Comparative Study of the Influence of Iem-1556 and Glatiramer Acetate (Copaxone) on the Severity of Neurologic Disorders and the Duration of Experimental Allergic Encephalomyelitis in the Rats

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Abstract
The effect of N-decyltropine chloride (IEM-1556) and the reference drug glatiramer acetate (GA) on the severity of neurological disorders and the duration of the experimental allergic encephalomyelitis (EAE), modeling the processes of neural inflammation, demyelination and neurodegeneration characteristic for multiple sclerosis were studied. EAE in female Wistar rats was induced by a single subcutaneous (SC) inoculation of the homologous spinal cord homogenate in complete Freund’s adjuvant. The test preparations were administered from 2 to 16 days after induction of EAE. The severity of the disease was assessed in scores (from 0 to 6) by the presence in animals of persistent paresis and paralysis. The course systemic administration of IEM-1556 in a dose of 3 mg/kg reduced the severity and duration of EAE in rats, comparable to GA. Advantage of IEM-1556 before GA is the possibility of non-invasive application, as well as the presence of analgesic, antiparkinsonian and antidepressant action. It is assumed that the therapeutic effect of IEM-1556 is related to its ability to release endogenous adenosine, which causes neuroprotective, analgesic, antiparkinsonian and antidepressant effects of the drug.

Keywords: Multiple sclerosis; EAE; Adenosine; IEM-1556; Copaxone

Introduction
Multiple sclerosis (MS) is a serious socially significant disease, as it affects able-bodied subjects; the number of cases of disease in childhood is growing. To date, there is not a very effective pathogenetic therapy for MS; all drugs used usually only reduce the severity of the course of MS. Among them, copaxone (non-proprietary name glatiramer acetate) has been used for more than 20 years. The main disadvantages of GA are the injectable method of application, the need for continuous use, insufficient effectiveness, skin reactions at the injection site, which limits the adherence to treatment with this drug, and the cost of the drug makes treatment costly [1], so the search for new highly effective and affordable medicines continues, still remains relevant.

The most common model of MS is experimental allergic encephalomyelitis (EAE) in laboratory animals, because it has clinical manifestations and pathogenetic mechanisms similar to multiple sclerosis [2,3]. A key role in the pathogenesis of MS and EAE is neuroinflammation and autoimmune reactions triggered by T-cell sensitized to brain antigens (encephalitogenic T-lymphocytes), which penetrate the brain in a large number as a result of increased BBB permeability, and the pro-inflammatory cytokines they synthesize (interleukin-1β [IL-1β], tumor necrosis factor α [TNFa], interferon γ [IFNγ], etc.) [4-6].

It is known that adenosine and A2A receptors are generally involved in the regulation of immune cell functions, the production of proinflammatory cytokines and the inflammation reaction. It was shown that the selective stimulant of A2 adenosine receptors CGS21680, as well as dipyridamole, which increases the level of endogenous adenosine in the brain during systemic administration, effectively reduce the severity of neurologic disorders and the duration of EAE in rats, reducing the proliferation of encephalitogenic T lymphocytes, the activity of microglia and the production of interleukin-1β and TNFa [7,8].

The synthesized compound N-decyltropine chloride (IEM-1556, Figure 1), when administered orally, promotes the release of endogenous adenosine, providing a neuroprotective effect [9] and therefore IEM-1556 was of interest for investigation in the model of PC-experimental allergic encephalomyelitis [10].

Methods
The work was performed on 90 female Wistar rats weighing 230-250 g (n. Rappolovo), following the principles of humanity (European Community Directive No. 86/609 EC) approved by the local ethics committee at the IEM (Institute for Experimental Medicine). Animals were kept in standard conditions with a light regime of 12 h a day, 12 h night, at an air temperature of 20–22°C, 4-5 individuals in one cage with free access to food and water. Female rats were used in the experiment in view of the fact that in males it is much worse than in females the progression of EAE is reproduced.

A validated model of EAE with a single subcutaneous inoculation of the encephalitogenic mixture (EGM) in Freund’s complete adjuvant (FCA) was used [4,11]. EGM was prepared from the calculation of 100

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mg homologous spinal cord homogenate; 0.2 ml of (FCA) (content of killed mycobacteria 5 mg/ml) and 0.2 ml of saline per animal. EGM was injected into the base of the tail under light ether anesthesia in a volume of 0.4 ml [11]. Then the animals were divided into 3 groups (Table 1). The test substances were administered to rats from 2 to 16 days after induction of EAE (latent phase-clinical phase before the end of the disease peak), once a day. Glatiramer acetate (GA, copaxone, Teva, Israel) was administered subcutaneously (s.c.), as used in the clinic, in a dose of 4 mg/kg, for which efficacy was previously shown in this model [12]. IEM-1556 (synthesized in IEM (Institute for Experimental Medicine)) was administered intraperitoneally (i.p.) in a dose of 3 mg/kg. The animals of the control group (with induced EAE) were injected with an apyrogenic physiological saline s.c. (n=15) or i.p. (n=15).

Daily for 30 days (mean duration of EAE) in animals evaluated the neurological status. The time of onset of the disease, its duration and the severity of neurological disorders were recorded. The severity of neurological disorders was assessed in points on the clinical index (C.I.), which was determined by the severity and prevalence of neurologic manifestations: muscle weakness of one limb- 0.5 points, paresis- 1 point and paralysis- 1.5. With the defeat of several extremities, the points were summed up. Absence of visible disturbances was taken for 0 points, in case of a lethal outcome- 6 points. Animals with C.I._max = 0.5-2.0 points were thought to be easily diseased; C.I._max = 2.5-3.5 points - of moderate severity; C.I._max = 4.0-6.0 points - seriously ill. For the integrative assessment of the severity of the EAE, a cumulative C.I. was calculated for each rat, which is the sum of the individual clinical indices for the entire period of the disease.

To evaluate the effectiveness of the neuroprotective effect of the test substances on the EAE model, the following indices were calculated for each group of animals: 1) the duration of the latent period of the EAE; 2) the total number of diseased rats and rats with different EAE weights (in% of the number of rats in the group); 3) the mean C.I. of the disease; 4) cumulative C.I.; 5) average duration of the disease; 6) the index of convalescence. All the indices were compared with the corresponding parameters of the animals in the control group (with induced EAE, but without the administration of drugs) and the comparison group (administration of GA).

For the comparative analysis of the indicators, a nonparametric test of multiple comparisons was used, for comparison of fractions, the Chi-square test. Statistical processing was carried out in the program Statistica [8]. Differences were considered valid for p<0.05.

### Results and Discussion

Inoculation of the homologous spinal cord homogenate in the FCA induced the development of neurological disorders in 100% of the female Wistar rats of the control group (i.p. or s.c. saline solution, Table 2), with only 10% of the disease in mild form (C.I._max=0.5-2.0 points). The mean clinical index in the control group was 3.4 points (Table 3). The first signs of EAE in the control group animals appeared on average 11 days later (duration of the latent period of EAE) after induction, and the peak of the disease was observed on day 13-15 and lasted 4-5 days. The mean duration of EAE in the control group was 19 days, and the average cumulative clinical index was 60.3 points (Table 3).

The course administration of GA prevented the development of EAE in 10% of rats, and increased the proportion of easily diseased rats to 20%. In this group there was a tendency to increase the latent period (p=0.1) and the duration of the disease decreased by 1.3 times (from 19 to 14.5 days). This drug reduced the mean C.I. to 1.9 points and the cumulative C.I. to 26.8 points (Table 3). In addition, 60% of the rats receiving GA completely recovered by the 30th day of the experiment (the convalescence index=0 points). Consequently, as expected, the reference drug-GA had a protective effect in this model of induced EAE in Wistar rats.

Course administration of IEM-1556 in a combined scheme with a dose of 3 mg/kg, as well as GA, completely prevented the development of EAE in 10% of rats. The proportion of rats with mild disease in this group was 30%. Only in rats that received IEM-1556, there were no lethal outcomes. The duration of the inductive phase was not significantly different from either the GA group or the control group, and the duration of the disease was reduced, as was the case with GA (13.5 and 14.5, respectively, versus 19, Table 3).

Although the dynamics of the severity of the disease in the three groups did not differ, the indices characterizing the severity of the disease when IEM-1556 was introduced-mean and cumulative C.I. - decreased significantly compared to the control group and did not differ from the group receiving GA. Thus, the mean C.I. was 1.8

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**Table 1: Characteristics of animals groups.**

| N group | Preparation | Dose (mg/kg) | Number of animals |
|---------|-------------|--------------|-------------------|
| 1       | Saline      | 0            | 30                |
| 2       | Glatiramer acetate (GA) | 4           | 30                |
| 3       | IEM-1556    | 3            | 30                |

*Note: saline solution was injected half of the animals intraperitoneally (IEM-1556, respectively), in the second half - subcutaneously (GA, respectively). Since there were no significant differences between the scores measured in these groups, they were grouped together.*

**Table 2: Proportion of diseased animals and animals with different severity of EAE in the course of administration of IEM-1556 and glatiramer acetate.**

| Preparation | Dose (mg/kg) | Proportion of diseased animals (%) | Total | Easily | Average | Severity |
|-------------|--------------|-----------------------------------|-------|--------|---------|----------|
| Control     | 0            |                                   | 100   | 10     | 30      | 60       |
| GA          | 4            |                                   | 90    | 20     | 30      | 40       |
| IEM-1556    | 3            |                                   | 90    | 30     | 20      | 40       |

Chi-square test: *p<0.05 in comparison with the control

**Table 3: Effect of course administration of IEM-1556 and glatiramer acetate on the severity of neurologic disorders, latent period and duration of induced EAE in female Wistar rats.**

| Preparation | Dose (mg/kg) | Duration of the latency period of the EAE (days) | Average clinical index (points) | Cumulative clinical index (points) | Duration of EAE (days) | Convalescence index (points) |
|-------------|--------------|-------------------------------------------------|--------------------------------|---------------------------------|-----------------------|-----------------------------|
| Control     | 0            | 11                                              | 3.4                            | 60.3                            | 19                    | 3                           |
|             |              | (11.0; 13.0)                                    | (21.4; 42)                      | (37.0; 80.5)                    | (16.0; 19.0)          | (1.5; 3.5)                  |
| GA          | 4            | 13                                              | 1.9                            | 28.6                            | 14.5                  | 0                           |
|             |              | (12.0; 23.0)                                    | (1.0; 3.3)                     | (3.0; 59.0)                     | (3.0; 18.0)           | (0.0; 2.0)                  |
| IEM-1556    | 3            | 14.5                                            | 1.8                            | 21.3                            | 13.5                  | 0.8                         |
|             |              | (11.0; 22.0)                                    | (0.8; 3.2)                     | (5.0; 54.0)                     | (7.0; 19.0)           | (0.0; 3.0)                  |

Data are presented in the form of a median with percentiles (25, 75)

Nonparametric test of multiple comparisons, *p<0.05; **p<0.01
points (against 3.4 and 1.9, respectively in the control group and the GA group), and the cumulative C.I. was 21.3 points (vs. 60.3 and 26.8, respectively). That is, in a dose of 3 mg/kg, the IEM-1556 does not concede in protective activity to the reference standard - glatiramer acetate- which also significantly reduced the severity of neurologic disorders and the duration of EAE in rats.

Earlier, we found that IEM-1556, when administered orally, has analgesic, antidepressant, antiepileptic, neuroprotective and anti-Parkinsonian effects as a result of increased release of endogenous adenosine [9]. Our results of the protective effect of IEM-1556 are in agreement with the data of other authors who used similar substances. Thus, with the systemic administration of selective stimulants A2 adenosine receptors, as well as dipyridamole, which increases the level of endogenous adenosine in the brain in rats, the severity of neurological disorders and the duration of EAE decreased [4,8,13].

Conclusion

The results of these experiments allow us to conclude that the liberator of endogenous adenosine IEM-1556 does not concede in protective activity to the reference drug glatiramer acetate (copaxone) in the EAE model in rats. The advantage of IEM-1556 over GA is the significantly lower cost due to the simplicity of industrial production (one-stage synthesis by the reaction of decyl chloride with the tropine), as well as the presence of antidepressant and analgesic effects [14].

All of the above allows us to consider IEM-1556 as a promising drug for the therapy of multiple sclerosis.

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