ANTI-INFLAMMATORY EFFECTS OF PROPOXAZEPAM ON DIFFERENT MODELS OF INFLAMMATION

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Background. *Propoxazepam, 7-bromo-5-(2-chlorophenyl)-3-propoxy-1H-benzo[e][1,4]diazepin-2(3H)-one, is a promising analgesic and anticonvulsant and is on preclinical trial.*

Objective. *The aim of the research was to study the anti-inflammatory and analgesic action of Propoxazepam.*

Methods. *The anti-inflammatory action was evaluated by carrageenan induced rat paw edema, formalin-induced paw licking response in mice and bradykinin-induced pain response in rat models.*

Results. *It was established for the first time that the administration of Propoxazepam caused a significant anti-inflammatory activity when tested in different in vivo chemical experimental models of induced inflammation, i.e. carrageenan-, bradykinin- and formalin-induced inflammation tests.*

Conclusions. *Propoxazepam significantly reduced acute and sub-acute inflammation and proved its efficacy and similar to anti-inflammatory action.*

KEY WORDS: ***propoxazepam; diclofenac sodium; anti-inflammatory effect; carrageenan; formalin.***

Introduction

Benzodiazepines are a large and still expanding group of synthetic heterocyclic derivatives. The wide spectrum of the pharmacological effects exhibited by these compounds makes them one of the most versatile class of drugs used in psychopharmacology. Classical 1.4-benzodiazepine (BDZ) drugs have sedative, anxiolytic and anticonvulsant properties, enhancing the gamma – aminobutyric acid (GABA)-ergic neurotransmission through binding to the specific BDZ recognition sites, within GABAA receptor-ion channel complex, and allosterically modulate its activity [1]. Almost since their introduction, there has been interest to the therapeutic use of the benzodiazepines for management of pain. As regarding many other drugs, initially developed and studied for indications other than pain, conclusive data regarding the analgesic activity of BDZ is lacking. A relevant aspect of neuroplastic changes in inflammatory and neuropathic conditions is reduction in inhibitory glycinergic and GABAergic control of dorsal horn neurons: a reduction in the GABAA-mediated endogenous inhibitory control within the central nervous system leads to exaggerated pain and hyperalgesia [2]. Potentiation of GABAA receptor-mediated synaptic inhibition by benzodiazepines reverses pathologically increased pain sensitivity in animal studies. Though BDZ and barbiturates have discriminating effects on pain, their pharmacological actions on the CNS are mediated through the GABA induced chloride currents [3]. In contrast to phenobarbitone, diazepam has also been reported to have anti-inflammatory action [4] and antipyretic action [5,6].

A number of 3-substituted 1.4-benzodiazepines have been synthesized at the Physico-Chemical Institute of the National Academy of Sciences of Ukraine and their structure – activity relationships, have been studied as well. Their pharmacological effect was unusual, because unlike most classical BDS, in the models of nociceptive and neuropathic pain these substances showed significant analgesic activity [7]. One of them, Propoxazepam, 7-bromo-5-(2-chlorophenyl)-3-propoxy-1H-benzo[e][1,4]diazepin-2(3H)-one, is a promising drug and is on preclinical trials [8]. Like gabapentin and pregabalin, which are well-known drugs used in general medical practice for treatment of neuropathic pain [9], Propoxazepam also has

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an anticonvulsant effect [10, 11], which explains the analgesic component of the pharmacological spectrum.

An inflammatory response and development of pain are interdependent processes. The uncontrolled inflammatory reaction triggers the process of chronic pain generation, which is associated with the phenomena of central sensitization and neuroplasticity. It becomes obvious that the main direction of complex analgesic therapy should be the targeted use of pharmacological agents possessing anti-inflammatory potential [4-6].

It has been established that acute diazepam (a BDZ derivative drug) treatment suppresses cell proliferation in rat thymus, decreases inter-leukin release from mouse macrophages [12] and decreases macrophage and neutrophil activities [13], most probably by affecting the peripheral-type benzodiazepine receptor (PBR) present in these immune/inflammatory cells. High doses of diazepam (10.0–20.0 mg/kg) have proved to reduce the volume of acute inflammatory paw edema in rats as a response to carrageenan administration [14]. This effect was associated with an action of diazepam on the PBR present in the adrenal and/or immune/inflammatory cells [15].

Therefore, the **objective of the study** was to investigate the possible anti-inflammatory effects of the innovative derivative of BDZ Propoxazepam on acute and chronic inflammatory responses in the rats exploring the possible underlying mechanisms involved in these anti-inflammatory effects.

Methods
Animals and Injection Procedures
Male Wistar rats (180-210 g in weight) and white mice (20-24 g in weight), took from Institute of Pharmacology and Toxicology of the NAMS of Ukraine, kept at the local animal facility, were used. The animals were exposed to a 12-hour light-dark cycle and were provided with food and water *ad libitum*. All experiments were conducted during the light part of the day (9.00-14.00). The experiments were carried out according to the recommendations of the Committee for Research and Ethical Issues of the IASP (1983) and were approved by the local ethical committee for animal research. All manipulations were performed to minimize animal suffering and to reduce the number of animals used.

The test compound was suspended in Tween 80 (1%) emulsion, and the control animals received corresponding amount of vehicle (1% Tween 80).

Drugs and Chemicals
Propoxazepam was synthesized according to the method described in [16]. The structure of the substance was determined and approved by a complex of physicochemical methods (IR and mass spectroscopy, as well as X-ray diffraction analysis) [17]. Chemical purity was confirmed by elemental analysis (99%). Sodium carrageenan and bradykinin were obtained from Sigma Chemical Co., USA. Diclofenac sodium salt (Merck). Various other chemicals and reagents were of analytical grade and got from local firms.

The doses of Propoxazepam for experiments on animals were chosen according to previous bioscreening and pharmacodynamic data, where the analgesic properties of Propoxazepam were estimated. For rat tests the mean effective dose (ED_{50}) in the tail flick test was 1.83 mg/kg, so in this species of animals for paw edema tests the doses were raised in 1.6 (for 3 mg/kg) and 5 times (for 10 mg/kg) in order to achieve expected 82 % (1 σ) and 95 % (2 σ) effect. Mice doses were derived according to allometric relations with regard to species and body weight coefficients.

Carrageenan-Induced Rat Paw Edema
Three groups of eight rats each were administered with the vehicle (Tween 80 (1%) emulsion, p.o.), the test compound (3 or 10 mg/kg, p.o.), and diclofenac sodium (10 mg/kg, p.o.). One hour following the treatment with various agents, edema was induced by a subplantar injection of 0.1 mL of 1% of freshly prepared suspension of carrageenan into the right hind paw of each animal. The volume of the injected paws was estimated at 0; 2 and 4 h following carrageenan injection utilizing a plethysmometer (Plethismometer, Ugo Basil, Italy). The edema development was determined by the increase in paw volume. The increase in percentage inhibition was calculated utilizing the following equations:

Percentage inhibition = $(\Delta V_{\kappa} - \Delta V_{A} / \Delta V_{\kappa}) \cdot 100\%$,
Where: ΔV_{κ} – mean paw size in the control group.

ΔV_{A} – mean paw size in the treated group.
Formalin-Induced Paw Licking Response in Mice
Mice were divided into four groups (n=6). Test drugs Propoxazepam (0.01, 0.1 and 3.0 mg/kg p.o.), diclofenac sodium (10 mg/kg p.o.) and control vehicle (1% Tween 80, p.o.) were administered 1 h before formalin injection into the

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animals in the first set (early phase) and 40 min before formalin injection into the animals in the second set (late phase), respectively. Mice were subcutaneously injected with 20 μl of formalin (1% in normal saline) into the right dorsal hind paw. The amount of time that the animal spent licking the injected paw was measured during the first 5 min (Phase 1, corresponding to the direct chemical stimulation of nociceptors) and 20-25 min after formalin injection (Phase 2, inflammatory).

Bradykinin-Induced pain response in rat

The possible contribution of bradykinin receptors in the antinociceptive effect of propoxazepam was evaluated by using the method described by Chau et al [18]. Bradykinin (0.01% solution) was injected to the subplantar area of rat right hind paw 0.1 ml/animal. Propoxazepam (1.83 mg/kg) was administered 2 hours prior to bradykinin injection. The degree of hyperalgesia was determined using a dolorimeter (Dolorimeter Baseline, USA) by determination of the threshold of pain sensitivity (TPS) – the minimum pressure on the lower surface of the rat's foot (g/mm^2), which caused pain in the animal (vocalization and/or withdrawal of the foot). Each animal was given 5 attempts; the threshold value was taken with such a pressure force, which caused a positive response in at least one attempt. The TPS was compared on intact and damaged limbs on the 14th day after tying (pathology without treatment), as well as on the injured limb in 2 hours (peak of action) after the drugs administration.

Data Analysis

Data are expressed as a mean and standard error mean (SEM) and a Student's t-test was used to compare the data of the control and standard groups. Probabilities (p) of <0.05 were considered statistically significant. Statistical analysis was performed using the standard statistical package of MS Excel.

Results

Effects of Propoxazepam on Acute Inflammation

A single sub-plantar injection of carrageenan induced an increase in the paw thickness within 24 hours. The rat group pretreated with Propoxazepam had a significantly reduced (p<0.05) increase in the paw thickness in 2 hours with 36.7%, (3.0 mg/kg) and 47.0% (10 mg/kg) reduction of the paw edema and in 4 hours – 25.0% (3.0 mg/kg) and 26.7% (10 mg/kg). While rats of the group pretreated with diclofenac sodium showed 47.0% and 26.7% (p<0.0001)

reduction of the paw edema, respectively (Table 1).

Effect of Propoxazepam on Formalin-Induced Inflammation

As shown in Figure 1, the i.p. treatment with Propoxazepam at the doses of 0.01, 0.10 and 3.0 mg/kg significantly inhibited the licking time in of both neurogenic (0-5 min), by 91.9% (p<0.001), 76.0% (p<0.001), and 15.0%, and inflammatory (15-30 min), by 98.4% (p<0.001), 54.0% (p<0.001), and 32.1% (p<0.01), phases of formalin-induced paw-licking test compare to the control group. Diclofenac sodium (10,0mg/kg, i.p.) significantly inhibited both phases of the test by 51.0 % and 53.6% (p<0.001), respectively.

### Propoxazepam Antinociceptive Action on the Bradykinin-Induced Hyperalgesia

Bradykinin injection to the rats induced statistically significant TPS decrease by 71.7% (Fig. 2). Under these conditions Propoxazepam induced prominent antibradykinin effect, since on the background of its administration bradykinin induced TPS decrease was threefold less that of in the control group (23.8% and 71.7% respectively).

Discussion

The study proves the anti-inflammatory and analgesic effect of Propoxazepam on different models of inflammation. The carrageenan-induced rat paw edema model is widely used to investigate mechanisms of inflammatory processes and to screen potential anti-inflammatory agents. This model has been extensively studied in the assessment of anti-inflammatory action of steroidal and non-steroidal drugs involving several chemical mediators such as histamine, serotonin, bradykinin and prostaglandins. The edema and inflammation induced by carrageenan is established to be mediated by histamine and 5-HT during the first hour, after which increased vascular permeability is maintained by the release of kinins up to 2.30 hours and from 2.30 to 6 hours, the mediators are found to be the prostaglandins, the release of which is closely associated with migration of leucocytes into the inflamed site [19, 20].

In carrageenan-induced paw edema test, the highest inhibitory activity was exhibited at the dose of 10 mg/kg compared to the other dose. This simply depicts that the higher the dose, the more the inhibition of the edema. Hence, an inhibitory activity of Propoxazepam is dose-dependent (Table 1).

The results of the study have revealed that Propoxazepam as well as diazepam has a sig-

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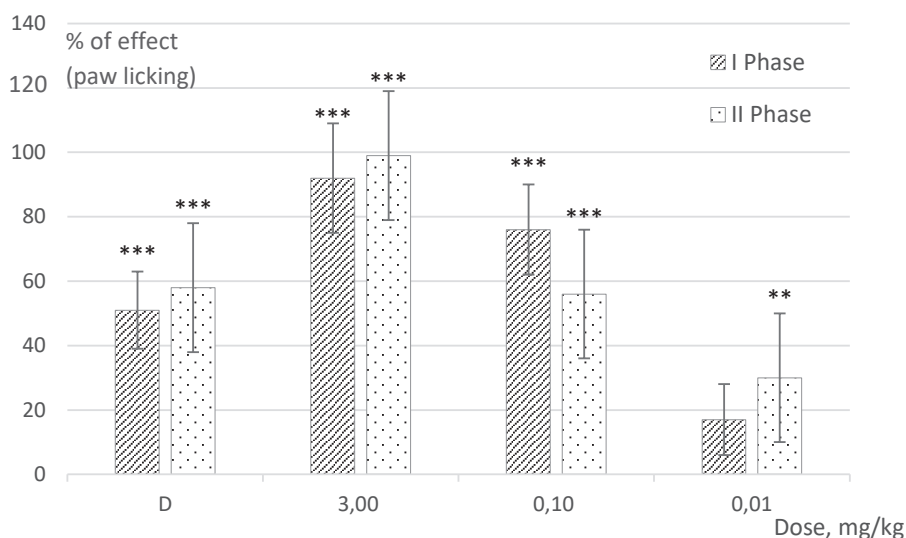
Figure 1. Effect of Propoxazepam and Diclofenac Sodium on the paw licking induced by a formalin injection in mice. Results are presented as mean±SEM (n=6).
** – p<0.01, *** – p<0.001, compared to the control.
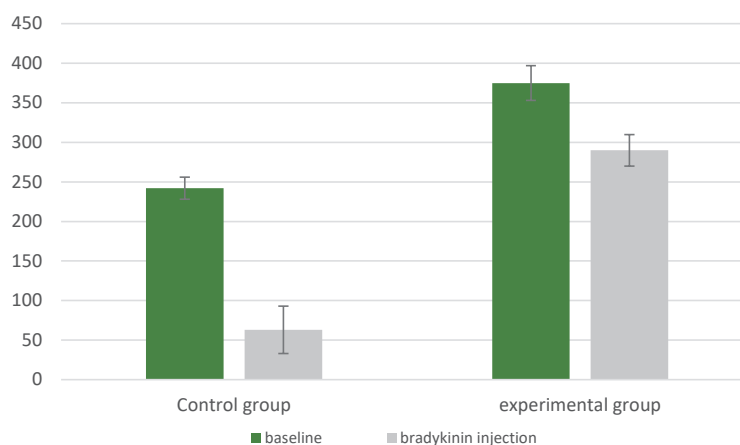
Figure 2. Effect of propoxazepam (2 mg/kg p.o.) on bradykinin-induced hyperalgesia in rats. Pressure induced hind paw withdrawal were evaluated before (baseline), and in 1 min after intraplantar bradykinin (0.01%) administration. Two hours before examination the control group received vehicle, and the experimental group received propoxazepam.
Data are presented as a mean±SEM, n=7. * – p<0.05 versus baseline, # – p<0.05 versus vehicle.

nificant anti-inflammatory effect on the different experimental models of inflammation either acute or chronic. In the acute inflammatory study, diazepam caused significant reduction of carrageenan-induced paw edema and serum NO_x levels in rats. While in the chronic inflammatory study, diazepam led to significant reduction of the paw thickness, significant reduction in the serum C-reactive protein, significant increase in serum albumin and significant increase in the serum corticosterone level [21, 22]. Similar pharmacological actions have been reported for chlordiazepoxide while, alprazolam is reported to be devoid of

Table 1. Change in paw size by carrageenan-induced paw edema in albino rats treated with increased doses of Propoxazepam

Treatment	Dose, mg/kg	Percentage inhibition (%) after 2 hours	Percentage inhibition (%) after 4 hours
Propoxazepam	3.0	36.7±4.1**	25.00±2.7*
	10.0	47.0±4.1***	26.70±5.4*
Diclofenac Sodium	10.0	43.0±7.0***	57.10±8.3***

*Notes. Values are expressed as Mean±SEM of eight rats, * – p<0.05, ** – p<0.001, *** – p<0.0001 (Student's t-test).*

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analgesic activity [23]. These reports clearly indicate that various benzodiazepines differ individually in their pharmacological profiles despite of the common chemical structure. Furthermore, the results of chronic inflammatory study showed that diazepam significantly increased serum albumin level and significantly decreased serum C-reactive protein, which is an acute phase protein commonly used to assess the disease activity in inflammatory rheumatic diseases, and this effect of diazepam could be explained by the fact that it can suppress secretion of pro-inflammatory cytokines from mouse macrophages such as TNF-α, IL-1, and IL-6, which is the main stimulator of acute phase protein synthesis in acute and chronic inflammation [24]. Structurally Propoxazepam is closer to diazepam and chlordiazepoxide, therefore their mechanisms of action coincide.

The formalin-induced licking response is used as a model for evaluation of new analgesic. This definitely proves whether the licking is genuinely due to formalin injected into the paw, because at times the animals lick the forepaw under normal physiological conditions. However, formalin test is sensitive to non-steroidal anti-inflammatory drugs and other mild analgesics. The test possesses two distinct phases, possibly reflecting different stages of pain. The early phase reflects a direct effect of formalin on nociceptors (non-inflammatory pain), whereas the late phase reflects inflammatory pain [25]. Moreover, formalin-induced nociception is also associated with direct action on a member of Transient Receptor Potential family (TRP) of cation channels denoted as TRPA1 receptor located in C fibers [26]. The results of the study show that the i.p. administration of Propoxazepam significantly and dose-dependently attenuates the nociceptive response in both neurogenic and inflammatory phases of the formalin-induced paw-licking test in mice at the level reference drug diclofenac sodium. The effect of diazepam is very similar to the effect of Propoxazepam in formalin-induced licking model [27]. The effect of diazepam is confined to increase the pain scores during the periods in 10, 15, and 20 min after formalin.

One of the cardinal features of inflammatory states is that normally innocuous stimuli produces pain [28]. Bradykinin, one of the peptide kinins, is an important inflammatory mediator. The main function of bradykinin is to increase the sensation of pain. A secondary function of bradykinin is to promote the production of histamine that is of increasing blood flow into the involved area by dilation of arteries and increased capillary vessel permeability [29].

Bradykinin diversely influences on the pathophysiological processes accompanying pain and inflammation. Its biological action is mediated by two established G-protein coupled receptors named B1 and B2. The bradykinin B2 receptor is constitutively expressed in most cell types and evokes acute pain responses following tissue injury, whereas the bradykinin B1 receptor is induced during inflammatory insults or painful stimuli [30].

The attained results show that Propoxazepam in this experiment reduces hyperalgesia on the in the model of bradykinin-induced edema. An additional argument for possible interaction of Propoxazepam with bradykinin receptors is the study [31] of compound influence on the maximal normalized speed of bradykinin-induced contraction of the rat stomach smooth muscles in the presence of gadolinium ions and verapamil. For Propoxazepam the statistically significant changes of the before-mentioned indicator have been shown as it is able to additionally inhibit the bradykinin-induced contraction in the presence of Gd^{3+} and verapamil by 19.3% and 32.0% respectively, and demonstrates the effects similar to those of des-Arg9-bradykinin-acetate (B2-bradykinin receptors concurrent antagonist) that proves either interaction with receptor or influence on signal transduction pathways.

Additionally, the mechanisms and antinociceptive effects of propoxazepam were studied on animal models of acute and chronic pain [7, 8]. The effects of Propoxazepam on pain responses were examined using tail-flick test (TFT) in rats, streptozotocin-induced rat model (SPZ) and sciatic nerve injury (SNI)-induced hyperalgesia in rats. Propoxazepam (3 mg/kg)) proved statistically significant analgesic effect compare to the control and ketorolac values after acute application in TFT and SNI-induced hyperalgesia in rats. Propoxazepam (2 mg/kg) compare to gabapentin (5 mg/kg) in greater degree after both single and chronic administrations showed analgesic action in SPZ-diabetic rats. Propoxazepam administration reduced bradykinin-induced (0.01 %) hyperalgesia. At a low dose (1 mg/kg) flumazenil diminished Propoxazepam antinociceptive effect while at a higher dose (10 mg/kg) had nearly no influence, possibly due to GABAA-receptor complex stabilization. This suggests

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that Propoxazepam causes both nociceptive and neuropathic analgesia in rats and $GABA_A$-receptor and bradykinin B-receptor are key sites of analgesic action of propoxazepam.

Conclusions

The study established the anti-inflammation effects caused by oral administration of a novel synthetic 1,4-benzodiazepine analogue, 7-bromo-5-(2-chlorophenyl)-3-propoxy-1H-benzo[e][1,4]diazepin-2(3H)-one (Propoxazepam) into the rats and mouse models with induced inflammation and nociception and explored potential mechanisms of its action. It was proved for the first time that the administration of Propoxazepam had significant anti-inflammatory action when tested in different in vivo chemicals experimental models of induced inflammation, namely carrageenan-, bradykinin- and formalin-induced inflammationtion tests.

The mechanism of Propoxazepam action might be the inhibition of synthesis or release of inflammatory mediators. As a result of the notable biological activity of Propoxazepam it would be applicable to conduct additional research to implement it into medical practice.

ПРОТИЗАПАЛЬНІ ЕФЕКТИ ПРОПОКСАЗЕПАМУ НА РІЗНИХ МОДЕЛЯХ ЗАПАЛЕННЯ

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ФІЗИКО-ХІМІЧНИЙ ІНСТИТУТ ІМ. О.В. БОГАТСЬКОГО НАЦІОНАЛЬНОЇ АКАДЕМІЇ НАУК УКРАЇНИ, ОДЕСА, УКРАЇНА

Вступ. *Пропоксазепам, 7-бром-5(2-хлорфеніл)-3-пропоксі-1Н-бензо[e][1,4]діазепін-2(3Н)-он розглядається, як перспективний анальгетик і антиконвульсант, і станом на сьогодні проходить доклінічні випробування.*

Мета. *Вивчити протизапальні властивості та аналгетичну активність пропоксазепаму.*

Методи. *Протизапальна активність була визначена на моделі карагінан-індукованого набряку лапи щурів, викликаної формаліном відповіді лизання у мишей та брадикінінової моделі болю у щурів.*

Результати. *Вперше було продемонстровано, що застосування пропоксазепаму викликало значний протизапальний ефект у різних тестах in vivo викликаного хімічними речовинами запалення, а саме карагінану, брадикініну та формаліну.*

Висновки. *Пропоксазепам значно зменшує інтенсивність гострого та підгострого запалення та проявляє протизапальну активність, співставну з референтними препаратами.*

КЛЮЧОВІ СЛОВА: **пропоксазепам; диклофенак натрію; протизапальна дія; карагінан; формалін.**

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