Etidronic acid adduct and bis (2-pyridyl-1,2,4-triazolyl-3) butane: properties and effect on pain sensitivity

I V Cheretaev¹, M Yu Ravaeva¹ and E N Chuyan¹

¹ Taurida Academy, Faculty of Biology and Chemistry, V.I. Vernadsky Crimean Federal University, 4 Academician Vernadsky Ave., Simferopol, the Republic of Crimea, Russia.

E-mail: cheretaev86@yandex.ru

Abstract. The article presents the results of experimental studies on the effect of etidrononic acid adduct and bis(2-pyridyl-1,2,4-triazolyl-3)butane (EA+BTB) on the pain sensitivity of male and female rats. This substance is created on the precursors basis, which have a pronounced analgesic activity in acute pain when injected once into male and female rats. Experiments were performed on 30 male and 30 female adult Wistar rats weighing 200–220 g. Pain sensitivity of animals was assessed 1 hour after a single intraperitoneal injection of the studied solution substances by acute pain tests (“tail-flick” test, Randall-Sellitto test, “hot plate” test). It was found out that EA+BTB, when injected once in doses of 5 and 50 mg/kg, significantly changes the pain sensitivity of male and female rats in acute pain tests, showing gender specificity of the analgesic effect with the participation of various pain mechanisms (spinal and supraspinal) and nociception components (perceptual and mechanical). The compound EA+BTB is recommended for further in-depth preclinical tests of its analgesic activity, decoding its physiological and molecular mechanisms in order to create an improved prototype of a new generation drug based on EA+BTB.

1. Introduction
According to statistics, 9 out of 10 diseases are accompanied by pain, and a fifth of the world's population experiences unpleasant sensations connected with the chronic pain [1]. Therefore, carrying out a primary screening on laboratory animals of promising substances with the purpose of searching for new analgesics is an important task of biomedical science. Full-fledged bioscreening of new substances in laboratory rodents should include mandatory experimental studies on males and females. Numerous data, collected by various researchers over the past 30 years, have shown significant differences between the sexes in response to pain stimuli, which are manifested in the thresholds of pain sensitivity, tolerance and response to pain [2].

Currently, there are many biologically active substances with high potential in terms of possible effects on the nervous system and, in particular, analgesic activity. These compounds include bisphosphonates and 1,2,4-triazoles. These substances are interesting because they are a suitable basis for assembling complex compounds with various ligands that have specific useful properties [3-5].

Analgesic effects were found in the etidronic acid and the representatives of 1,2,4-triazole bis(2-pyridyl-1,2,4-triazolyl-3)propane, as well as in some new complex derivatives created on their basis [4-9].

The huge potential of synthetic possibilities for modifying the 1,2,4-triazole molecule opens up great prospects for the synthesis and research of the biological activity of its new derivatives with...
bisphosphonates to obtain effective and safe analgesics of a new generation. One of these derivatives is an adduct of etidronic acid and bis(2-pyridyl-1,2,4-triazolyl-3)butane (EA+BTB).

The aim of the work is to evaluate the effect of EA+BTB on the pain sensitivity of male and female rats in doses of 5 and 50 mg/kg with a single injection.

2. Material and methods

2.1. Animals

All studies on the animals were carried out in compliance with the principles set out in Directive 2010/63/EU of the European Parliament and of the Council of 22.09.2010 on the protection of animals used for scientific purposes. The experiments were performed on 30 male and 30 female adult Wistar rats weighing 200-220 gr (“FSUE Nursery of laboratory animals “Rappolovo”, Saint-Petersburg, Russia). All the rats had free access to food and water. One week before the experiment, the animals with an average motor activity in the “open field” test and not exceeding 8 sec latent period of pain reaction (LPPR) in the “hot plate” test were selected. All the animals were used in experiment after one acclimation week to the housing conditions. The animals were kept in 12-hour light-dark cycle conditions in a room with a constant temperature (22 °C) and humidity (60%).

2.2. Design of research EA+BTB on pain sensitivity, used research and statistical methods.

Pain sensitivity of rats was studied in acute pain tests (“tail-click” test, Randall-Selitto test, “hot plate” test), which are used for screening analgesic activity of various substances [2, 6-17]. The pain sensitivity of animals was evaluated after 1 hour of single intraperitoneal injection of the studied solutions of substances. Two experimental groups of males and two experimental groups of females (n=10 in each group of animals) received intraperitoneal injections of 0.2 ml EA+BTB in doses of 5 and 50 mg/kg respectively. The males (n=10) and the females (n=10) of control groups of animals received single intraperitoneal injections of 0.2 ml 0.9% NaCl solution. All the experiments to determine the threshold of pain sensitivity in rats in the listed tests were conducted from 09:00 to 15:00 h at the Department of Human and Animal Physiology and Biophysics (Faculty of Biology and Chemistry) and the Center for Collective Use of scientific equipment “Experimental physiology and Biophysics” of Taurida Academy (structural division), V.I. Vernadsky Crimean Federal University. EA+BTB was synthesized at the Department of General and Inorganic Chemistry (Faculty of Biology and Chemistry) of Taurida Academy (structural division), V.I. Vernadsky Crimean Federal University. The chemical purity of EA+BTB was at least 98%.

In the “tail-click” test (LE7106 Tail-flick Meter, Pan Lab Harvard Apparatus, Spain), the latent period of tail retraction (LPPR) in rats was recorded, which was determined by the time (s) of the tail flick. On the tail of each rat, sitting in the fixator, three presentations of a thermal stimulus were performed, followed by the calculation of the average value of LPPR in seconds for each animal. This test is based on a spinal flexor reflex that occurs in response to a local force on the tail by high temperature, and allows us to judge the pain sensitivity of animals mainly at the spinal level [7-12].

In the Randall-Selitto test (an experimental apparatus analgesia meter BIO-RP-R Rodent pincher, Bioseb, France), the device displays the applied force (in gr) at which the animal reacts to a gradually increasing mechanical compression of the tail – the pain threshold (PT) [13]. We performed three mechanical compressions of the tail of each rat sitting in the retainer with tongs, followed by calculating the average PT value in grams for each animal.

In the “hot plate” test (an experimental apparatus Cold and hot plate CHP, Bioseb, France), the LPPR of the animal was recorded, which was determined by the value of the time (s) of the reaction manifestation of withdrawal and licking of the limbs and (or) vocalization from the heated surface. This indicator was measured after placing the animal on a hot metal surface with a temperature of + 55 °C. The test allows to judge the pain sensitivity of animals with the participation of supraspinal mechanisms [7-9, 14-16].
The reliability of statistical differences between the control and experimental groups with different doses of EA+BTB (5 and 50 mg/kg) was determined by using a one-way analysis of variance (ANOVA) with a posteriori Tukey test and Dunn’s test of multiple comparisons.

3. Results and discussion
In the "tail-flick" test in male rats (Figure 1A), EA+BTB in a dose of 50 mg/kg caused a significant increase in LPTR by 19.5 % (p≤0.05, n=10) relatively to control (n=10). This indicates the presence of an analgesic effect of adduct EA+BTB in a dose of 50 mg/kg, implemented with the participation of the perceptual component of nociception and the spinal mechanism for regulating pain sensitivity. In female rats (n=10) in this test, EA+BTB (Figure 1A) did not significantly affect the value of LPTR.

![Figure 1. Latent period of tail retraction in rats (median, interquartile range from 25 to 75 %) in the "tail-flick" test in the control group and after intraperitoneal injection of adduct of ethidronic acid and bis(2-pyridyl-1,2,4-triazolyl)butane (EA+BTB) in doses of 5 and 50 mg/kg: (A) males, (B) females.](image)

In the Randall-Sellitto test in male rats (Figure 2A, n=10), EA+BTB did not significantly change PT compared to the control (n=10). In female rats in this test, EA+BTB in a dose of 5 mg/kg (Figure 2B) significantly increased PT by 24.2 % (p≤0.01, n=10) relatively to control (n=10) in response to the mechanical tail compression. This indicates the effect of EA+BTB (5 mg/kg) on the mechanical component of nociception.

In the "hot plate" test in male rats (Figure 3A), EA+BTB in a dose of 50 mg/kg significantly increased LPPR by 57.1 % (p≤0.01, n=10) compared to the control (n=10). In female rats (Figure 3B), EA+BTB in a dose of 5 mg/kg also significantly increased LPPR by 37.8 % (p≤0.05, n=10) relatively to control (n=10). Consequently, both male and female rats showed analgesic effect of EA+BTB, which is implemented with the participation of supraspinal mechanisms in the pain sensitivity regulation.

![Figure 2. Pain threshold in rats (median, interquartile range from 25 to 75 %) in the Randall-Selitto test in the control group and after intraperitoneal injection of adduct of ethidronic acid and bis(2-pyridyl-1,2,4-triazolyl)butane (EA+BTB) in doses of 5 and 50 mg/kg: (A) males, (B) females.](image)
Figure 3. Latent period of pain reaction in rats (median, interquartile range from 25 to 75 %) in the “hot plate” test in the control group and after intraperitoneal injection of adduct of ethidronic acid and bis(2-pyridyl-1,2,4-triazolyl)butane (EA+BTB) in doses of 5 and 50 mg/kg: (A) males, (B) females.

Thus, both studied doses of EA+BTB, either in male or female rats in certain acute pain tests, showed an analgesic effect. However, the studies have shown that gender-specific manifestations of the analgesic effect of EA+BTB are observed. This may be conditioned by different tolerance of the receptors and structures of the nervous system to pain in male and female rats. According to the assumptions [2], male mammals, including humans, have more pronounced activation of somatosensory regions of the cortex when experiencing pain, while females have more pronounced activation of the structures responsible for the perception of the affective – emotional component of pain. Therefore, males are easier to perceive the emotional-affective component of pain, determine the source of pain irritation better and have a more clearly directed motivation to get rid of it. The authors of the review [2] believe that initially there are pronounced gender differences in the perception of pain in males and females, due to significant psychophysiological and biological differences in the functioning of the central and peripheral nervous systems. We associate the differences in the effects of different doses of EA+BTB on pain sensitivity in male and female rats exactly with these gendered features of the functioning of the rat nervous system. This is partly confirmed by the fact that BP and pain tolerance in females are lower during the mechanical compression [17]. Lower PT in females in control than in males were demonstrated in the present study in the Randall-Selitto test (Figure 2).

4. Conclusion

It was found that EA+BTB when injected once in different doses significantly changes the pain sensitivity of male and female rats in acute pain tests, showing gender specificity of the analgesic effect with the participation of various pain mechanisms and nociception components.

In the “tail-flick” test, EA+BTB in a dose of 50 mg/kg has an analgesic effect, significantly increasing LPTR in male rats. This confirms the participation of the perceptual component of nociception and the spinal mechanism of pain sensitivity regulation in the implementation of the analgesic properties of EA+BTB in a dose of 50 mg/kg in male rats. In females, the analgesic properties of EA+BTB (5 and 50 mg/kg) were not detected.

In the Randall-Sellitto test, reflecting the participation of the mechanical component of nociception in the analgesic effect of substances, in female rats EA+BTB in a dose of 5 mg/kg shows analgesic activity, and in males the effect of this substance is not detected.

In the “hot plate” test in both male and female rats, EA+BTB has an analgesic effect, which is implemented with the participation of supraspinal mechanisms for regulating pain sensitivity. This effect is gender-specific: in males, EA+BTB increases LPPR in a dose of 50 mg/kg, and in female rats – in a dose of 5 mg/kg.
The reason for the observed gender specificity of the analgesic effect of EA+BTB in male and female rats in various acute pain tests may be different tolerance of receptors and structures of the nervous system to pain, different pain perception with the participation of various nociception components in male and female rats. This is due to significant psychophysiological and biological differences in the functioning of the central and peripheral nervous systems in males and females.

The compound EA+BTB is recommended for further in-depth preclinical tests of its analgesic activity, decoding its physiological and molecular mechanisms in order to create an improved prototype of a new generation drug based on EA+BTB.

Acknowledgment
The study was carried out with the financial support of the Russian Science Foundation in the framework of the scientific project No 18-13-00024.

References
[1] Bondarenko D A, D'jachenko I A, Skobcov D I and Murashev A N 2011 Biomedicina 2 84
[2] Reshetnyak V K and Reshetnyak D V 2014 Rossiyskiy Zhurnal Bol i3–4 54
[3] Kuźnik A, Październik-Holewa A, Jewula P and Kuźnik N 2020 Eur J Pharmacol 866 172773
[4] Sarigol D, Uzgoren-Baran A, Tel B C, Somuncuoglu E I, Kazkayasi I, Ozadali-Sari K, Unsal-Tan O, Okay G, Ertan M and Tozkoparan B 2015 Bioorg Med Chem 23 2518
[5] Thakur A, Gupta P S and Shukla P K 2016 Int J Curr Res Acad 4 2 277
[6] Cheretaev I V, Chuyan E N, Ravaeva M Yu and Shulgin V F 2019 Int Res J 7(85) 192
[7] Cheretaev I V, Ravaeva M Yu, Dzheldubaeva E R, Chuyan E N, Shulgin V F, Sheichmambetov N, Palaevskaya M V 2019 Scientific notes of the Crimean Federal University named after V.I. Vernadsky. Biology, Chemistry 5 (71) 2 162
[8] Cheretaev I V, Ravaeva M Yu, Dzheldubaeva E R, Chuyan E N, Shulgin V F, Sheichmambetov N, Palaevskaya M V 2019 Scientific notes of the Crimean Federal University named after V.I. Vernadsky. Biology, Chemistry 5 (71) 3 199
[9] Chuyan E, Ravaeva M, Cheretaev I, Kornilenko O, Furkovskaya A and Birukova E 2019 Adv Biol Sci Res 7 82
[10] Smith E S, Lewin G R 2009 J Comp Physiol A Neuroethol Sens Neural Behav Physiol 195 12 1089
[11] Gulati A, Bhalla S, Matwyshyn G, Zhang Z, Andurkar S V 2011 Pharmacology 83 45
[12] Xu F, Zhang B, Li T 2013 Pharmacology 27 4 427
[13] Randall L O, Selitto J J 1957 Arch Int Pharmacodyn Ther 111 4 409
[14] Espejo E F, Mir D 1993 Behav Brain Res 56 2 171
[15] Woolfe G, Macdonald A D 1944 J Pharmacol Exp Ther 80 3 300
[16] Rogozhin EA, Kisil O V, Cheretaev I V, Zavriev S K 2017 Antibiots Khimioter 62 9-10 3
[17] Riley J L, Robinson M E, Wise E A, Myers C D and Fillingim R B 1998 Pain 74 2-3 181