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Immunoregulatory *In vitro*/*In vivo* Effects of 2,3-Secotriterpene Acetylhydrazone

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Abstract: Immunity is a defensive reaction of an organism to suppress alien bodies (antigens), such as bacteria and viruses. Natural triterpenoids and their semisynthetic derivatives have a wide range of biological activities, including effects on immune system. This study was aimed to determine immunotropic properties of the semisynthetic A-secotriterpenoid with antiviral activity-acetylhydrazone of 1-cyano-19β, 28-epoxy-2, 3-seco-2-nor-18αH-olean-3-al (ASO). The research included the study of ASO acute toxicity and its effects on humoral and cellular immunity in the local/systemic administration of the antigen and evaluation of proliferative response of mouse splenocytes *in vivo* and cytokine production of immunocompetent cells *in vitro*. ASO has been classified as a low-toxic compound (LD>1 g kg^{-1}) having different effects on humoral and cellular immunity. ASO showed dose-dependent (1.0-100.0 mg kg^{-1}) stimulatory effect on the formation of Plaque-forming Cells (PFC) under local/systemic immunization conditions and the inhibitory effect on the intensity of Delayed-type Hypersensitivity response (DTH). At concentrations 30 nM, ASO demonstrated *in vivo* inhibitory properties on production of IL-2, IL-4 and IFN-γ, while not affecting the synthesis of IFN-α. So it is concluded that, ASO is an attractive low-toxic A-secotriterpenoid with differently directed properties which suppresses the *in vitro* production of IL-2, IL-4 and IFN-γ cytokines, whereas *in vivo* this compound stimulates intensity of the humoral immune response.

Key words: Antibody-genesis, DTH, IL-2, IL-4, IFN-γ, triterpenoids, acetylhydrazones

INTRODUCTION

Development of efficient drugs based on low-molecular natural metabolites is a trend of current importance in modern medical chemistry. Among these, a plant triterpenoid betulin is regarded as a promising source. Examples of chemical modifications of betulin into biologically active derivatives are presented in numerous publications and reviews (Tolstikova *et al*., 2006a, b). An approach contributing to cytotoxic and antiviral actions of betulin and of its derivatives is related to the bond cleavage of the triterpene A ring (Urban *et al*., 2004, 2005; Wei *et al*., 2009; Akihisa *et al*., 2002; Tolmacheva *et al*., 2009).

Earlier, we had synthesized from betulin 2,3-secotriterpene hydrazones of the lupane and 18αH-oleanane types featured by a high level of inhibitory activity against the Vesicular Stomatitis Virus (VSV, Indiana strain) (Tolmacheva *et al*., 2010; Galayko *et al*., 2010). Antiviral properties of the synthesized hydrazones had been studied using two models of the virus infection: (1) Protection of a cell system against VSV affection and (2) The suppression of the VSV reproduction in primarily infected cells. The acetylhydrazone of 1-cyano-19β, 28-epoxy-2, 3-seco-2-nor-18αH-olean-3-al (ASO, Fig. 1), combining prophylactic (ED_{50} 0.00016 μg mL^{-1}) and therapeutic (ED_{50} 0.21 μg mL^{-1}) activities, had been selected (Galayko *et al*., 2010) (Fig. 1).

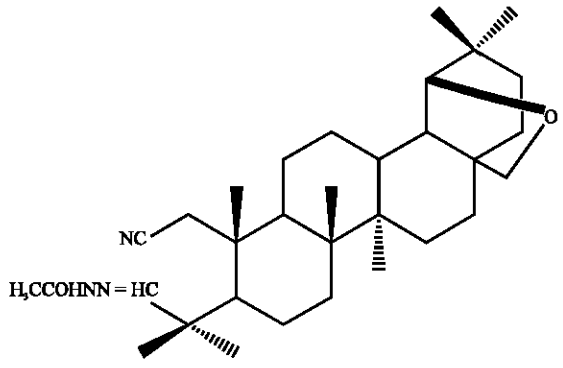

Fig. 1: Structure of acetylhydrazone of 1-cyano-19β, 28-epoxy-2,3-seco-2-nor-18αH-olean-3-al (ASO)

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Practical significance of the triterpenoids as potential therapeutically active agents is in many cases conditioned by their poly-targeted properties. So, several studies have revealed that some triterpenoids, additionally to the basic activity, can show a stimulating or a suppressive effect on the immune system (Rios, 2010). The discovered prophylactic anti-VSV action of ASO enables to assume the compound to have an additional immunstimulating activity. The aim of this work is to evaluate the influence of ASO on the indices of humoral and cell-mediated immunity under conditions of local/systemic administration of an antigen, as well as a proliferative *in vivo* response of mouse splenocytes and *in vitro* production of cytokines by immunocompetent cells.

MATERIALS AND METHODS

Animals: Outbreed male mice of 17-22 g b.wt. were used in the study. Animals were kept on a 12 h light-dark cycle with water and food *ad libitum* and were housed 5 mice to a cage. Mice were allowed to acclimate to the laboratory conditions for a minimum of 10 days before experimental manipulations. Procedures involving animals and their care were in conformity with the institutional guidelines, which are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, December 12, 1987).

Preparation of the synthetic secotriterpenoid ASO: Synthesis, physical and chemical characteristics of ASO has been described by Tolmacheva *et al*. (2010). Working concentrations of ASO were prepared in 0.2% solution of starch slime in the *in vivo* experiments and 10% alcohol solution (1% final concentration of alcohol in a well) of ASO was used in the *in vitro* experiments.

Toxicity study of ASO: Acute toxicity was determined by single intraperitoneal introduction of just-prepared suspension of ASO (1.0, 0.5 or 0.1 g kg^{-1} doses). In the acute toxicity tests, each dose was tested on 6 animals. Coextensive quantities of starch slime were injected into the control mice. The animals were monitored for 14 days. During this period, death-rate, variations in appearance, weight and behavioral reactions of the animals were registered.

Delayed-type hypersensitivity (DTH) reaction: To conduct immunological investigations, single 1, 10 or 100 mg kg^{-1} doses of ASO were intraperitoneally injected into the mice. Coextensive quantities of starch slime were injected into the control mice. In 1 h after introduction of ASO, the first group of animals was immunized by Sheep Red Blood Cells (SRBC) (10^8 cells/0.02 mL subcutaneously into plantar surface of the right foot to evaluate the local immune response, 10^8 cells/0.02 mL intraperitoneally-to evaluate the systemic immune response). In order to model the local immune response, the antigen was re-injected into plantar surface of foots on the 4th day of the immune response development. On the 5th day, the etherized animals were put out of the experiment. The regional popliteal lymph node or the spleen was extracted, whereupon the cell number of the organs and the PFC number (Jerne and Nordin, 1963) were evaluated. In addition, the DTH reaction was determined using measuring the foot size with an engineering micrometer. The DTH reaction index was calculated as per the formula:

$$\text{DTH index} = \frac{(P_o - P_k)}{P_k} \times 100\%$$

where, P_0 is the size of the experimental extremity, P_k is the size of the control extremity.

Proliferative activity: The second group of the experimental animals was put out of the experiment in 1 h after injection of ASO. Splenocytes were extracted and cultivated in round-bottom 96 well culture plates (Medpolymer, Russia) at 37°C for 72 h. Each well contained 5×10^5 cells in 0.2 mL of a complete cultural medium which was prepared *ex tempore* on the basis of the RPMI 1640 medium (Biolot, Russia) with added 10 mM HEPES (Sigma, USA), 2 mM L-glutamin (Sigma, USA), 100 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% FCS (ICN, USA). The Con A (20 μg mL^{-1}, Sigma, USA) was used as a mitogen. Eighteen hours before the end of cultivation, 10 μL (2 μCi) [^{3}H]-thymidine was added to each well to estimate cell proliferation. Radioactivity of the samples was measured using the Guardian scintillation counter (Wallac, Finland).

Cytokine detection: The samples of peripheral blood were harvested from a group of healthy volunteers aged 20-35 years, who confirmed their consent by signature. Leucocytes of peripheral blood were the object of the *in vitro* investigation. Mononuclear cells were isolated on Ficoll-verografin density gradient and cultivated (10^6 cells mL^{-1}) in 96-well plates (Medpolymer, Russia) in the 199 medium with added 10 mM HEPES (Sigma, USA), 2 mM L-glutamin (Sigma, USA), 100 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% FCS (ICN, USA) for 24 or 48 h. Simultaneously, spontaneous and PHA-induced

production of cytokines was estimated. The PHA (20 μg mL^{-1}) was introduced into the cultures concurrently with ASO (30 nM and 40 μM) under investigation.

The frozen supernatants of the cell cultures were stored at -20°C. Determination of the IL-2, IL-4, IFN-γ and IFN-α concentrations in the cell cultures' supernatants was performed using the sets as recommended by the manufacturer (Vector-Best, Russia).

Investigations were performed in accordance with national standards and requirements of the Rules of Laboratory and Good Clinical Practice in the Russian Federation.

Statistical analyses: The data were analyzed using one-way ANOVA followed by *post-hoc* LSD-test. Values of $p<0.05$ were taken as significant. All the data in the figures are presented as average values and their standard errors.

RESULTS

It has been ascertained that the investigated ASO has low toxicity and in dose-dependent manner *in vivo* (1,0-100,0 mg kg^{-1}) activates the humoral immune response under local and systemic immunization. Also, ASO inhibits cell-mediated immune reactions by suppressing DTH manifestation. At concentrations 30 nM and 40 uM *in vitro*, ASO supresses production of IL-2, IL-4 and IFN-γ without affecting the synthesis of IFN-α.

Toxicity study: The first study investigation had included determination of the ASO's toxicity under animal model conditions while taking into account appreciable *in vitro* level of the cytotoxic activity of ASO towards the finite swine embryo kidney cells (cytotoxic dose CD_{100} 62.5 μg mL^{-1}) (Galayko *et al*., 2010).

As it had been revealed in investigations into acute toxicity, ASO doses (1.0, 0.5 or 0.1 g kg^{-1}) did not influence the central nervous system, did not evoke either agitation or suppression of animals' activity. Death of animals was not registered in any group under investigation for the subsequent 14 day rehabilitation period. It had been ascertained that the tested doses of ASO did not cause inappropriate behavior of the animals during the entire experiment. Thus, ASO under investigation is classified as a low-toxic compound (4 toxicity class, according to Sidorov (1973)).

Delayed-type hypersensitivity reaction: In further experiments, 1, 10 and 100 mg kg^{-1} doses of ASO were tested. Our study has shown that intraperitoneal introduction of ASO under local immunization conditions leads to a dose-dependent stimulation of both relative (Fig. 2a) and absolute (Fig. 2b) quantities of Plaque-forming Cells (PFC) in a regional lymph node. At the same time, ASO was observed to suppress intensity of the Delayed-type Hypersensitivity (DTH) reaction at the 100 mg kg^{-1} dose as compared with the control animals (Fig. 3a) and did not influence a cell number in regional popliteal lymph nodes (Fig. 3b).

Introduction of ASO was also leading to an appreciable increase in the PFC number in the spleen, against a background of the systemic immune response (Fig. 4a, expressed relatively). The absolute values

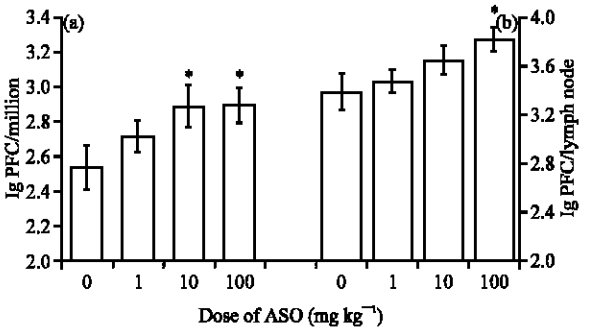

Fig. 2(a-b): Influence of ASO on (a) Relative and (b) Absolute number of PFC in regional lymph node of the mice locally immunized with SRBC (n = 7-8). *$p<0.05$ according to the Fisher LSD-criterion, n: No. of animals in groups, PFC: Plaque-forming cells

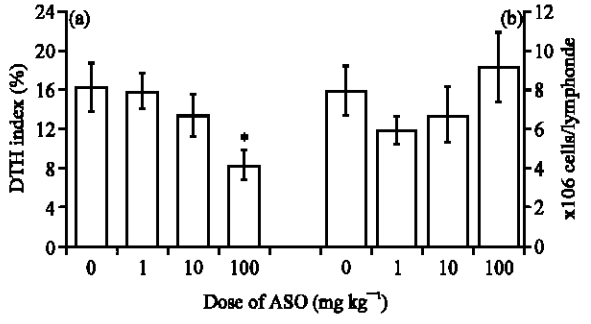

Fig. 3(a-b): Influence of ASO on (a) Intensity of the DTH reaction and (b) No. of nucleus-containing cells in the regional lymph node of the mice locally immunized with SRBC (n = 7-8). *$p<0.05$ according to the fisher; LSD: Criterion, DTH: Delayed-type hypersensitivity

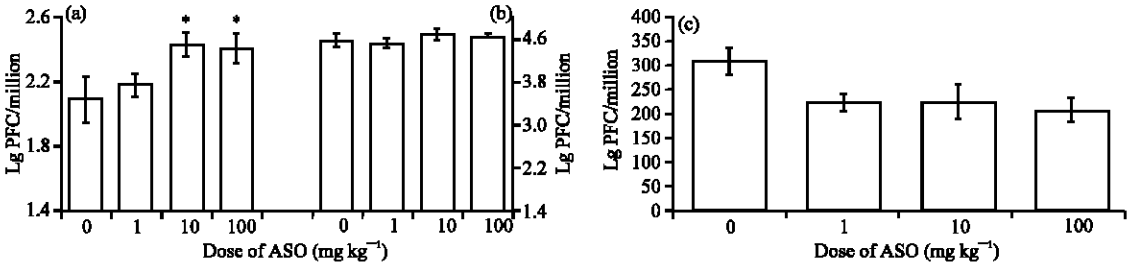

Fig. 4(a-c): Influence of ASO on (a) Relative, (b) Absolute number of PFC and (c) Total number of nucleus-containing cells in the spleen of the mice immunized with ram erythrocytes (n = 7-8). *p<0.05 according to the Fisher LSD: Criterion, PFC: Plaque-forming cells

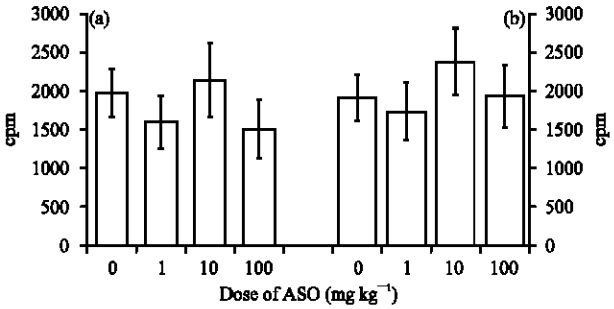

Fig. 5(a-b): Effect of ASO on (a) Spontaneous and (b) Con A-induced proliferative activity of mouse splenocytes (n = 11-12). *p<0.05 according to the Fisher LSD: Criterion, cpm: Intensity (imp min^{-1}) of ^{3}H-thymidine actuation

(the PFC number per organ) did not reveal any statistically significant intergroup discrepancies under the action of ASO (Fig. 4b). Simultaneously, the injected animals had evinced a clear trend in the decrease in total number of nucleus-containing spleen cells as compared to the control mice (Fig. 4b). This fact, most probably, explains the absence of a statistically significant effect of ASO on the AFC number expressed absolutely. Thus, ASO demonstrates *in vivo* a marked stimulating effect on the humoral component of the immune system, the mechanism of which is dependent on how the antigen is introduced into an organism.

Proliferative activity: The next series of experiments had evinced that ASO did not exhibit any significant influence on the proliferative activity of mouse splenocytes harvested in 1 h after injection of ASO. Our results had shown that stimulation of the cells with concanavalin A (Con A) immediately after extraction did not induce any appreciable variation in the radioactive marker's uptake by the test groups as compared with the values registered in the control groups of cells (Fig. 5).

Cytokine detection: ASO in 30 nM and 40 μM concentrations was used to evaluate secretory activity of leucocytes *in vitro*. The data in Fig. 5 show that introduction of ASO into the cell cultures does not significantly influence spontaneous production of IFN-α, IFN-γ, IL-2 and IL-4 by the cells. Statistically appreciably decreasing production of IFN-γ, IL-2 and IL-4 was detected in mitogen-stimulated leukocyte cultures treated with ASO in the concentration under investigation, while not influencing secretion of IFN-α (Fig. 6).

DISCUSSION

The maintenance of immune homeostasis is realized due to continuous interaction between various types of cells in the process of the immune response. Selectivity and flexibility in the immune reactions' progress, necessary to control intercellular interaction, are provided by expression of a wide spectrum of cytokines and of their receptors. Activation process of innate immunity cells, accompanied by releasing proinflammatory cytokines, plays a key role in induction and activation process of effector clones of T-and B-lymphocytes to eliminate an antigen. As a consequence, the cytokine network, responsible for induction and regulation of the immune response, is a logical target for development of drugs of synthetic, biological or microbial origin featured by the immunomodulating action.

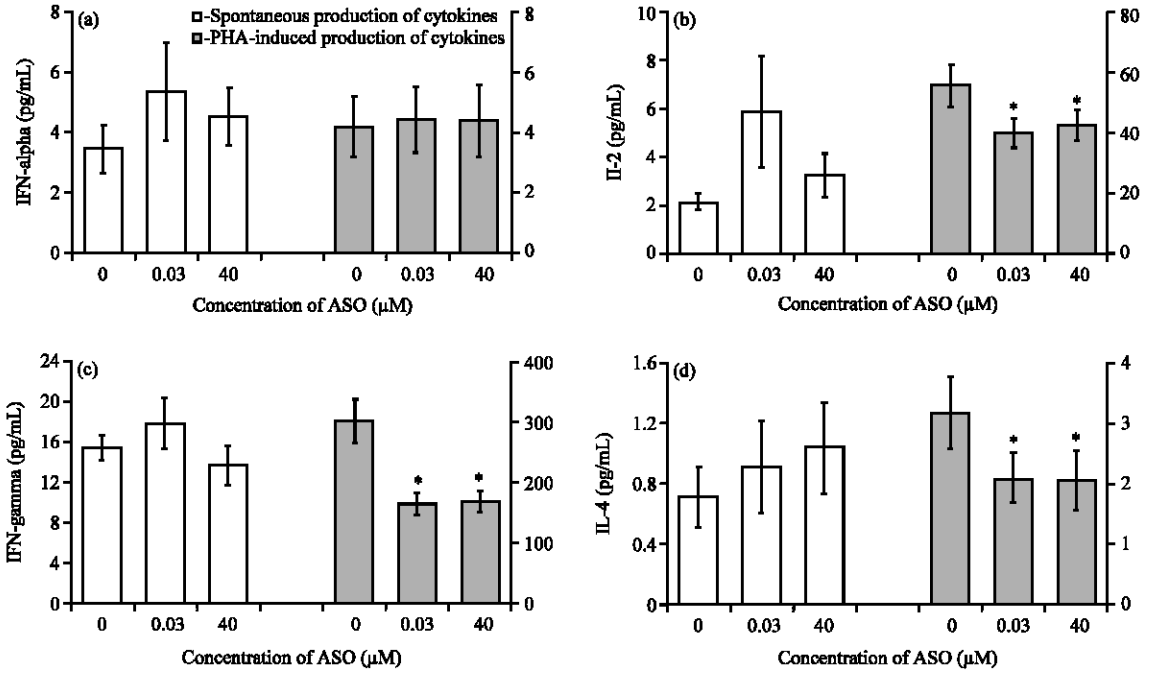

Fig. 6(a-d): Influence of ASO on spontaneous and PHA-induced production of (a) IFN-α, (b) IL-2, (c) IFN-γ and (d) IL-4 (d) by mononuclears of peripheral human blood (n = 14-15). IFN-γ and IL-4 were determined in the 48 h cultures. IFN-α and IL-2 were determined in the 24 h cultures. Mononuclear cells were isolated on Ficoll-verografin density gradient and cultivated (10^6 cells mL^{-1}) in 96-well plates in a complete cultural medium. The PHA (20 μg mL^{-1}) was introduced into the cultures simultaneously with ASO (30 nM and 40 μM). IFN-γ and IL-4 were determined in the 48 h cultures. IFN-α and IL-2 were determined in the 24 h cultures. ASO: Acetylhydrazone of 1-cyano-19β,28-epoxy-2,3-seco-2-nor-18αH-olean-3-al, IFN-α-interferon-α, IFN-γ-interferon-γ, IL-2-interleukin-2, IL-4-interleukin-4

Recent reports (Rios, 2010) have demonstrated polycyclic triterpenoids, incl. originally and structurally close to ASO, to exhibit regulatory effect on functions of immune system cells, in particular, of proinflammatory cytokines. For example, betulin is a modest TNF-α inductor of production of TNF-α in mitogen-induced blood cells (Zdzisinska *et al*., 2003). Betulinic acid exhibits modulating action on production of the IL-1β, TNF-α (Yun *et al*., 2003), IL-2, IL-12 (Mashitoh *et al*., 2012) cytokines, dose-dependently stimulates proliferation of mouse thymocytes, splenocytes and human mononuclears (Mashitoh *et al*., 2012).

Anti-inflammatory effects of oleanolic acid and of its derivatives (Brinker *et al*., 2007) are realized by inhibition of the TNF-α, IL-1β, IL-6 (Marquez-Martin *et al*., 2006), IL-2, IL-4, IL-17 and IFN-γ (Nataraju *et al*., 2009) cytokines release. In addition, oleanolic acid selectively inhibits the classical route of complementary activation *in vitro* by inhibiting the C3-convertase (Kapil and Sharma, 1994). Oleanolic acid contributes *in vivo* to the increase in the phagocytic index and exhibits dose-dependent stimulating effect towards macrophages while conditioning the increase in the humoral and cell-mediated immune response (Khajuria *et al*., 2007).

The analysis of properties of ASO, betulin, betulinic and oleanolic acids show that suppressing *in vitro* production of IFN-γ, IL-2 and IL-4. ASO is close to the 18βH-oleanane triterpenoid oleanolic acid, albeit ASO's molecule contains the 18α-oriented hydrogen atom and the epoxy cycle. At the same time, ASO was intensifying formation of PFC under conditions of systemic/local immunization and suppressing intensity of the DTH reaction. However, it did not modulate cell number in lymph nodes and in the spleen, as well as proliferation of mouse splenocytes in response to the T-cell mitogen (Con A). Our results demonstrate, that ASO shows differently directed *in vitro*/*in vivo* effects on intensity of immune reactions, while activating predominantly the humoral immune response, independently from antigen's introduction route into the organism.

CONCLUSION

Thus, we have discovered the oleanane A-secotriterpenoid's ability of suppressing *in vitro* production of IL-2, IL-4 and IFN-γ, whereas *in vivo* of stimulating intensity of the humoral immune response. The obtained data evince expediency of further investigations in biological properties of ASO, combining specific anti-VSV action and immunomodulating activity.

ACKNOWLEDGMENTS

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