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Anti-Inflammatory Effect and a Possible Mechanism of Action of Ethowurtzine from the Class of Hexaazaizowurtzitane Derivatives

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ABSTRACT

Aim. To assess the anti-inflammatory and gastroprotective effects of ethowurtzine compared to the reference drug diclofenac in a rat model of chronic inflammation; to evaluate the influence of ethowurtzine on nitric oxide production as a possible mechanism of its anti-inflammatory effects.

Materials and methods. The object of the study was a new patented ethowurtzine from the class of hexaazaizowurtzitane derivatives with an acceptable safety profile.

The gastroprotective and anti-inflammatory effects of ethowurtzine compared to diclofenac were studied in a model of chronic inflammation using 69 female SD rats. The compound (12.5–100 mg / kg) and a non-selective COX inhibitor diclofenac (5 mg / kg) were administered intragastrically for 7 days, 1 hour before subcutaneous implantation of a cotton swab. On day 8, the proliferative response (%), the exudative response (%), and the ulcerogenic effect of the compounds were assessed.

Nitric oxide (NO) synthesis by macrophages obtained from peritoneal cavity of 25 C57Bl/6 mice (*in vitro* inflammation model) was evaluated by the concentration of nitrites in the cell supernatant after incubating cells in the presence of ethowurtzine and / or lipopolysaccharide (LPS) for 48 hours. The classical MTT test (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA)) was used to assess the effects of ethowurtzine on macrophage proliferation.

Results. A comparative study of ethowurtzine and diclofenac in a rat model of chronic inflammation revealed the predominant anti-exudative effect of the new substance and a suppressive effect on granulation tissue proliferation comparable to that of NSAID.

The macroscopic examination of the gastric mucosa in rats receiving ethowurtzine did not reveal any ulcer damage. On the contrary, in 30% of the rats receiving diclofenac, the severity score of ulcer was 2. In the *in vitro* inflammation model, the addition of LPS to the macrophage culture resulted in a significant increase in NO synthesis. The introduction of ethowurtzine at different concentrations together with LPS dose-dependently reduced the NO production. A statistically significant decrease in the NO synthesis was noted at high doses of the test substance compared to the group of isolated LPS use. However, the introduction of ethowurtzine at different concentrations together with LPS did not cause statistically significant changes in the proliferation of macrophages compared to the group with the isolated LPS use.

Conclusion. The newly synthesized ethowurtzine had a pronounced anti-inflammatory effect and caused a significant decrease in granulomatous infiltration and exudative edema in the chronic inflammation model. Suppression of the NO synthesis is one of the possible mechanisms in the anti-inflammatory effect of ethowurtzine. The obtained data allow to suggest possible administration of the new patented analgetic ethowurtzine in chronic pain treatment associated with inflammation.

Keywords: hexaazaisowurtzitane, ethowurtzine, inflammation, gastric toxicity, nitric oxide

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Противовоспалительный эффект и возможный механизм действия анальгетика этовюрцина из класса гексаазаизовюрцитанов

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РЕЗЮМЕ

Цель: изучение противовоспалительного и гастропротективного действия этовюрцина в сравнении с диклофенаком на модели хронического воспаления; исследование его влияния на продукцию оксида азота.

Материалы и методы. Объект исследования – впервые синтезированный этовюрцин из класса гексаазаизовюрцитанов с приемлемым профилем безопасности.

Гастрозащитное и противовоспалительное действие этовюрцина в сравнении с диклофенаком исследовали на модели хронического воспаления у 69 самок крыс стока SD. Вещество (12,5–100 мг/кг) и нестероидное противовоспалительное средство (НПВС) (5 мг/кг) вводили *per os* в течение 7 сут, начиная за 1 ч до подкожной имплантации ватного тампона. На 8-й сут эксперимента оценивали пролиферативную (%) и экссудативную реакцию (%), ульцерогенное действие веществ.

На модели воспаления *in vitro* продукцию оксида азота (NO) макрофагами (МФ), полученную из перитониальной полости 25 мышей линии C57Bl/6, оценивали по концентрации нитритов в супернатанте клеток после 48-часового культивирования в присутствии различных концентраций этовюрцина и (или) липолисахарида (ЛПС). Влияние этовюрцина на пролиферацию макрофагов определяли в тесте MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, США)) в лизате МФ.

Результаты. Сравнительное исследование этовюрцина и диклофенака на модели хронического воспаления у крыс выявило преимущественное антиэкссудативное действие вещества при сравнимом с НПВС подавляющем влиянии на пролиферацию грануляционной ткани.

В слизистой оболочке стенки желудка у крыс, получавших этовюрцин, не обнаружено язвенных деструкций. Напротив, у 30% животных, получавших диклофенак, тяжесть ульцерогенного повреждения составила 2 балла. На модели воспаления *in vitro* внесение этовюрцина в различных концентрациях совместно с ЛПС снижало выработку оксида азота, а при использовании высоких доз вещества наблюдалось статистически значимое уменьшение продукции NO по сравнению с группой применения ЛПС. Однако исполь-

зование этовюрцина совместно с ЛПС не оказывало статистически значимого влияния на пролиферацию макрофагов относительно группы применения ЛПС.

Заключение. Сравнительное исследование противовоспалительного действия впервые синтезированного этовюрцина на модели хронического воспаления у крыс выявило его преимущественную активность относительно диклофенака и отсутствие гастротоксичности. Полученные результаты свидетельствует об его альтернативном механизме противовоспалительного действия, не связанном с ингибированием циклооксигеназы. Подавление выработки оксида азота может быть одним из механизмов его противовоспалительного действия.

Ключевые слова: гексаазаизовюрцитан, этовюрцин, воспаление, гастротоксичность, оксид азота

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INTRODUCTION

Inflammation is a universal pathological process that underlies many nosologies, varies in clinical manifestations, and is one of the central problems in the treatment of pain syndromes of various etiologies [1–4]. The modern concept of analgesic therapy involves an integrated approach with the use of drugs and treatment methods and affects different chains of the pain pathogenesis [1–6]. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) as first-line therapy is associated with a big role of inflammation in the development of pain responses [1, 3, 6]. According to the literature, hundreds of substances containing steroidal and non-steroidal anti-inflammatory agents have been synthesized worldwide over more than 140 years to control the inflammatory process [3–6].

Despite the fact that drugs from other pharmaceutical groups, in particular some psychotropic, neurotropic drugs, antihistamines, alpha- and beta-receptor agonists, and others, somewhat resemble NSAID action [4, 5], NSAIDs still remain the main drugs for suppressing inflammation, pain, and fever [1, 6]. Nonetheless, the risk of developing dangerous adverse effects, primarily in the gastrointestinal tract [2] and the cardiovascular system (gastroduodenopathy, nephro- hepato-, and hematotoxicity, cardiovascular diseases, etc.) significantly limits the use of NSAIDs even with high therapeutic efficacy [1–3]. Searching for and designing new analgesics with different action mechanisms and combined anti-inflammatory and analgesic effects, which can become a safe alternative to NSAIDs, remain urgent.

The development of a prototype of the efficacious non-toxic analgesic thiowurtzine (120 mg capsules) based on a first-in-class molecule from the hexaazaisowurtzitane class has marked the beginning of a priority national research area – modeling pharmacologically active candidate molecules from a pharmacophore – a high-energy compound 2,4,6,8,10,12 hexaazatetracyclo[5,5,0,$0^{3,11}$,$0^{5,9}$] dodecane (hexaazaizowurtzitane) [7, 8]. Within this research area, the compound 4,10-di(ethoxyacetyl)-2,6,8,12-tetraacetyl-2,4,6,8,10,12-hexaazatetracyclo[5,5,0,$0^{3,11}$,$0^{5,9}$] dodecane (ethowurtzine) from the class of hexaazaizowurtzitanes was first synthesized at the Institute for Problems of Chemical and Energetic Technologies [9]. The analgesic effect of the substance is comparable and superior in some parameters to the activity of the reference agent tramadol in models of the somatogenic pain of various origin [8, 9]. The statistically significant anti-exudative activity of ethowurtzine was revealed to be comparable to that of diclofenac only in carrageenan-

induced edema when evaluating the anti-inflammatory effect of ethowurtzine in models of carrageenan- and histamine-induced inflammation [9, 10]. In a model of chronic inflammation, the substance is not inferior to the selective cyclooxygenase (COX) inhibitor meloxicam in terms of the studied parameters of anti-inflammatory action, while demonstrating no ulcerative damage to the gastrointestinal mucosa secondary to the death of animals in the NSAID group due to severe gastrotoxicity [10]. It is advisable to examine experimentally the anti-inflammatory and ulcerogenic effects of ethowurtzine compared to the non-selective COX inhibitor diclofenac and a possible mechanism of the specific activity of the substance.

The aim of this study was to assess the anti-inflammatory and gastroprotective effects of ethowurtzine compared to the reference drug diclofenac in a rat model of chronic inflammation, as well as to evaluate the influence of ethowurtzine on nitric oxide production as a possible mechanism of its anti-inflammatory effects.

MATERIALS AND METHODS

The experiments were carried out on 69 mature female SD rats of the first category (172–176 g), which were obtained from the Department of Experimental Biomodeling of Goldberg Research Institute of Pharmacology and Regenerative Medicine of Tomsk NRMC. Animal housing and the experimental design were approved by the Bioethics Committee of Goldberg Research Institute of Pharmacology and Regenerative Medicine of Tomsk NRMC (IACUC Minutes No. 19212021) and complied with Directive 2010/63/EU of the European Parliament and the European Union Council and GOST R no. 33044-2014 “Guidelines for Good Laboratory Practice” dated August 01, 2015.

The experimental design, sample size, experimental protocol, and choice of statistical analysis methods were determined optimally for this type of study and allowed for the acquisition of reliable data for result interpretation. The animals were divided into groups randomly using body weight (±10%) as a criterion. The animals were euthanized in a CO_2 chamber.

The substrate for the study was 4,10-di(ethoxyacetyl)-2,6,8,12-tetraacetyl-2,4,6,8,10,12-hexaazatetracyclo[5,5,0,$0^{3,11}$,$0^{5,9}$]dodecane (hereinafter referred to as "the ethowurtzine") from a new class of hexaazaizowurtzitanes, with an acceptable safety profile (LD_{50} > 2,000 mg/kg with no animal lethality). This molecule is a colorless crystalline product with an assay of 99.6% (as per the HPLC method) and a melting point of 230.5–231.5 ºC. The ethowurtzine is water-insoluble.

Ethowurtzine was administered *per os* through an atraumatic probe in the dose range of 12.5–100 mg/kg [11]. The reference drug diclofenac (Ozon LLC, Russia) was administered *per os* at a dose of 5 mg/kg, equivalent to the average daily dose for humans; purified ampoule water was used as the solvent [5]. Purified water was used as the solvent for the ethowurtzine, with an addition of 20 µl Twin-80 (Polysorbate LAUROPAN T/80, Italy) per 1 mL water. The substances were administered at 0.7 mL solvent / 200 g rat body mass. The animals in the control group had a water – Twin-80 solution received in a similar fashion.

Chronic inflammation [11] was modeled in female SD rats. A sterile cotton swab (13 mg) was implanted under the spinal skin using a needle (A1-20 x 40-117I25). The ethowurtzine and diclofenac were administered intragastrically an hour before placing the cotton swab for 7 days. On day 8, the rats were euthanized. The cotton swabs with granulation tissue around them were excised, weighed on an Adventurer electronic balance (USA), and dried in a thermostat at 60 ºC to the constant weight. The proliferative response (%) was evaluated by the difference between the mass of the dry granuloma tissue and the initial mass of the cotton swab. The exudative response (%) was evaluated by the difference between the wet and dry weights of the granuloma tissue (exudate).

ULCEROGENIC EFFECT OF ETHOWURTZINE

The ulcerogenic effect of the ethowurtzinewas examined upon completion of the chronic inflammation modeling in female SD rats by the standard method [11]. At day 8, during autopsy, the rat stomachs were excised using tweezers and sharp-ended scissors, dissected along the lesser curvature, rinsed with cold normal saline to remove the contents, spread flat on a white substrate, and examined macroscopically using a special backlighting lens to assess the ulcerogenic effect by a 4-grade scale: 0 – no damage, 0.5 – hyperemia, 1 – single minor damage (1 or 2 hemorrhages), 2 – multiple hemorrhages (erosions, single hemorrhages); 3 – significant and multiple damage (erosions, single hemorrhages), 4 – extensive damage encompassing the whole mucosa (massive hemorrhages, erosions, and perforations). The average number of ulcerations per animal in the group and the percentage of animals with ulcers were estimated. The criterion for the ulcerogenic effect

was considered to be manifestations corresponding to grade 2 and higher.

In an *in vitro* inflammation model, the nitric oxide (NO) production by macrophages isolated from the peritoneal cavity of 25 intact C57Bl/6 mice was evaluated by the concentration of nitrites in the cell supernatant following 48-h cell incubation in the presence of various concentrations of the ethowurtzine and/or lipopolysaccharide (LPS) by adding the Grace's insect medium (Sigma-Aldrich, USA) in a 1:1 ratio and measuring the optical density of the solution on the Titertek Multiskan® MCC multichannel spectrophotometer (Labsystems, Finland) at a wavelength of 540 nm [12]. Macrophages ($2.5–3 \times 10^6$) were cultured for 48 h (37 °C, 5% CO_2, 100% humidity) in a complete growth medium (CGM) RPMI-1640 (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, UK), 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES, Sigma, USA), 0.5 mM 2-mercaptoethanol (Sigma, USA), 50 μg/ml gentamicin (Sigma, USA), and 2 mM L-glutamine (Sigma, USA) in 96-well plates in the presence of various concentrations of ethowurtzine and/or 0.1 μg/ml LPS (Sigma, USA).

The effect of the ethowurtzine on the macrophage proliferation was determined by the classical MTT test (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA)) in the macrophage lysate by adding it 4 h before the end of the incubation at a final concentration of 200 μg/ml. The supernatant was then dissolved with dimethyl sulfoxide (Sigma, USA), and the optical density was measured on the Titertek Multiskan® MCC multichannel spectrophotometer (Labsystems, Finland) at a wavelength of 540 nm.

The statistical analysis of the obtained data was carried out using Statistica 8.0 software. The Shapiro – Wilk test was used to check for normality of distribution of random variables. The distribution in the *in vivo* experiment was non-normal; therefore, the Kruskal–Wallis test was used for multiple comparisons, the Wilcox–Mann–Whitney *U*-test was applied for intergroup comparisons, and the Fisher's angular transformation (φ) was used to find statistical significance of qualitative variables. The distribution of random variables in the *in vitro* experiment was normal; therefore, the one-way ANOVA and the Dunnett's test for multiple comparisons were employed to compare group means of several experimental samples with a control. For each sample, the mean and the standard error of the mean ($X \pm m$) were estimated, which are presented in the summary tables together with the value n (the number of variants). In all cases, the null hypothesis was rejected at $p < 0.05$ [13].

RESULTS

The potential analgesic agents may have an inhibitory effect on the development of inflammatory reactions, regardless of the nature of the damaging factor, phase, and stage of the process [1, 6], which explains the relevance of studying their action in modeling chronic inflammation in comparison with NSAIDs of varying selectivity [1, 6, 11].

As can be seen from Table 1, the administration of the ethowurtzine at all doses in the chronic inflammation model resulted in a significant decrease in the exudate burden: by 1.7 times at a dose of 12.5 mg/kg ($p < 0.01$, 40%), by 1.5 times at 25 mg/kg ($p < 0.01$, 35%), by 1.5 times at 50 mg/kg ($p < 0.01$, 32%), and by 1.5 times at 100 mg/kg ($p < 0.01$, 34%) compared to the controls. It should be noted that the anti-exudative effect of the ethowurtzine was significantly superior (44.5%) to that of diclofenac (8.7%).

Table 1

Parameters of Anti-Inflammatory and Anti-Ulcer Effects of Ethowurtzine (in the Dose Range of 12.5–100 Mg / Kg, *Per Os*) Compared to Diclofenac (5 Mg / Kg, *Per Os*) in the Modified Proliferative Inflammation Model in Female SD Rats

Study group, dose	Exudate weight $X \pm m$	Exudation suppresion, %	Weight of dry granuloma, $X \pm m$	Weight of granuloma tissue $X \pm m$	Proliferation suppression, %	Number of rats with ulcers per group, %
1.Control, $n = 19$	144.1 ± 10.9	0	97.2 ± 9.7	84.2 ± 9.7	0	0
2. Diclofenac, 5 mg/kg, n = 10	127.0 ± 6.8	12	68.1 ± 9.4 1–2*	55.1 ± 9.4 1–2*	35	30% 1–2**
3. Ethowurtzine, 12.5 mg/kg, $n = 10$	86.7 ± 5.0 1–3** 2–3**	40	54.9 ± 6.2 1–3**	41.9 ± 6.2 1–3**	50	0
4. Ethowurtzine, 25 mg/kg, $n = 10$	93.3 ± 6.4 1–4** 2–4**	35	56.6 ± 5.5 1–4**	43.6 ± 5.5 1–4**	48	0

End of table 1

Study group, dose	Exudate weight $X \pm m$	Exudation suppresion, %	Weight of dry granuloma, $X \pm m$	Weight of granuloma tissue $X \pm m$	Proliferation suppression, %	Number of rats with ulcers per group, %
5. Ethowurtzine, 50 mg/kg, $n = 10$	98.6 ± 6.7 1–5** 2–5**	32	58.6 ± 5.5 1–5**	44.5 ± 6.1 1–5**	47	0
6. Ethowurtzine, 100 mg/kg, $n = 10$	95.7 ± 4.5 1–6** 2–6**	34	63.5 ± 6.9 1–6**	50.5 ± 7.0 1–6**	40	0

Note: the numbers of comparison groups are given in front of the significance level, n – the number of animals. * $p < 0.05$, ** $p < 0.01$.

The statistically significant decrease in the weight of fibrotic granuloma tissue showed that the ethowurtzine in the dose range of 12.5–100 mg/kg effectively suppressed proliferation of crude granuloma burden by 50 and 40%, respectively, versus 35% for diclofenac (Table 1). However, the detected activity of the compound did not differ significantly from that of diclofenac. In previous studies, the anti-inflammatory activity of the compound in the chronic inflammation model was comparable to that of the selective COX inhibitor meloxicam [11], however, the current summary dataset indicated that the compound exhibited a better effect than the non-selective COX2 inhibitor diclofenac. It should be noted that the chemical and physical properties make it impossible to calculate the ED_{50} due to the dose-independent effect of the compound [8, 10].

The gastric mucosa of the rats that received the ethowurtzine had no ulcers. The dataset clearly indicated no gastrotoxicity of the compound and supposedly no COX2-dependent mechanism of its anti-inflammatory effect (Table 1). In contrast, in 3 out of 10 animals (30%, $p < 0.01$) who received the non-selective COX inhibitor diclofenac in the same administration regimen, grade 2 ulcers were revealed.

The LPS added to the macrophage culture caused a significant increase in NO production in the *in vitro* inflammation model (Table 2). The added ethowurtzine at various concentrations together with LPS reduced the NO production in a dose-dependent manner, and the NO production decreased significantly compared to the group of LPS alone when the test compound (750 and 1,000 μg/ml) was administered at high doses (control 2).

The proliferative macrophage activity decreased both when LPS alone and the ethowurtzine + LPS were added compared to macrophage parameters with no drugs used (control 1) (Table 2). However, the use of the compound at different concentrations together with LPS did not induce any significant changes in the macrophage proliferation relative to control 2.

Table 2

The Effect of Ethowurtzine at Different Concentrations on the Nitric Oxide Synthesis by Peritoneal Macrophages of the Intact C57BL/6 Mice, $X \pm M$

Test compound	Concentration, μg/ml	Nitrite concentration, μM	Proliferation, optical density units
Control 1 (macrophages + medium)	–	5.58±0.21	0.514±0.005
Control 2 (macrophages + LPS)	0.1	87.13±0.53*	0.446±0.008*
Ethowurtzine (+LPS)	50	81.52±0.28*	0.449±0.005*
	100	75.52±0.40*	0.466±0.007*
	200	75.86±0.23*	0.462±0.002*
	300	75.68±0.36*	0.455±0.003*
	500	75.13±0.26*	0.480±0.002*
	750	67.09±0.40*#	0.477±0.002*
	1,000	63.70±0.27*#	0.449±0.002*

Note: number of wells $n = 5$. * $p < 0.05$ compared to control 1; # $p < 0.05$ compared to control 2.

DISCUSSION

The overproduction of pain and inflammation mediators, such as interferon gamma, tumor necrosis factor, prostaglandins, interleukins-1,6, and bacterial lipopolysaccharides that induce peripheral and central pain sensitization, has a decisive role in the development of a subclinical inflammatory reaction [1, 4, 14]. It has now been proven that these substances are activators of the inducible calcium-independent NO synthase isoenzyme, thereby enhancing the NO production at the inflammation site [14], which in turn stimulates the synthesis of proinflammatory cytokines

and increases exudation, leukocyte migration, and connective tissue proliferation [15].

Evidence behind the effect of ethowurtzine on the production of NO, which is a pro-inflammatory mediator and a key player in the pathogenesis of inflammation [14], was obtained in the *in vitro* inflammation model. The addition of the ethowurtzine with LPS to the culture of peritoneal macrophages of the experimental animals suppressed the NO production in a dose-dependent manner, which may be one of the mechanisms of the identified anti-inflammatory effect of the substance.

CONCLUSION

The newly synthesized analgesic ethowurtzine outperformed the non-selective COX inhibitor diclofenac by its anti-exudative effect in the chronic proliferative inflammation model while inhibiting the granulation tissue proliferation to the same extent as NSAIDs. A lack of ulcerogenic effect of the ethowurtzine, as confirmed in this study, may indicate an alternative mechanism of its anti-inflammatory action not related to the blockade of COX-2. The results obtained in the *in vitro* inflammation model backed this assumption. The addition of the ethowurtzine together with LPS to the culture of peritoneal macrophages of the test animals inhibited the production of nitric oxide which is a pro-inflammator mediator and a key player in the pathogenesis of inflammation. The revealed activity of the test substance may be one of its anti-inflammatory action mechanisms.

The analgesic and anti-inflammatory effects of the ethowurtzine combined with its authentic action mechanism and the lack of gastrotoxicity substantiate the relevance of its further preclinical trials, while the design of a drug on its basis for the treatment of chronic inflammatory diseases associated with pain may become a safe alternative to NSAIDs.

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