EAS Journal of Pharmacy and Pharmacology
Abbreviated Key Title: EAS J Pharm Pharmacol
ISSN: 2663-0990 (Print) & ISSN: 2663-6719 (Online)
Published By East African Scholars Publisher, Kenya

OPEN ACCESS

Volume-2 | Issue-3 | May-June: 2020 | DOI: 10.36349/easjpp.2020.v02i03.005

Review Article

The Discovery and Development of Propoxazepam, A Novel Analgesic and Anticonvulsant with Multimodal Mechanism of Action: Review of Own Preclinical Data

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Article History
Received:03.06.2020
Accepted: 16.06.2020
Published: 21.06.2020

Journal homepage:
https://www.easpublisher.com/easjpp

Quick Response Code

Abstract: Propoxazepam, 7-bromo-5-(*o*-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one, in the models of nociceptive and neuropathic pain showed significant analgesic activity and also has an anticonvulsant effect, which explains the analgesic component of the pharmacological spectrum. Mechanism of propoxazepam anticonvulsant properties includes GABAergic and glycinergic systems. Antibradykinin and antileukotriene action, dopaminergic system, NMDA, and alpha-1 adrenergic receptors, except the prostaglandin component, are involved in the mechanisms of propoxazepam analgesic effect. Previous pharmacokinetic studies in mice have shown that [2^{14}C]Propoxazepam evacuation from stomach (intragastral administration, 10 mg/kg) described as two-phase process (first phase with k_{el} -0,68 h^{-1}, the second of k_{el} = 0,0094 h^{-1}). Total dose quantity, absorbed during experiment, was ~ 80%, with absorption constant of 0,371 ± 0,098 h^{-1}. Similar values of distribution volume (743 ± 195 sm^3/kg and 1090 ± 421 g/kg for blood and brain respectively) let suggest intensive mass transfer. LD_{50} of propoxazepam is greater than 5000 mg/kg and it therefore, belongs to the category V of relatively non-toxic substances according to the GHS. In the acute toxicity study, neither mortality no significant change in the body weight and the relative organ weights were recorded in all treated mice and rats. Propoxazepam did not show the ability to induce gene mutations in the microplate version of the test (Muta-ChromoPlate kit) on the strains *Salmonella typhimurium* TA 98 and TA 100. The metabolic activation system was also not effective, that is, Propoxazepam is neither a "direct" nor an "indirect" mutagen for Ames strains. The present investigation demonstrates the analgetic and anticonvulsant effects, good bioavailability and safety of propoxazepam suggesting its promising potential for pharmaceutical uses.

Keywords: Propoxazepam, nociceptive and neuropathic pain, anticonvulsant effect, mechanism of action, pharmacokinetic, toxicological studies.

INTRODUCTION

In recent years, advances in biochemistry, neuropharmacology and other biological fields have been responsible for new insights in the high complexity multifactorial pathophysiological hallmarks of epilepsy and pain. The limitations imposed by the low therapeutic efficacy against most of these diseases, coupled with the apparent reduction in the approval of new bioactive chemical entities, despite significant investments in the Pharmaceutical Industry, have reinforced the need to look up new strategies for planning and discovery of more effective and safer drugs (Stahl, S. M. 2009). At the same time the understanding was achieved in complexity and multifactoral origin of chronic deceases. It promoted the knowledge that paradigm "one drug-one targed" have to be changed to that of polypharmacology – simultaneous influence on many targets (Morphy, R., & Rankovic, Z. 2009). This approach has the potential to enhance drug efficacy, improve dosing regimens and reduce side effect profiles.

Based on the concept 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 were studied. Their pharmacological effect was unusual, as, unlike most drugs in this class, in the models of nociceptive and neuropathic pain these substances showed significant analgesic activity; one of them, propoxazepam, 7-bromo-5-(*o*-chlorophenyl)-3-propoxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one, is considered as a promising drug and is undergoing preclinical trials. Similar to gabapentin and pregabalin, which are well-known drugs used in general medical practice in the treatment of neuropathic pain (Kong, V.

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K. F., & Irwin, M. G. 2007), propoxazepam also has an anticonvulsant effect, which explains the analgesic component of the pharmacological spectrum.

In this review, we have focused our attention on simple 1,4-benzodiazepine heterocyclic structure (Propoxazepam), recently used in the design, synthesis and biological evaluation as a multimodal approach to epileptic and pain medicine and management.

Synthesis of propoxazepam and [^{14}C]propoxazepam.

Propoxazepam was synthesized according to the method described in (Pavlovskyi V. I. *et al.,* 2015). 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). Chemical purity was confirmed by elemental analysis (99%).

In our study (Reder, A. *et al.,* 2019), we also prepared propoxazepam of various particle sizes and assessed the effects of particle size the on physiological activities, such as the anticonvulsant activity in mice. The obtained sample has a lower particle size but remains unnchanged in polimorphous form and chemical structure. The decreased particle size leads to increase in solubility and pharmacological effect manifestation.

A method (Andronati, S. A. *et al.,* 2019) has been developed for the synthesis of isotopically labelled propoxazepam in a six-step synthesis starting from [1^{14}C]Glycine with the 30 % yield. [2^{14}C]Propoxazepam was isolated with a specific activity of 2,68μCi/mol, a chemical purity of 99.0%, and a radiochemical purity of 89.8%. In the model experiments there were rationed the quantity of sequential extractions and extragents volumes ratio to provide 90%, 95% or 99% of significance level extraction. The estimated analytical characteristics of the method (coefficient of variation ω_s=3, 33%, relative error ε=9,53 %) make it possible for use in pharmacokinetic studies in animals.

The targeting strategies for propoxazepam and selection of targets for development

Identifying drug targets is a key aspect of modern drug discovery. Traditionally, the drug discovery process is related to a ligand and a high binding affinity to a target. Similarly to the experimental process of drug discovery, the current computational drug design methods focus on maximizing ligand efficiency, increasing his potency towards targets. In recent years, computational tools have helped to define as well as visualize target-compound interaction and have been effective instruments in the identification active molecules.

In Vitro Radioligand Binding Studies

In order to clarify the mechanism of action and definition of biotarget, radio-receptor studies were conducted (Golovenko, N. Y. *et al.,* 2018). The binding of the compound was determined by the method of displacement of the sites of specific binding [^{3}H]flumazenil to the synaptosomal fraction of the membranes of the brain. In our radio-receptor studies, it was found that affinity (K_i is the inhibition by the propoxazepam binding of [^{3}H]flumazenil with synaptic membranes of the rat brain) is 3.5 ± 0.3 nMol. Compared with other benzodiazepine drugs, this is a fairly significant value. For diazepam, chlordiazepoxide, nitrazepam and oxazepam, these figures are 6.3. 220; 6.4; 14.0, respectively (Golovenko, N. Y. *et al.,* 2018). The internal activity of the compound, determined by the magnitude of the GABA-shift of the radioligand displacement curve by the investigated ligand in the absence and presence of GABA (1×10^{-4} Mol) have shown that GABA-shift for propoxazepam is 1.9, which allows it to be attributed to a full agonist of GABA-receptor (GABA-R).

Molecular Docking Studies

The characteristics propoxazepam binding to GABA-R are determined by the geometry of the ligand-receptor complexes based on the use of experimental data on the conformation of the compound and the three-dimensional structure of the ligand-binding center of the macromolecule followed by docking (Kemp, J. A. *et al.,* 1987). Thus, by the method of molecular docking it has been shown that there are several sites of binding of propoxazepam to the part of GABA-R. The largest contribution to the formation of the complex is carried out by the residues of polar amino acids (serine, asparagine, methionine and arginine), which create a polar subcenter of binding). For individual conformers, aromatic amino acids, preferably phenylalanine (Phe-31 and Ala-135 - hydrophobic subcenter binding), play a significant role (Golovenko, M. Y. *et al.,* 2018).

Antiepileptic mechanism of action

Although the identification of molecular targets has been made possible by modern cellular, neurophysiological, and biochemical approaches, *in vitro* testing (binding studies) of new AEDs is not likely to replace preclinical animal models of epilepsy as the first stage of identification. The preclinical animal models *in vivo* enable selection of molecules that demonstrate anticonvulsant activity and are most often performed prior to the determination of their pharmacodynamics. The results indicate a high protective activity of propoxazepam based on the data of the dose-effect curves: picrotoxin (PCT) 1.67 ± 0.09, pentylenetetrazole (PTZ) 0.9 ± 0.04, strychnine (STR) 14.24 ± 0.47, maximal electroshock (MES) 0.57 ± 0.23, thiosemicarbazide (TSC) 0.18 ± 0.09, 4-aminopyridine (4AP) 37.3 ± 7.9 mg/kg (Golovenko, N. Y. *et al.,* 2017; & Golovenko, N. Y. *et al.,* 2018b). Changing the time of onset seizures and their redistribution in the course of an epileptic syndrome makes the compound promising

in the epilepsy treating. The specific feature of the used convulsive agents is the different structure of different seizures representation. For nearly all the chemical convulsants used (except 4AP), typically the tonical component is represented as major (more than 70 %). It is possible that such ratio is determined by the peculiarities in their action mechanisms and the rate of primary focal epilepsy center transformation to the dominant one with paroxysmal activity. For example, the most myoclonic component representation is noted for PCT, whose action mechanism is chloride ions current through the ionophore channel of GABAA receptor with the simultaneous decrease of its average open state time. PTZ is the most similar to PCT by its action mechanism, being the nondirect concurrent GABAA receptor antagonist, having the binding sites in the cavity of the ionophore channel. Thus, for receptor channel blocking by PTZ, the primary activation of a re ceptor is necessary, which explains the nearly absence of the clonic component in a PTZ induced structure of seizures. For TSC, which blocks the GABA reserve refilling, the fast generalization of excitation, leading to the development of tonic seizures (mostly 100 % of seizures) is the main feature. Despite the BMG action also develops through the GABAergic system (barbitura tebinding site blocker), there is also a little manifestation of the myoclonic component (2 %). The higher part of the myoclonic component (5.8 %) is in STR induced seizures (concurrent antagonist of glycine receptors, mainly concentrated in the spinal cord and controlling peripheral signals transmission). Convulsive action of MES develops through the total depolarization of brain neurons, caused by electric discharge, which generates the tonical component of seizures. Among the described convulsants, the myoclonic component is mostly represented (67 %) in the 4AP induced seizures, whose action mechanism is in the potassium channels blocking and repolarization inhibition. The results obtained provide the possibility to determine the propoxazepam antiseizure profile in various models of epileptic seizure, taking into account that the development of tonic seizures is associated with more severe and generalized CNS excitations, which is more difficult to block as compared to the formation of primary epileptopoietic cells (which are accompanied by myoclonic types of convulsions).

The results of the study prove the high safety of propoxazepam use for the treatment of GABA associated pathologies and primary generalized seizures (status epilepticus) in the tests of TSC and MES induced seizures, as well as MES induced partial paroxizmas**.**

Anti-nociceptive mechanisms

The mechanisms and antinociceptive effects of propoxazepam were studied on animal models of acute and chronic pain (Golovenko, N. Y. *et al.,* 2018c; & Golovenko, M. *et al.,* 2019). 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)) produced statistically significant analgesic effect compared to the control and ketorolac values after acute application in TFT and SNI-induced hyperalgesia in rats. Propoxazepam (2 mg/kg) in compare to gabapentin (5 mg/kg) in greater degree after both single and chronic administrations produced analgesic action in SPZ-diabetic rats. Propoxazepam administration reduced bradykinin-induced (0.01 %) hyperalgesia. At low dose (1 mg/kg) flumazenil diminished propoxazepam antinociceptive effect while at higher dose (10 mg/kg) had nearly no influence, possibly due to GABA-receptor complex stabilization. It suggests that propoxazepam causes both nociceptive and neuropathic analgesia in rats and $GABA_A$-receptor and bradykinin B-receptor are a key sites of the analgesic action of propoxazepam. There was also studied (Voloshchuk, N. I. *et al.,* 2017) the possible role of different receptors on propoxazepam effects. Naloxon, prazosin, clonidine, yohimbine, isoproterenol, propranolol, nalbuphine, chlorpromazine, dopamine, memantine, magnesium sulfate, phenylephrine were used as the pharmacological analyzers. Propoxazepam was administered orally (1.83 mg/kg, ED_{50} of analgesic activity). A detailed study of realization mechanisms of propoxazepam analgesic action was conducted under bradykinin, zymosan and carrageenan-induced hyperalgesia and «tail-flick» model.

The results showed (Voloshchuk, N. I. *et al.,* 2017) that propoxazepam reduced pain reaction caused by bradykinin and zymosan, and almost had no effect on carrageenan-induced hyperalgesia. On the «tail-flick» model, it was proved that opioid system was not involved in the action of this compound. Our study has demonstrated that dopaminergic and adrenergic system were also involved in the mechanisms of propoxazepam activity, particularly alpha-adrenergic receptors. Using of NMDA-receptors antagonist of magnesium sulfate, but not memantine under conditions of its compatible introduction with propoxazepam, increased its analgesic effect.

Bradykinin injection to rats induced statistically significant TPS decrease on 71.7 %. 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 control group (23.8 % and 71.7 % respectively). An additional argument for possible interaction of propoxazepam with bradykinin receptors is the study (Virych, P. A. *et al.,* 2017), dedicated to the investigation 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 if the noted indicator have been shown, as it is able to additionally inhibit the

bradykinin-induced contraction in the presence of Gd^{3+} and verapamil on 19.3 % and 32.0 % respectively, and demonstrates the effects similar to those of des-Arg^9-bradykinin-acetate (B2-bradykinin receptors concurrent antagonist), which proves either interaction with receptor, or influence on signal transduction pathways.

Application of pharmacokinetics in early propoxazepam development

During the early phases of drug discovery, in modern drug development processes, it becomes necessary to evaluate the bioavailability of the new compounds in parallel to investigation of their efficacy. The obtainment of appropriate pharrnacokinetics and pharmacodynamics is critical to achieving an efficacious and safe clinical dose range. Therefore, the combination of pharmacokinetic and pharmacodynamic considerations at the preclinical discovery stage should lead to drugs with optimum performance characteristics in the clinic. Indeed, the pharmacokinetic phase, incorporating the absorption, distribution and clearance of the compound can have a profound impact on the in vivo potency, duration and selectivity of the compound being tested.

Establishment of pharmacokinetic parameters for propoxazepam

Previous pharmacokinetic studies (Golovenko, N.Ya. *et al.,* 2017; & Valivodz, I. *et al.,* 2020) in mice have shown that [2^{14}C]propoxazepam evacuation from stomach (intragastral administration, 10 mg/kg) described as two-phase process (first phase with k_{el} = 0,68 h^{-1}, the second of k_{el} = 0,0094 h^{-1}). The metabolism process of propoxazepam includes both classical pathways (hydroxylation and methylation) and the elimination of the alkoxy radical forming a 3-hydroxy derivative. The distribution of propoxazepam in the internal organs and tissues in the dose range of 10-45 mg/kg can be determined as a linear process of mass transfer with a rapid redistribution of the compound between the organs and the blood. The absorption degree reaches ~ 85% the small intestine was defined as a nonspecific "absorption window". Prolonged propoxazepam administration does not change the parameters of its excretion. The low impact on the enzyme systems that carry out its biotransformation is confirmed by the absence of statistically significant changes in the elimination constant before and after the course administration.

Toxicological studies of propoxazepam

In order to explore clinical potential of propoxazepam for long term human administration, toxicology testing in laboratory animals using well-accepted international guidelines is required. Therefore, the present study aimed to evaluate the possible toxicological profiles of propoxazepam single dose (acute oral toxicity) and repeated dose (90-days, subchronic) toxicity, by assessing its physical (body weight gain and organ weights) and physiological (food and water consumption) parameters in rats and mice.

Acute and subacute toxicity profiles of propoxazepam

Acute toxicity tests were conducted by the oral administration of 2500; 3500; 4000; 4500 and 5000 mg/kg body weight to male and female mice and rats for a period of 3, 7 and 14 day (Golovenko, N.Ya. *et al.,* 2020). In subacute study, male rats were administered with various doses of propoxazepam (0.9, 4.5, and 9.0 mg/kg) to evaluate its toxicity for a period of 90 days. The effect of propoxazepam on body weight gain and organ weights, food and water consumptions were analyzed. From the present study, it can be concluded that the acute (3, 7 and 14 days) and subchronic (90 days) oral administrations of propoxazepam did not produce any clinical signs of toxicity or mortality of the male and female mice and rats. These results revealed that the LD_{50} of propoxazepam is greater than 5000 mg/kg and it therefore, belongs to the category V of relatively non-toxic substances according to the GHS. In the acute toxicity study, neither mortality no significant change in the body weight and the relative organ weights were recorded in all treated mice and rats. In the acute toxicity study, no mortality, no significant change in the body weight and the relative organ weights were recorded in all treated mice and rats. The result indicated that the oral administration of propoxazepam did not produce any significant toxic effect in mice and rats and the substance can be safely used for therapeutic use in pharmaceutical formulations.

Assessment of mutagenic effect of propoxazepam

In our studies (Golovenko, M. *et al.,* 2020), the possible induction of gene mutations by the effects of propoxazepamon the S. typhimurium TA98 (mutation by the type of shift of the reading frame) and TA 100 (mutations of the substitution of base pairs) without and with metabolic activation (fraction S9) which is important to prove its safety.

Analysis of the results showed that propoxazepam did not show the ability to induce gene mutations in the test used by us. The metabolic activation system was also not effective, that is, propoxazepam is neither a "direct" nor an "indirect" mutagen for Ames strains. Thus, the data obtained during the microplate version of the test (Muta-ChromoPlate kit) on the strains *Salmonella Typhimurium* TA 98 and TA 100 indicate that mutagenic activity of Propoxazepam in the concentrations studied is not known. In this regard, the presence of carcinogenic properties associated with genetic toxicity in Propoxazepamis also unlikely.

CONCLUSION

The present investigation demonstrates, at least, the analgetic and anticonvulsant effects, good bioavailability and safety of propoxazepam suggesting its promising potential for pharmaceutical uses**.**

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