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THERAPEUTIC EFFECT OF NUCLEOSIDE ANALOGS ON EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN DARK AGOUTI RATS

DANIJELA STOJKOV[1*], IRENA LAVRNJA[1], SANJA ŠUBAŠIĆ[2], IVANA BJELOBABA[1], SANJA PEKOVIĆ[1], IVANA GAĐANSKI[1], MARIJA MOSTARICA-STOJKOVIĆ[3], STANISLAVA STOŠIĆ-GRUJIČIĆ[1], LJ. RAKIĆ[4] and MIRJANA STOJILJKOVIĆ[1]

[1] *Department of Neurobiology and Immunology, Siniša Stanković Institute for Biological Research, University of Belgrade,* Bulevar Despota Stefana 142, 11060 Belgrade, Serbia and Montenegro
[2] *Faculty of Biology, University of Belgrade,* Studentski trg 3, 11000 Belgrade, Serbia and Montenegro
[3] *Institute of Microbiology and Immunology, School of Medicine,* Dr Subotića 1, 11000 Belgrade, Serbia and Montenegro
[4] *ICN Galenika Pharmaceutical Works,* 11000 Belgrade, Serbia and Montenegro

Abstract - Experimental autoimmune encephalomyelitis (EAE) is a commonly used animal model of the human neurological disorder multiple sclerosis. The purpose of the present study was to investigate the effect of combined treatment with two nucleoside analogs, ribavirin and tiazofurin, on development of EAE actively induced in highly susceptible dark agouti rats. The obtained results showed that ribavirin and tiazofurin applied either separately or in combination from the onset of the firstsymptoms of EAE after its induction (therapeutic treatment) significantly suppressed EAE's clinical symptoms. However, the most pronounced effect was gained with combined treatment, probably as a result of synergistic/additive action.

Key words: Experimental autoimmune encephalomyelitis, multiple sclerosis, ribavirin, tiazofurin, therapeutic treatment

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INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is an organ specific autoimmune demyelinating disease of the central nervous system (CNS). It can be induced in susceptible animal strains either by active immunization with CNS antigens or by passive transfer of encephalitogenic cells into naive animals (for a review, see P e n d e r, 1995). Experimental autoimune encephalomyelitis is a prototypical animal model of multiple sclerosis (MS), one of the most common chronic and disabling disorders of the CNS in humans. However, although distinct variants of EAE exist, no single model reflects all aspects of multiple sclerosis. Nevertheless, it is still widely used as a model for this human disease on the basis of clinical and histopathological resemblances (t ' - H a r t and A m o r, 2003). The generally accepted concept of pathogenesis in both EAE and MS suggests that encephalitogenic proteins in the periphery activate myelin-specific T cells, which cross the blood-brain barrier (BBB) and migrate into the CNS. In CNS tissue, T lymphocytes are reactivated by autoantigens that cause expression of T-cell effector functions through the cytokine network (L o c k *et al.* 2002), producing inflammation and neurological defects (for a review, see W h e e l e r and O w e n s, 2005). The histological characteristics of EAE are infiltration of T-cells and macrophages into the CNS, focal demyelization, and a variable degree of axonal loss (B a u e r *et al.* 2001).

So far, current therapies of MS have limited efficiency, and many new treatment protocols are currently under trial (N e u h a u s *et al.* 2003). Based on its similarities with MS, EAE might be helpful in preclinical testing of various substances that could be considered for treatment of this human CNS disease.

Ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-

carboxamide) and tiazofurin (2-β-D-ribofuranosylthiasole-4-carboxamide) are synthetic nucleoside analogs, that act as inosine monophosphate dehydrogenase (IMPDH) inhibitors through binding to the enzyme at different sites. The active tiazofurin metabolite thiazole-4-carboxamide adenine nucleotide (TAD) binds at the NAD^+/NADH ligand site on the enzyme molecule (Yamada *et al.* 1988), while the active ribavirin metabolite ribavirin monophosphate (RMP) binds at the IMP/XMP ligand site (Yamada *et al.* 1988), with higher affinity than the natural substrates and products. Inhibition of IMPDH, a rate–limiting enzyme in the *de novo* purine synthesis pathway (Weber *et al.* 1997) leads to depletion of the guanylate pools (Franchetti and Grifantini, 1999). Reduction in guanine nucleotide levels causes various disturbances in sensitive cells such as inhibition of DNA/RNA and protein synthesis (Weber *et al.* 2003), glycosilaton of adhesion molecules (Sokolski and Sartorelli, 1985), interruption of signal transduction (Weber *et al.* 2003; Vallee *et al.* 2000), etc. What is very important for MS and EAE is that among other cell types, activated T lymphocytes are more sensitive to this IMPDH inhibition because salvage pathways for the production of guanine nucleosides do not operate in them (Allison and Eugui, 2000). This fact recommends ribavirin and tiazofurin as possible immunosuppressive/immunomodulatory drugs.

Recently it has been shown that each of these substances given preventively or therapeutically reduces clinical symptoms of EAE in DA rats (Stošić-Grujičić *et al.* 2002; Milićević *et al.* 2003). Moreover, proceeding from the fact these drugs affect IMPDH by different mechanisms, we previously showed that of ribavirin and tiazofurin, given in combination preventively had a more potent effect in attenuating clinical and histological symptoms of the disease (Lavrnja *et al.* 2005). Accordingly the purpose of the present study was to determine whether combined treatment with ribavirin + tiazofurin, starting precisely at the appearance of the first clinical symptom, would have the same suppressing effect on the development of EAE.

MATERIALS AND METHODS

Experimental animals

Male dark agouti (DA) rats, from 8 to 14 weeks of age, were used throughout the experiments. The animals, obtained from the animal colony maintained at the Siniša Stanković Institute for Biological Research in Belgrade, were divided into groups of 3-5 per cage, matched by age and weight (150-220 g). Housed under conventional conditions with laboratory food and water *ad libitum*, they were watered by hand during the period of paralysis. The experimental protocols were approved by the Local Animal Care Committee and conformed to the recommendations given by the National Research Council (1996).

Active induction and clinical evaluation of EAE

The rats were immunized by an encephalitogenic emulsion prepared by mixing equal amounts of rat spinal cord tissue homogenate (50% w/v in saline) and complete Freund's adjuvant containing 0.5 mg/ml *Mycobacterium tuberculosis* (CFA, from Sigma, St. Louis, USA, or ICN Pharmaceuticals, Costa Mesa, CA, USA). Each animal was given 0.1 ml of the emulsion by a single intradermal injection in the hind footpad. Immunized animals were daily monitored for clinical symptoms of EAE, which were graded on a scale of 1-5 as follows: (0) no clinical symptoms; (1) flaccid tail; (2) hind limb paresis; (3) hind limb paralysis; (4) moribund state; (5) death of the animal. Several parameters of the disease were examined to evaluate the severity of EAE and the efficacy of therapeutic treatment with ribavirin and tiazofurin. These parameters were: incidence (the number of rats within a group that developed a clinical score of 1 or greater in comparison with the starting number of animals in the group); daily mean clinical scores (the mean clinical scores for all rats within a group on a given day); mean maximal severity score (the mean of the maximal clinical score that each rat in a group developed over the course of the experiment); mean day to onset (the mean day on which affected rats within a group first developed clinical symptoms of the disease); and duration of paralysis (the mean number of days for which the rats had a score of 3 or more). All animals were observed for 26 days after immunization.

Treatment procedure

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) were provided by ICN Pharmaceuticals (Costa Mesa, CA). In order to analyze their therapeutic effect on development of EAE, the immunized rats were randomly divided in four groups: saline- (control), ribavirin-treated, tiazofurin-treated and ribavirin + tiazofur-

in-treated. The first group of treated animals received only ribavirin in a daily dosage of 30 mg/kg *i. p.*; the second group received only tiazofurin in a dosage of 10 mg/kg *i. p.* every other day, and the third group was treated with a ribavirin + tiazofurin combination *i. p.* in a dosages of 30 mg/kg/day and 10 mg/kg every other day, respectively. Control EAE-immunized rats were given 0.2 ml/kg/day of saline *i. p.* In all groups, treatment started when the first symptoms of EAE appeared (day 9 after immunization) and lasted to the end of the observation period (day 26 after immunization).

Statistical analysis

The results are expressed as mean ± SEM. Significant differences in disease parameters were evaluated by two-way ANOVA (daily mean clinical score, mean maximal severity score) and Student's t-test (mean day to onset). Differences were considered statistically significant at *p<0.05 and **p<0.005.

RESULTS

To analyze whether the nucleoside analogs ribavirin and tiazofurin given solely or in combination can modulate the development of EAE, we used as a model the actively induced disease in highly susceptible DA rats. In the control group, all immunized animals developed clinical symptoms of the disease (incidence of 10/10 – Table 1), and the first symptoms were noticed on day 9 after immunization (Figs. 1A, B, and C). Disease symptoms in this group reached a peak at day 14 (mean maximum severity score was 2.5 ± 0.2, Table 1), recovery began at day 16, and all animals completely recovered by days 23-26 (Figs. 1A, B, C). Therapeutic treatment with ribavirin and tiazofurin separately or in combination was initiated from the onset of the first clinical symptoms of EAE. The mean day of onset varied between days 10 and 14 (Table 1) after immunization. The ribavirin-treated group of animals developed EAE at day 9 (Fig. 1A), but incidence of the disease was lower compared with the control group of animals (incidence of 3/7 – Table 1). In this experimental group recovery began at day 15, and no clinical symptom was noticeable in any of the animals after day 19 (Fig. 1A), meaning that duration of the disease was shortened in comparison with the control rats. Ribavirin also considerably ameliorated the severity of clinical symptoms (mean maximum severity score - Table 1). The first signs of EAE in the tiazofurin-treated group of animals were spotted at day 11 (Fig. 1B), and the disease was developed in four out of seven animals (incidence – Table 1). Therapeutic treatment with tiazofurin delayed the disease

Table 1. Effect of *in vivo* treatment with ribavirin and tiazofurin on active EAE.

† Parameters of disease were evaluated as described in Materials and methods. Data are presented as mean ± SEM

[a] Control EAE-immunized rats were given 0.1 ml saline as a daily i.p. injection from the onset of the first clinical symptoms

[b] Daily i.p. injections of 30 mg/kg ribavirin were given from the onset of the first clinical symptoms

[c] I.p. injections of 10 mg/kg tiazofurin were given every other day from the onset of the first clinical symptoms

[d] I.p. injections of 30 mg/kg/day ribavirin + 10 mg/kg/every other day tiazofurin were given from the onset of the first clinical symptoms

**P<0.005, *P<0.05 refers to control saline treated EAE rats (Student t-test).

Treatment / Parameters of disease†	Saline [a]	Ribavirin [b]	Tiazofurin [c]	Ribavirin [d] + tiazofurin
Number of animals	10	7	7	6
Incidence	10/10	3/7	4/7	2/6
Mean days to onset	11.1 ± 0.6	11.3 ± 1.2	13.8 ± 1.0	13.0 ± 2.0
Mean clinical score	0.4 ± 0.1	0.1 ± 0.04*	0.2 ± 0.1	0.1 ± 0.03*
Duration of paralysis	0.8 ± 0.2	0	0.6 ± 0.6	0
Mean maximum severity score	2.5 ± 0.2	0.7 ± 0.4**	1.0 ± 0.4*	0.5 ± 0.3**

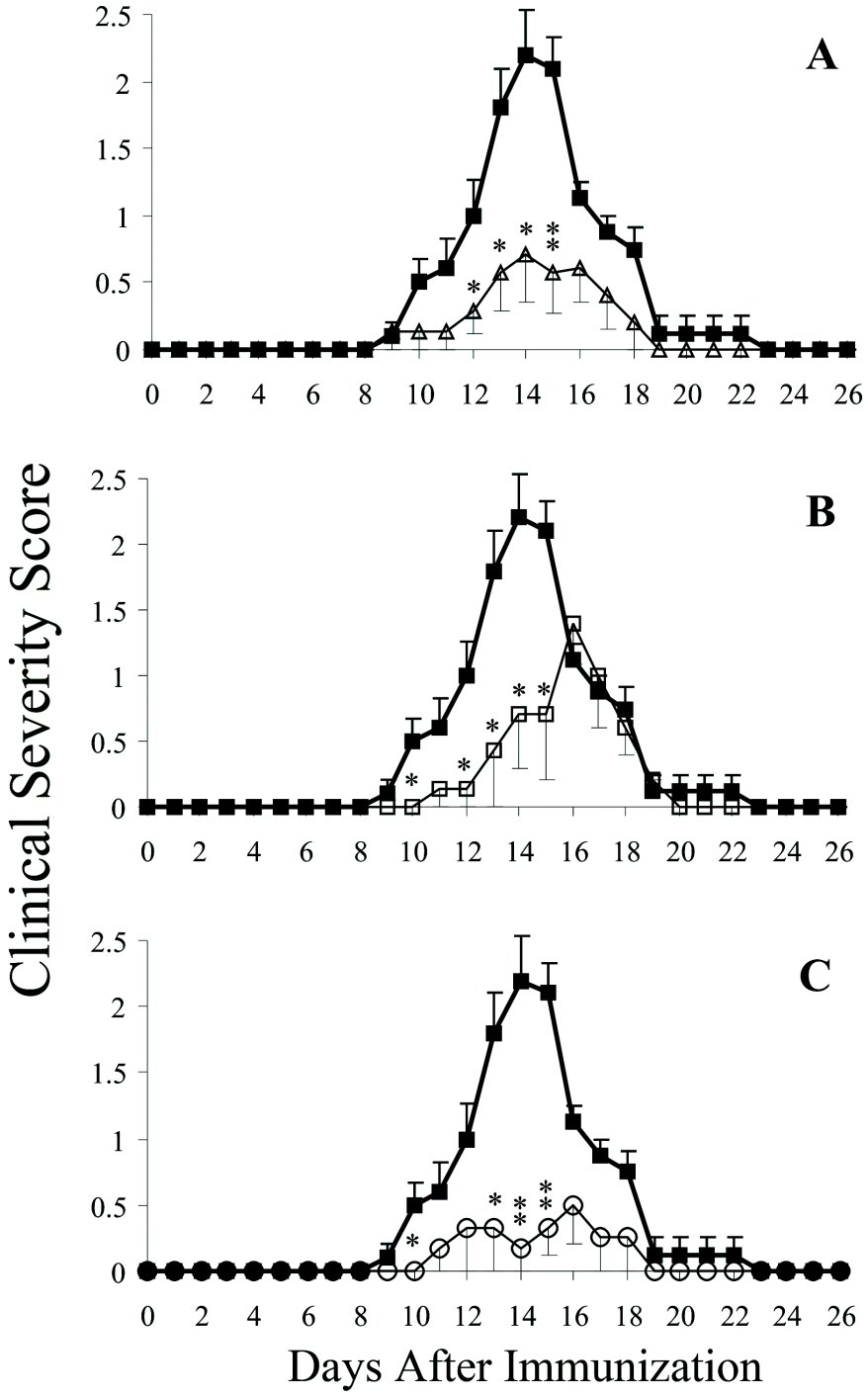

Fig. 1. Effect of ribavirin and tiazofurin treatment on supression of EAE clinical symptoms.

Ribavirin and tiazofurin suppress the course of EAE in DA rats. Daily mean clinical scores in rats treated with saline (control group - < -) and **A**: 30 mg/kg/day i.p. ribavirin (- r -) from the onset of the first EAE clinical symptoms to day 26; **B**: 10 mg/kg i.p. tiazofurin (- £ -) every other day from the onset of the first EAE clinical symptoms to day 26; **C**: 30 mg/kg/day ribavirin + 10 mg/kg tiazofurin every other day (- • -) from the onset of the first EAE clinical symptoms to day 26. Data points are presented as the mean ± SEM for 6-10 animals per group. **P < 0.005 and *P < 0.05 refers to control saline treated EAE rats (two-way ANOVA).

peak for two days (Fig. 1B), and lowered the mean maximum severity score (Table 1). Furthermore, complete recovery was faster in tiazofurin-treated than in control animals (at day 20, Fig. 1B). In the group treated with a combination of ribavirin + tiazofurin, the first clinical symptom of EAE was observed on day 11 (Fig. 1C), but incidence of the disease was very low, only 2/6 (Table 1). Treatment with ribavirin + tiazofurin significantly attenuated clinical symptoms of EAE compared to the control (saline-treated) rats (the mean maximum severity score was 0.5 ± 0.3 vs. 2.5 ± 0.2 in the control group, $p<0.005$, Table 1) and accelerated recovery (no clinical symptoms were detectable after day 19 following the immunization, Fig. 1C). As indicators of ribavirin and tiazofurin efficacy in therapy of EAE, other parameters of disease severity were also followed, namely the duration of paralysis (in days, Table 1) and the number of animals that developed this severe EAE symptom. Paralysis was noticed only in the control group, in six out of 10 rats (duration of paralysis was 0.8 ± 0.2 days, Table 1) and in the tiazofurin-treated animals, in one out of seven rats (duration of paralysis was 0.6 ± 0.6 days, Table 1). In the other two experimental groups of animals, ribavirin- and ribavirin + tiazofurin-treated, no one animal developed this severe EAE symptom (Table 1).

DISCUSSION

Therapeutic effects of the nucleoside analogs ribavirin and tiazofurin given separately or in combination on development of EAE were investigated in the present study. We have shown that administration of these drugs from the onset of first clinical symptoms of the disease until the end of observation (at day 26 after immunization) reduced its manifestation with different effectiveness. The most pronounced effect was observed with the ribavirin + tiazofurin combination.

The effect of nucleoside analogs on EAE was examined in DA rats, which are highly susceptible to EAE induction (Vukmanović *et al.* 1990), even without any adjuvant (Stošić-Grujičić *et al.* 2004) and therefore represent an appropriate rodent model of experimental autoimmunity of the CNS for studying novel therapeutic strategies in MS.

Ribavirin was developed principally as an antiviral drug (Sidwell *et al.* 1972; Witkowski *et al.* 1972) that is effective against a broad spectrum of DNA/RNA viruses (Tam *et al.* 2001). On the other hand, the efficacy of tiazofurin as an anticancer drug is based on its antiproliferative and maturation-inducing effects, which have been demonstrated both *in vitro* and *in vivo* (Timar *et al.* 1996; Tovari *et al.* 1996; Weber *et al.* 1996). The antiviral and anticancer effects of these nucleoside analogs can be primarily ascribed to inhibition of IMPDH at different binding domains on the enzyme molecule. As a result of the same mode of action, ribavirin and tiazofurin may have an immunosuppressive/immunomodulatory effect and thereby potential application in the therapy of autoimmune diseases. Indeed, an immunosuppressive effect of ribavirin was reported in murine lupus nephritis (Klassen *et al.* 1979) and in the experimental autoreactive form of animal myocarditis (Maisch *et al.* 1995). Also, in a previous study of ours, we reported that ribavirin applied from the day of EAE induction significantly reduced clinical symptoms, lowered mononuclear cell infiltration, and prevented demyelination in the CNS (Milićević *et al.* 2003). In addition, we previously demonstrated that tiazofurin given continuously after EAE induction or following the onset of EAE symptoms, down-regulates severity of the disease (Stošić-Grujičić *et al.* 2002). These findings led us to investigate the effect of preventive treatment with a combination of ribavirin and tiazofurin combination applied immediately after EAE induction, but in lower dosages than in the above mentioned studies (Lavrnja *et al.* 2005). The obtained results provided evidence suggesting that a combination of these nucleoside analogs given in lower doses could be more effective in prevention of disease development than each drug used alone. In the present study, we wanted to verify whether combined treatment with ribavirin + tiazofurin would suppress ongoing EAE if their application started when clinical symptoms of the disease were clearly expressed (therapeutic treatment). We therefore initiated administration of ribavirin and tiazofurin, separately and in combination, from the onset of the first signs of EAE after induction. The results confirmed the beneficial effects of ribavirin and tiazofurin in reducing all disease parameters. Again, the best effects were gained in the ribavirin + tiazofurin-treated group, especially in regard to EAE incidence. To explain this observation, two facts must be taken into consideration: 1. In the present study, animals developed moderate EAE with variable beginning of clinical symptoms between days 9 and 16 after immunization; and 2. We started with treatment when the onset of EAE appeared in the first animal in all four groups. Consequently, it is possible that ribavirin and tiazofurin in some animals were given preventively, with the result

that they completely prevented clinical manifestations of EAE and caused a wide range of mean days to the onset and very low incidence in all nucleoside analog-treated groups, especially in the ribavirin + tiazofurin treated group. These protective effects of ribavirin and tiazofurin might result from suppression of events associated with activation of autoreactive T cells and their passing through the BBB. On the molecular level, ribavirin and tiazofurin act via their active metabolites, RMP and TAD, respectively. The primary target of these active metabolites is IMPDH, a key enzyme in the pathway of *de novo* purine nucleotide synthesis (Franchetti *et al.* 1996). Inhibition of IMPDH leads to depletion of the guanylate and ATP pools, consequent impairment of specific signal transduction pathways, and blocking of *de novo* DNA synthesis (Weber *et al.* 2003). While other cell types can use purine synthesis salvage pathways, T cells (including the $CD4^+$ subpopulation of encephalitogenic lymphocytes) completely depend on *de novo* purine synthesis (Allison and Eugui, 2000). Additionally, it has been shown that ribavirin can arrest the cell cycle in the G_0/G_1 phase (Li *et al.* 1999; Vallee *et al.* 2000), induce cell differentiation, and cause suppression growth (Majumdar *et al.* 1995). It is therefore possible that ribavirin and tiazofurin prevent the development of EAE by inhibiting autoreactive T cell proliferation. However, such an effect is less likely, to judge from a previous study of ours (Stošić-Grujičić *et al.* 2002.) showing that tiazofurin administration *in vivo* did not inhibit the *in vitro* T cell proliferative response to specific antigens or concanavalin A. As demonstrated in the present study, ribavirin and tiazofurin also suppressed clinicalsymptoms of EAE when their application started in the effector phase of the disease after the appearance of the first clinical symptom, indicating that another mechanism for mechanisms must also be taken into account. Several lines of evidence suggest that interactions between autoreactive T-cells and endothelial cells of the BBB mediated by adhesion receptors and ligands are of great importance in both the initiation and the effector phases of immune responses (Allison and Eugui, 2000). It has been shown that the depletion of GTP by mycophenolic acid, which is the other known inhibitor of IMPDH, suppresses glycosylation and the binding activity of adhesion molecules on lymphocytes and monocytes (Allison *et al.* 1993; Laurent *et al.* 1996; Allison and Eugui, 2000). This is consistent with previous findings indicating that mononuclear cells from tiazofurin-treated animals displayed a reduced capacity for intercellular adhesion or adhesion to plastic (Stošić-Grujičić *et al.* 2002). Thus, it is possible that ribavirin and tiazofurin - as strong IMPDH inhibitors - through GTP depletion and consequent limiting of glycosylation of T cell adhesion molecules can terminate further lymphocyte infiltration in the CNS. These data suggest that suppression of adhesive cell-cell interactions by ribavirin and tiazofurin might be a component of the observed therapeutic action. In addition to their ability to prevent the initial migration of T cells into the CNS, they may also reduce the potential of T cells to elicit inflammation in the brain, which results in secondary infiltration of macrophages and T cells.

In conclusion, the present study provides clear evidence that lowered doses of ribavirin and tiazofurin given in combination can be more effective in suppression of the ongoing disease, than each drug used alone. Our results point to synergistic/additive action of these two nucleoside analogs. Also of great importance is their beneficial effect in the late phase of the immune response, which recommends them as suitable candidates for a new approach in MS therapy.

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ТЕРАПИЈСКИ ЕФЕКАТ НУКЛЕОЗИДНИХ АНАЛОГА НА ЕКСПЕРИМЕНТАЛНИ АУТОИМУНСКИ ЕНЦЕФАЛОМИЈЕЛИТИС КОД DARK AGOUTI ПАЦОВА

ДАНИЈЕЛА СТОЈКОВ[1], ИРЕНА ЛАВРЊА[1], САЊА ШУБАШИЋ[2], ИВАНА БЈЕЛОБАБА[1], САЊА ПЕКОВИЋ[1], ИВАНА ГАЂАНСКИ1, МАРИЈА МОСТАРИЦА-СТОЈКОВИЋ[3], СТАНИСЛАВА СТОШИЋ-ГРУЈИЧИЋ[1], Љ. РАКИЋ[4] и МИРЈАНА СТОЈИЉКОВИЋ[1]

[1] *Одељење за неуробиологију и имунологију, Институт за биолошка истраживања «Синиша Станковић», Универзитет Београду,* Булевар Деспота Стефана 142, 11060 Београд; Србија и Црна Гора

[2] *Биолошки факултет, Универзитет Београду,* Студентски трг 3, 11000 Београд; Србија и Црна Гора

[3] *Институт за микробиологију и имунологију, Медицински факултет,* Др Суботића 1, 11000 Београд; Србија и Црна Гора

[4] *Галеника а. д.,* 11000 Београд; Србија и Црна Гора

Експериментални аутоимунски енцефаломијелитис (ЕАЕ) је анимални модел који се обично користи као прототип хуманог неуролошког обољења – мултипле склерозе. Циљ ове студије био је испитивање ефекта комбиноване терапије са два нуклеозидна аналога, рибавирина и тиазофурина, на развој ЕАЕ, активно изазваног код веома осетљивог дарк агути соја пацова. Добијени резултати показали су да рибавирин и тиазофурин, примењени појединачно или у комбинацији, од појаве првог симптома ЕАЕ (терапијски третман), значајно супримирају тежину ове болести. Међутим, најбољи ефекти су постигнути комбинованим третманом, што је вероватно последица синергистичког или адитивног деловања рибавирина и тиазофурина.