smo SN modulaciju urođenog upalnog odgovora citokina ljudskih PBMC stimuliranih sa LPS in vitro. Nadalje, istraživali smo imunomodulacijske učinke SN kod miševa izloženih LPS-om E. Coli in vivo, da bi istražili mogući novi pristup terapiji prekomjerne upale, koji se upliće u odgovor endotoksina i medijatora upale. Naši rezultati su pokazali da je SN učinkovit u sprečavanju sepse na mišjem modelu LPS-om inducirane sepse. Promjene inducirane SN-om uključuju smanjenje produkcije plazmatskih upalnih citokina. Slično smo dokazali i sniženje TNF-á, IFN-ã i IL-6 odgovara kod ljudskih LPS-stimuliranih PBMNC. SN je stoga prikazan kao obećavajući inhibitor multiple upalne citokinske sekrecije.