IN VITRO BIOLOGICAL PROPERTIES OF PYRIDOXINE AND KETOROLAC CONJUGATES

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Prodrugs based on pyridoxine and ketorolac (the most potent analgesic NSAIDs) exhibit analgesic activity comparable to ketorolac *in vivo* and significantly higher safety and prolonged action. In this study the antioxidant and protective properties, inhibitory activity against cyclooxygenase (COX) and intracellular permeability for two prodrug bipharmacophoric conjugates based on pyridoxine and ketorolac have been investigated *in vitro*. Their inhibitory activity towards the COX-1 and COX-2 enzymes was comparable to that of ketorolac (the IC_{50} values ranged from 12.0 μM to 34.7 μM). These compounds markedly protected albumin against thermal and chemical (urea and citric acid) treatments and demonstrated the cell-penetrating ability through passive diffusion.

Keywords: pyridoxine; ketorolac; conjugate; protective action; cyclooxygenase; permeability

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INTRODUCTION

Ketorolac is a member of a broad pharmacological group of nonsteroidal anti-inflammatory drugs (NSAIDs); it is used primarily for the short-term relief of moderate to acute pain [1, 2]. In contrast to opioid analgesics, ketorolac does not affect opioid receptors, thus eliminating their characteristic side effects, such as respiratory depression and the development of drug dependence [3, 4]. The pharmacological effect is due to the non-selective inhibition of cyclooxygenases (COX-1 and COX-2), which leads to the suppression of prostaglandin synthesis and, consequently, key phases of inflammation, providing a strong analgesic and moderate anti-inflammatory and antipyretic effect [5, 6]. However, the use of ketorolac is associated with a high risk of adverse reactions in the gastrointestinal tract, cardiovascular system, liver, and kidneys due to non-selective COX inhibition and the direct ulcerogenic effect of the drug; this limits the possibility of its long-term use, particularly in chronic pain syndromes [7, 8]. In this regard, the development of NSAIDs with an improved safety and efficacy profile is one of the key challenges in modern pharmacology [9].

Recently, our research group synthesized prodrug bipharmacophoric conjugates based on pyridoxine and ketorolac (compounds **1** and **2**, Figure 1). The analgesic and anti-inflammatory activity of the obtained

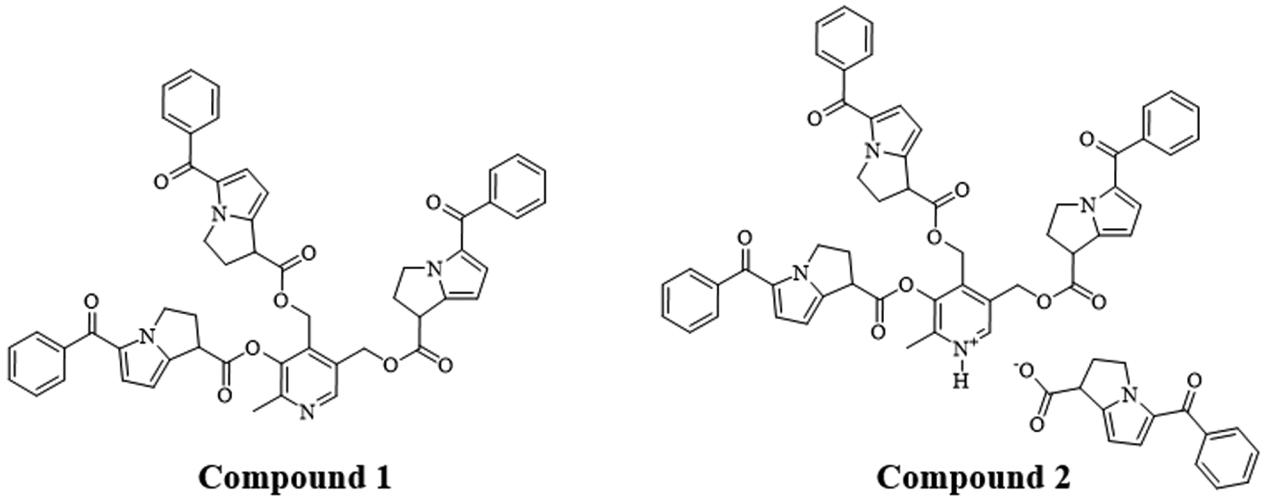

Figure 1. Structures of compound **1** (5-((5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carbonyl)oxy)-6-methylpyridine-3,4-diyl)bis(methylene)bis(5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylate) and compound **2** (3-((5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carbonyl)oxy)-4,5-bis(((5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carbonyl)oxy)methyl)-2-methylpyridinium 5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylate) [10].

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compounds was studied, and their safety was assessed *in vitro* and *in vivo* [10, 11]. According to *in vivo* results, compounds **1** and **2** are comparable to ketorolac tromethamine in analgesic and anti-inflammatory activity in thermal and chemical irritation tests ("hot plate", "carrageenan edema", "vinegar writhing", "acute exudative inflammation") and exhibit a more prolonged effect [10, 11]. Furthermore, the studied compounds were significantly safer both after single (LD_{50} of 2000 mg/kg, compared to LD_{50} of 189 mg/kg for ketorolac) and after daily intragastric administration for 14 days. In this study we have investigated the factors influencing the favorable safety profile previously established in the course of *in vivo* studies, and also elucidated their mechanism of action.

MATERIALS AND METHODS

The Compounds under Study

In this study, bipharmacophoric prodrug conjugates based on pyridoxine and ketorolac, **1** and **2**, were studied (Fig. 1). Compounds **1** and **2** were synthesized using the method described in [10].

Cell Lines and their Cultivation Conditions

Caco-2 and MCF-7 cell lines (obtained from ATCC, American Type Culture Collection) were cultured according to ATCC recommendations using 75 cm^2 culture flasks at 37°C, 95% humidity, and 5% CO_2. Cells were passaged twice at a frequency of approximately once per week (~$(0.25\text{-}1.0)\times10^6$ cells) using Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine (0.584 g/l), 10% fetal bovine serum (v/v), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) [12, 13].

Reagents and Equipment

The following equipment was used: Mikro 200R centrifuge (Hettich, Germany); Tecan Infinite 200 PRO (Tecan, Switzerland) and Varioskan LUX (Thermo Scientific, USA) plate spectrophotometers; MCO-15AC incubator (Sanyo, Japan); S30 Elmasonic ultrasonic bath (Elma, Germany); EVOM2 epithelial volt/ohm meter with STX2 electrode (WPI, USA); Agilent 1260 Infinity chromatograph (Agilent Technologies, USA) coupled with ABSciex 5600 mass spectrometer (SCIEX, Singapore); CFX96 Touch Bio-Rad thermal cycler (Bio-Rad, USA); Sonopuls HD ultrasonic homogenizer (Bandelin, Germany).

Reagents used in the study included: nutrient medium (PanEco, Russia); penicillin-streptomycin (PanEco); L-glutamine (PanEco); fetal bovine serum (Hyclone, USA); trypsin-EDTA (PanEco); Human Cyclooxygenase (COX) ELISA Kit (Abclonal, China); protease inhibitor cocktail (Thermo Scientific); phosphate buffered saline (PBS) and HEPES buffer (PanEco; EcoSERVIS, Russia); NP 40 detergent (Sigma, USA); CHAPS detergent (Sigma); ABTS (2,2′-azino-bis-(3-ethylbenzthiozoline-6-sulfonic acid) diammonium salt (Macklin, China); albumin/lecithin (Servicebio, China); SYPRO Orange Protein Gel Stain (Lumiprobe, Russia); dimethyl sulfoxide (DMSO) (Servicebio); trichloroacetic acid (Macklin); thiobarbituric acid (Macklin); ferrous sulfate (Macklin); Tris-HCl buffer (Servicebio); potassium chloride (Macklin); chloroform (Tatkhimprodukt, Russia); hydrochloric acid (Tatkhimprodukt); urea (Servicebio); citric acid (Macklin); methanol (Biosolve, France); formic acid (Sigma), verapamil (Sigma), ranitidine (Sigma), propranolol (Sigma).

Determination of COX Inhibitory Activity in MCF-7 Cells Based on ELISA

MCF-7 cells (3.5×10^6) were washed with PBS buffer (pH 7.3) and sedimented using a Hettich Mikro 200R centrifuge (5 min, 2000 rpm). The procedure was repeated twice. To disrupt the cells, a lysis buffer (containing PBS pH 7.3, 1% (v/v) NP40, 4% (w/v) CHAPS, and protease inhibitors) was added to the pellet. The lysate was mixed and the cells were placed in an ultrasonic bath for 20 min (GT SONIC-P20, 10 min at room temperature, 40 kHz frequency) and centrifuged for 15 min at 15,000 rpm (Hettich Mikro 200R). The supernatant was then collected, placed on ice and used to study the inhibitory activity. The reagents included in the Human Cyclooxygenase (COX) ELISA Kit were prepared according to the kit instructions. Stock solutions of the test compounds and the reference drug (ketorolac tromethamine) were diluted in 85% (v/v) DMSO so that the concentrations in the reaction mixture were 100 μM, 25 μM, 6.25 μM, and 1.56 μM, respectively. The reaction mixture included 93 μl of the cell lysate, 5 μl of the test substance, 2 μl of the COX isoform inhibitors included in the kit (SC560/Celecoxib), or an equal volume of diluted standards from the commercial kit. Samples were incubated for 2 h at 37°C, and unbound components were removed by washing. Biotinylated antibodies were then added and samples were incubated for 1 h at 37°C; unbound components were removed by washing. After addition of HRP-streptavidin samples were incubated for 1 h at 37°C. After another washing step, a solution of the TMB (3,3′,5,5′-tetramethylbenzidine) substrate included in the kit was added and samples were incubated in the darkness for 20 min at 37°C. The reaction was stopped by adding the supplied stop solution, and samples were immediately read spectrophotometrically at 450 nm. The experiment was performed three times under identical conditions. Using the standard curve, the percentage of COX inhibition was calculated (Annova software, chi-square test, absorbance values for untreated cell lysate were defined as 100%). An inhibition curve was then constructed using the available data

to determine the IC_{50} values of the test compounds. The significance of differences in mean IC_{50} values was determined using the Mann-Whitney U test ($p \leq 0.01$) with a Bonferroni correction. Graphical and table data are expressed as mean values and standard deviations.

The Permeability Study Using the Caco-2 Intestinal Cell Model

A cell suspension (at least 70,000 cells) has been placed in the upper compartment of a 12-well plate with inserts (Nest, China). The bottom of the well consists of a semipermeable filter on which a cell monolayer is formed: membrane surface area 1.2 cm^2, pore size 0.4 nm. 2 ml of medium was added to the lower compartment, the plate was covered with a lid, and the cells were incubated for 21 days with medium change every 2 days. The last medium change was performed no later than 24 h before the experiment. The integrity of the monolayer was checked by measuring the electrical resistance of the electrode using an EVOM2 device.

The following solutions were prepared: 10 mM HEPES, pH 7.4 (buffer I); 10 mM HEPES containing 1% (v/v) DMSO, pH 7.4 (buffer II); 0.2 mM stock solutions of compounds **1**, **2**, and reference drugs (ketorolac tromethamine, propranolol, ranitidine, verapamil) in 5 ml DMSO. The stock solutions were diluted 200-fold with buffer I to a final verapamil concentration of 10 μM and 1 μM (final concentrations) solutions of compounds **1** and **2**, ketorolac tromethamine, ranitidine, and propranolol. Buffer I was then heated to 37°C and 300 μl were added to each insert and 1800 μl to each well, the plate was incubated for 20 min at 37°C. Using an aspirator, the medium was carefully removed from the plate inserts and wells (washing was repeated twice). The transport of the test compounds and ketorolac tromethamine was determined using the control compounds propranolol (high-permeability control) and ranitidine (low-permeability control). Transport rates were determined in the A→B (from the apical to basolateral compartment) and B→A (from the basolateral to apical compartment) directions.

To determine transport rates, 300 μl of buffer II were added to filter inserts and 1800 μl of the test compounds and ketorolac tromethamine in buffer I were added to the wells of the plate. The plate was then covered with a lid and incubated for 1.5 h at 37°C. After incubation, 200 μl of a precipitating solution (acetonitrile/methanol (3:1, v/v) and 0.1% (v/v) formic acid) were added. The samples were vortexed, centrifuged at 10,000 rpm for 10 min, the supernatant was collected in chromatographic vials for subsequent and HPLC-MS analysis was performed. Tables 1 and 2 show the chromatographic parameters and MS detection parameters, respectively.

The concentration of the test substance in the samples was determined from the area of the chromatographic peak for the selected ion (XIC). Concentration calculations were performed using MultiQuant software (ABSciex, v. 3.0.2) using the MQ4 algorithm. Permeability (P_{app}, cm/s) through a Caco-2 cell monolayer was estimated using the formula:

$$P_{app} = (V_A / \text{Area} \times \text{Time}) \times (C_{A(t)} / C_{D(0)}),$$

where V_A is the volume (ml) in the acceptor well (0.6 ml or 0.1 ml depending on the transport direction); Area is the surface area (cm^2) of 0.33 cm^2 for SPLInsert™ Hanging 24-well plate inserts; Time is the transport time (7200 s); $C_{A(t)}$ is the A/IS of the substance in the acceptor well after the experiment; $C_{D(0)}$ is the A/IS of the initial solution of the substance in the donor well.

Efflux was estimated using the ratio of P_{app} values calculated based on permeability data both in the absence and presence of verapamil:

$$\text{Efflux} = P_{app}(B \rightarrow A) / P_{app}(A \rightarrow B).$$

*Antioxidant Activity of Compounds **1** and **2** in the ABTS Assay*

Aliquots (260 μl) of the test and control compounds (ascorbic acid, quercetin) were added to the wells of a 96-well plate (SPL, Korea) using methanol/water (1:1, v/v) as a solvent.

Table 1. Chromatographic parameters the analyzed compounds

Solvent A	0.005 M ammonium formate in water/formamide (99:1, v/v)			
Solvent B	acetonitrile			
Chromatographic column	ZIC-HILIC column: size 2×20 mm, particle size 3.5 μm (Merck KGaA, Germany), column temperature 40ºC, total analysis time 7 min			
Gradient	Time, min	Flow rate, ml/min	A, % (v/v)	B, % (v/v)
	0.0	0.6	0	100
	0.1	0.6	0	100
	1.1	0.6	80	20
	2.8	0.6	95	5
	2.9	0.6	0	100
	7.0	0.6	0	100

Table 2. Parameters of MS detection of the studied compounds

Type of ion source / Ionization mode	Turbo Ion Spray / Negative
Ion source temperature, ºC / Ion source voltage (IS), V	550 / 4500
Nebulizer gas (GS1), psi / Heater gas (GS2), psi	55 (air)
Data collection mode	TOFMS + HRMRM via SWATH
MS/MS mode	High sensitivity
Declustering potential (DP) in the TOFMS mode, eV	-80
Collision energy (CE) in the TOFMS mode, eV	-10
Signal accumulation time in the TOFMS mode, s	0.05
Isolated precursor ions, (*m/z*)/ Isolation window size	244.04 / 3 Da
MRM transitions	262.04→244.03 172.01→79.96
Declustering potential (DP) in HR MRM + SWATH for the ion with *m/z* = 262.04, eV	-80
Collision energy (CE) in HR MRM + SWATH for the ion with *m/z* = 262.04, eV	-20
Declustering potential (DP) in HR MRM + SWATH for the ion with *m/z* = 172.01, eV	-80
Collision energy (CE) in HR MRM + SWATH for the ion with *m/z* = 272.01, eV	-40
Signal accumulation time in the MRM + SWATH mode, s	0.025

The concentrations of the test compounds and ketorolac tromethamine varied from 5.5 μM to 700 μM. After addition of 260 μl of 0.7 mM ABTS to each well, the samples were incubated for 15 min at 37°C, and then absorbance was read at 734 nm. The experiment was performed three times under identical conditions. The results were processed using Microsoft Excel 2013 and Origin 8 Pro. The percentage of ABTS radical neutralization was calculated as the ratio of the optical density of the test samples to the control (absorbance of the reaction mixture without the test substance). To determine the IC_{50}, a dependence of oxidation inhibition on test substance concentrations was plotted.

Antioxidant Activity of the Test Compounds in a Phosphatidylcholine Liposome Model System

Egg lecithin was dissolved in chloroform and the solvent was evaporated until a uniform lecithin film was formed. After addition of Tris-HCl/KCl buffer (pH 7.4) was added the mixture was then sonicated in pulsed mode at medium power for 90 s. The concentrations of the test compounds and ketorolac tromethamine varied in the range of 5.5–700 μM. The suspension obtained from lecithin and solutions of the test substances, as well as solutions of ascorbic acid and ferrous sulfate, were placed in the wells of a 96-well plate, and the mixture was incubated for 1.5 h at 37°C. After incubation, the reaction mixture from the plate was transferred to Eppendorf tubes and a mixture of acids (7.5% trichloroacetic, 0.375% thiobarbituric, and 0.25 molar hydrochloric) was then added. The reaction mixture was incubated for 15 min at 85°C and then centrifuged at 4°C (5 min, 10,000 rpm). The resultant supernatants were transferred to 96-well plates (SPL) and read at 535 nm (with a reference wavelength of 600 nm). The experiment was performed three times under identical conditions. The results were processed using Microsoft Excel 2013 and the Origin 8 Pro software package. The percentage of lipid peroxidation (LPO) inhibition was calculated as the ratio of the absorbance of the samples to the control, which was the absorbance of the reaction mixture in the absence of the test compound in an aqueous DMSO solution. To determine the IC_{50} value, a dependence of LPO inhibition on test substance concentrations was plotted.

The Study of the Protective Properties of a Model Protein under Thermal Exposure

Stock solutions of the test compounds were dissolved in 85% (v/v) aqueous DMSO. In the absence of direct light, concentrated SYPRO Orange Protein Gel Stain (5000×) was diluted 100-fold with distilled water. A reaction mixture was prepared in PBS buffer (pH 6.5) containing 10 μl of protein solution (the protein content in the reaction mixture was 4 μg) and 12.5 μl of each buffer and test compound (final concentrations of 500 μM and 250 μM). DMSO was added to control wells instead of the test compounds. Each well was then thoroughly mixed with 2.5 μl of a 50× dye solution. Fluorescence was then measured (excitation wavelength 470 nm, emission wavelength 570 nm) using a CFX 96 system. Signal changes were recorded as the samples were sequentially heated from 20°C to 95°C at a heating rate of 0.5°C/5 s.

The experiment was performed three times under identical conditions. The results were processed using Microsoft Excel 2013 and the Anova chi-square test. The percentage reduction in denatured protein in the reaction mixture was calculated, with the signal values in the control wells defined as 100%. Data given in tables are expressed as mean and standard deviation.

The Study of the Protective Properties of a Model Protein under Chemical Treatment

The reaction mixture consisted of an ovalbumin solution (1 μg/μl) and the test compounds (final concentrations of 500 μM or 250 μM). Buffer was added to the control instead of the test compounds. The samples were incubated for 30 min at 37°C. In the absence of direct light, SYPRO Orange Protein Gel Stain (50×) was added, along with citric acid (final concentration 1 mM, in PBS, pH 5), and urea (final concentration 500 mM, in PBS buffer, pH 7.5). Fluorescence intensity was measured at an excitation wavelength of 470 nm and an emission wavelength of 570 nm for 150 min, recording the signal every 5 min. The experiment was performed three times under identical conditions. The results were processed using Microsoft Excel 2013 and Origin 8 Pro. The mean fluorescence value was calculated for each time point, and then graphs were plotted to demonstrate time-dependence of signal changes. The significance of differences in mean values was determined using the Mann-Whitney U test ($p \leq 0.01$) with a Bonferroni correction.

RESULTS AND DISCUSSION

*COX Inhibitory Activity of Compounds **1** and **2** in MCF-7 Cell Lysates*

It is known that the action of almost all NSAIDs is based on inhibition of COX-1 and COX-2, the key enzymes, involved in biosynthesis of prostaglandins (PGE) [14, 15]. Since compounds **1** and **2** are prodrugs, their effect on COX activity was studied based on residual prostaglandin content in MCF-7 cell lysates. To assess the selectivity of the test compounds against various COX isoforms, a specific COX-1 (SC560) or COX-2 (Celecoxib) inhibitors were also added to the samples.

According to the obtained results, 25 μM ketorolac tromethamine and the same concentration of compounds **1** and **2** inhibited COX-1 by 56%, 50%, and 44%, and COX-2 by 71%, 73%, and 76%, respectively (Fig. 2).

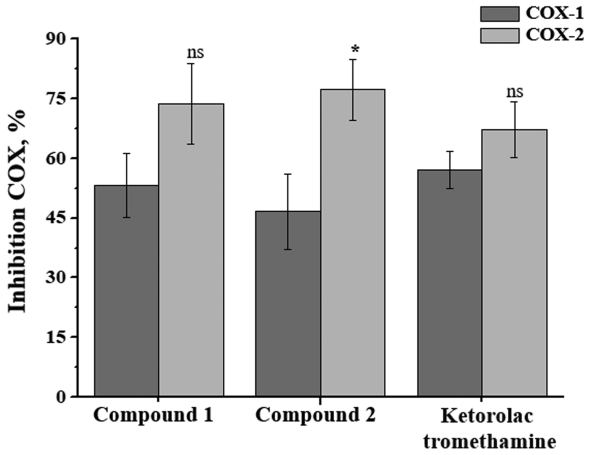

Figure 2. Inhibition of COX-1 and COX-2 in MCF-7 cell lysates by 25 μM ketorolac tromethamine and 25 μM compounds **1** and **2**. Significant differences are indicated by asterisks (* $p < 0.05$), ns ($p \geq 0.05$) – no significant differences.

The IC_{50} values for COX-1 inhibition by ketorolac and compounds **1** and **2** were 21.90±2.30 μM, 23.00±3.00 μM, and 34.70±2.90 μM, respectively; in the case of COX-2, and the IC_{50} values were 13.50±2.10 μM, 12.30±1.50 μM, and 12.02±0.12 μM, respectively. The obtained data suggest that pyridoxine and ketorolac conjugates act as rather non-selective inhibitors of COX-1 and COX-2 (Table 3).

It should be noted that the COX inhibitory activity of compounds **1** and **2** is basically the same (or even exceeds) the COX inhibitory activity of other known commercial NSAIDs such as diclofenac (IC_{50} for COX-1 and COX-2 of 35 μM and 41 μM, respectively), indomethacin (IC_{50} for COX-1 and COX-2 of 10 μM and 660 μM, respectively), and ibuprofen (IC_{50} for COX-1 and COX-2 of 2400 μM and 5700 μM, respectively) [15, 16].

*The Study of Compound **1** and Compound **2** Permeability through a Monolayer of Caco-2 Intestinal Adenocarcinoma Cells*

To study the intracellular transport of the test compounds, we have used the Caco-2 cell model, applicable for evaluation of various transport mechanisms, including passive diffusion and active transport [17]. Caco-2 intestinal epithelial adenocarcinoma cells are morphologically and functionally similar to the intestinal barrier epithelium; they are successfully used to assess permeability and absorption in the gastrointestinal tract [18].

Table 3. The IC_{50} values (μM) for inhibition of Cox-1 and Cox-2 in MCF-7 cell lysates by compound **1** and compound **2**

Compounds	IC_{50}, COX-1, μM	IC_{50}, COX-2, μM
Ketorolac tromethamine	21,90±2,30	13,50±2,10
Compound **1**	23,00±3,00	12,30±1,50
Compound **2**	34,70±2,90	12,02±0,12*

The asterisk shows differences in inhibitory potency of compound **2** towards COX-1 and COX-2: *$p \leq 0.01$.

These cells also contain transporters, including P-glycoprotein (Pgp), and can be used to study the active transport of substances across the cell monolayer. In this study, using a specific Pgp inhibitor (verapamil), we have assessed the contribution of these transporters to the transport of the test compounds across the cell monolayer. Good evidence exists that the data obtained using this model correlate well with results obtained *in vivo* [19].

The transport of compounds **1** and **2**, as well as ketorolac tromethamine, was studied using a 21-day Caco-2 culture by incubating the test compounds at a concentration of 1 μM for 1.5 h. The low- and high-permeability compounds ranitidine and propranolol, respectively, were used as reference drugs (Table 4).

The data obtained indicate that all the test compounds are capable of penetrating the model lipid bilayer via passive diffusion, which determines their basic bioavailability. However, an additional effect was observed for compound **1**: the efflux coefficient > 2 ($P_{app}(B\rightarrow A)/P_{app}(A\rightarrow B)$), which decreased with the addition of the Pgp inhibitor verapamil. This suggests that compound **1** is a Pgp substrate involved in active transport.

*Antioxidant and Inhibitory Activity of Compounds **1** and **2** in the ABTS Assay and LPO Assay*

Inflammation leads to suppression of natural antioxidant systems, which inevitably results in an overall increase in the number of molecules capable of causing oxidative damage to cells [20, 21]. In this case, the additional use of antioxidants helps to prevent oxidative damage and to protect cells and tissues. Since compounds **1** and **2** contain a pyridoxine fragment with antioxidant properties [22], it seemed relevant to study their antioxidant and inhibitory in both the ABTS assay and the LPO assay using a phosphatidylcholine liposome model. However, in both experiments, compounds **1** and **2** were inactive, with IC_{50} values exceeding 700 μM.

*Protective Effects of Compounds **1** and **2** during Thermal Treatment of a Model Protein*

Attempts to study the protective effects of NSAIDs to identify compounds capable of protecting endogenous proteins from denaturation have been known for quite some time [23, 24]. However, such activity has not yet been documented for existing NSAIDs due to limitations of their long-term use associated increased risks of side effects [25].

The protective effect of compounds **1** and **2** in a model of thermal denaturation of egg albumin was studied using a specific fluorescent dye SYPRO Orange [26, 27]. Under conditions of sequential heating of albumin from 37°C to 85°C in the presence of compounds **1** and **2**, as well as the reference drug ketorolac tromethamine, changes in dye fluorescence were recorded in real time relative to control samples containing only protein.

The results presented in Table 5 show that compounds **1** and **2** exhibited some protective activity in the thermal denaturation model and reduced albumin denaturation by 7–11%, in contrast to the reference drug, which lacked such effect.

Table 4. The values of apparent drug permeability coefficients ($P_{app}\times10^{-6}$, cm/s) and efflux of compounds studied

Compound	A→B $P_{app}\times10^{-6}$, cm/s	B→A $P_{app}\times10^{-6}$, cm/s	Efflux*
Compound **1**	0.100	6.080	60.800
Compound **1** + Verapamil	0.110	0.640	5.800
Compound **2**	0.100	1.340	13.400
Compound **2** + Verapamil	0.110	1.800	16.400
Ketorolac tromethamine	1.030	3.280	3.180
Ketorolac tromethamine + Verapamil	1.000	4.000	4.000
Ranitidine	0.006	—	—
Propranolol	30.750	—	—

*Efflux is expressed ad the efflux coefficient value.

Table 5. The protective effect of compound **1** and compound **2** in the model of egg albumin thermal denaturation

Temperature range, °C	Fluorescence intensity (*I*), relative units		
	Ketorolac tromethamine	Compound **1**	Compound **2**
40–50	1.34±0,23	9.30±2.60	6.90±1.30
50–60	0.78±0,11	11.00±3.00	8.80±1.40
60–70	0.45±0,08	8.80±1.30	7.60±1.70

Fluorescence intensity is expressed as mean values and standard deviations.

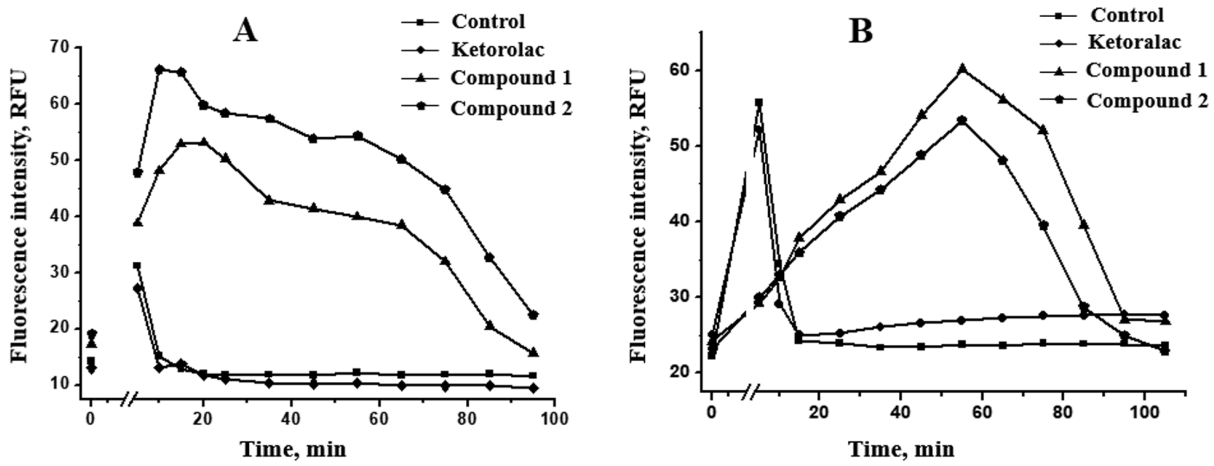

Figure 3. Fluorescence intensity of the SYPRO Orange dye in the presence of compounds **1** and **2** and ketorolac under conditions of chemical treatment of ovalbumin: **A** – urea (500 mM, 37°C, PBS, pH 7.5), **B** – citric acid (1 mM, 37°C, PBS, pH 5.0).

*Protective Effects of Compounds **1** and **2** during Chemical Treatment of a Model Protein*

Various chemical agents (e.g. alkalis, acids, detergents, urea, etc) cause protein denaturation and gradual unfolding. In this study citric acid and urea were used as denaturing chemical agents. Urea cleaves hydrogen bonds, weakens electrostatic and hydrophobic interactions, while acids alter the charge of protein molecules and ionize acidic groups [28]. The study was conducted under conditions close to physiological ones (37°C, PBS buffer, pH 5 and pH 7.5). The concentration of urea in the reaction mixture was 500 mM, and the concentration of citric acid was 1 mM.

Results have shown that protein denaturation in control samples occurred very rapidly, and fluorescence intensity decreased to a minimum within 10–15 min of incubation. A clear protective effect was observed in the presence of the test compounds (Fig. 3). In the case of citric acid exposure in the presence of compounds **1** and **2**, the time to reach maximum fluorescence increased by 40 min, while in the case of urea, it increased by 10–20 min compared to the control. The results obtained indicate a pronounced protective effect of compounds **1** and **2** on the model protein during its exposure to chemical agents; in these context compounds **1** and **2** may be considered not only as effective agents for treatment of acute pain or inflammation, but also for treatment of diseases associated with protein condensation (rheumatic diseases, cataracts, Alzheimer's disease, etc.) [25].

CONCLUSIONS

Pyridoxine-ketorolac conjugates **1** and **2** exhibit high *in vitro* inhibitory potency against COX-1 and COX-2, comparable to that of ketorolac tromethamine. A model of intestinal epithelial permeability demonstrated the ability of compounds **1** and **2** to passively diffuse across the lipid bilayer. Additional possibility also exists that transport of compound **1** may also involve active transport mechanisms. These data suggest the high absorption of the conjugates upon intragastric administration. An additional factor contributing to the favorable safety profile previously established during *in vivo* studies is the presence of pronounced protective properties in the test compounds. These results suggest that pyridoxine-ketorolac conjugates may be promising, highly effective, and safe analgesic drug candidates.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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БИОЛОГИЧЕСКИЕ СВОЙСТВА КОНЪЮГАТОВ ПИРИДОКСИНА И КЕТОРОЛАКА *IN VITRO*

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Пролекарственные бифармакофорные конъюгаты на основе пиридоксина и наиболее мощного из всех известных НПВС анальгетика кеторолака *in vivo* проявляют сопоставимую с кеторолаком анальгетическую активность, но при этом обладают значительно более высокой безопасностью и пролонгированностью действия. В настоящей работе *in vitro* исследованы антиоксидантные и протекторные свойства двух пролекарственных бифармакофорных конъюгатов на основе пиридоксина и кеторолака, их ингибирующая активность в отношении циклооксигеназы (ЦОГ), а также внутриклеточная проницаемость на модели клеток кишечника линии Caco-2. Показано, что данные соединения ингибируют ЦОГ-1 и ЦОГ-2 на уровне кеторолака со значениями IC_{50} в интервале от 12,0 мкМ до 34,7 мкМ. Они оказывают выраженное протекторное действие в условиях теплового и химического воздействия мочевины и лимонной кислоты в отношении альбумина и могут проникать в клетки посредством пассивной диффузии.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: пиридоксин; кеторолак; конъюгат; протекторное действие; циклооксигеназа; проницаемость

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