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Sodium (2-(2-hydroxyphenoxy) acetyl)-L-prolinate is a Novel Proline-Derived Compound for Treatment of Indomethacin-Induced Gastric Ulcer in Rats.

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ABSTRACT

This study was designed to improve pharmacological properties of recently discovered small-molecule compound KUD259 influencing the pathogenesis of gastric lesions. To achieve the goal, the novel agent sodium (2-(2-hydroxyphenoxy)acetyl)-L-prolinate (KUD869) was synthesized, characterized by physico-chemical methods, and evaluated *in vivo* at indomethacin-induced ulcer conditions. 3-day therapeutic administration of 1mg/kg of KUD869 decreases stomach ulceration in rats by approximately 4 times. The study reveals the possible mechanisms involved in KUD869 gastroprotective effects. Found that KUD869 possesses the antioxidant properties and protects gastric mucosa from lipid peroxidation. Also it was established the attenuation of inflammation induced by indomethacin under KUD869 action. KUD869 elevated the level of gastroprotective prostaglandin E_2 in rats serum treated with indomethacin and preserved the integrity of gastric mucosa that was proved by the reducing in products of depolymerization of collagen and non-collagen proteins of gastric mucosa.

Keywords: Gastric ulcer healing, NSAID, indomethacin, superoxide dismutase, catalase, xanthine oxidase.

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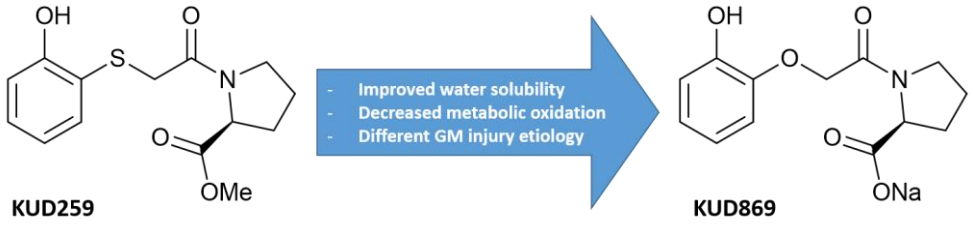
INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are abundant medications with tens million annual prescription cases and billions dollars profit for drug-makers [1-3]. Both the therapeutic and sides effects of NSAIDs are generated by inhibition the ability of cyclooxygenase (COX) to produce prostaglandins from arachidonic acid. This leads to suppression of inflammation as a desired property and causes commonly gastrointestinal tract injures as a by-effect variant. The gastrointestinal NSAIDs side effects vary from stomach upset to fatal-diaphragm-like colonic strictures. Proton pump inhibitors (PPIs) have been utilized extensively as cotherapy to prevent NSAID-induced peptic ulcers [4]. Despite proven efficacy for inhibition of acid secretion, PPIs are associated with increased level of interaction with co-administrated drugs, bacterial overgrowth, transepithelial leaks, particularly in long-term users [5,6]. Considered complications stimulate the search of novel agents for the NSAID-caused ulcer prevention.

Recently we discovered KUD259 (Figure 1), an efficient prophylactic agent for stress-induced stomach ulceration in rats [7]. With the aim to improve water solubility, suspend thioether fragment metabolic oxidation and expand ulcers etiology, we designed the novel small-molecule agent sodium (2-(2-hydroxyphenoxy)acetyl)-L-prolinate (KUD869) (Figure 1) and studied preventing influence and therapeutic pathways of KUD869 on indomethacin-caused gastrointestinal ulceration.

Figure 1: Structural formula of proline-based antiulcer agents KUD259 and KUD869.



MATERIALS AND METHODS

Synthesis of sodium (2-(2-hydroxyphenoxy)acetyl)-L-prolinate KUD869.

Reagents were obtained from Alfa Aesar and used without further purification unless otherwise stated. Solvents were dried using standard procedures. Reactions were monitored by thin layer chromatography (TLC) on precoated silica gel plates (Sorbfil) with a UV indicator. Melting points were determined in open capillary and are uncorrected. ^{13}C NMR spectra were recorded with a Bruker Avance 400 MHz spectrometer. The chemical shifts (δ) are reported in parts per million upfield using residual signals of solvents as internal standards. Combustion analyses were performed with a Carlo Erba CHN analyzer.

L-proline (0.380 g, 3.33 mmol) was added to the stirred solution of 0.500 g (3.33 mmol) of benzo[*b*][1,4]dioxan-2(3*H*)-one in 20 mL of tetrahydrofuran (THF). The reaction mixture was stirred for 12 h at +60 ÷ +70 °C. After cooling to rt the reaction mixture was filtered, the filtrate was evaporated under vacuum and redissolved in 20 mL of methanol. Sodium methylate (4.25 mL of 0.782 M solution in methanol, 3.33 mmol) was added, the mixture was agitated and evaporated under vacuum. The solid was treated with 20 mL of acetone, filtered, rinsed with 2 x 10 mL of acetone and dried. Yield 58% (0.560 g), white powder, mp 165-170 °C. ^{13}C NMR (100 MHz; DMSO-d_6): δ 173.95, 167.88, 156.46, 148.66, 147.34, 122.76, 118.69, 117.30, 116.45, 69.73, 61.01, 46.39, 31.18, 21.78. Anal.Calcd for $C_{13}H_{14}NNaO_5$: C, 54.36; H, 4.91; N, 4.88. Found: C, 54.24; H, 5.14; N, 4.56.

Animal tests

The study was carried out on 30 male albino Wistar rats weighing 200-250 g in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with the approval from the Animal Ethical Research Committee of Taras Shevchenko National University of Kyiv (Protocol number: 20/2013). The rats were kept in collective cages at controlled conditions of temperature (22±3 °C), light (12 h light/dark cycle) and relative humidity (60±5 %). The animals were fed

with laboratory chow and tap water ad libitum. The rats were divided into three groups of 10 animals in each: intact control, indomethacin and indomethacin+KUD869 groups. 24 Hours before an exposure to the ulcerogenic factor animals were housed in wire-net bottom cages to avoid coprophagy and were provided with free access to tap water. Formation of erosive and ulcerative gastric lesions was induced with 200 µl of indomethacin solution per 100 g of animal weight administered intragastrically. Ulcerogenic dose of indomethacin was 20 mg/kg [8]. Indomethacin solution consisted of 89.5% water, 10% ethanol and 0.5% carboxymethylcellulose. After 6 hours after administration of indomethacin rats were given with food. The animals were sacrificed 3 days after administration of indomethacin. To study the therapeutic effect of compound KUD869, rats of the indomethacin group were injected with saline at a volume 2 ml/kg and rats of the indomethacin+KUD869 group were injected with saline solution of KUD869 in dose 1 mg/kg once a day 3 days after administration of indomethacin.

Evaluation of gastric mucosal lesions

At the final stage, animals were decapitated. The stomachs were removed, cut along the lesser curvature, turned out (mucosa out), and thoroughly washed with physiological saline. The severity of mucosal lesions was visually inspected and digitally photographed. The length and width of each lesion, including epithelial cell damage, glandular disruption, vasocongestion, hemorrhage and deep necrosis were measured and the total area of the lesions in one stomach was assessed by planimetry. The ulcers estimation was performed by a protocol-blind researcher. After that, gastric tissues were fixed with 10% formalin, dehydrated and imbedded in paraffin wax. Paraffin sections of 5 µm were cut and stained with hematoxylin and eosin. Histological changes were checked under a microscope XS-4130 MICROmed.

Measurement of products of lipid peroxidation

In the rat GM we estimated the level of H_2O_2, diene conjugates (DC), TBA-active products and Schiff bases (SB) by standard biochemical methods. H_2O_2 was detected by the reaction with sorbitol that produces peroxyl radicals which convert Fe^{2+} into Fe^{3+}. Fe^{3+} with xylenol orange dye forms the colored complex [9,10]. In brief, 2 ml of reagent (1:100 solution A (25 mM $(NH_4)_2Fe(SO_4)_2 \times 6H_2O$ + 2.5M H_2SO_4) and solution B (100 mM sorbitol + 125 µM xylenol orange)) was added to 400 µl of a sample containing 200-400 µg of protein. After 20 minutes the optical density was measured at 540 nm. The concentration of H_2O_2 was expressed as nmol/mg of protein.

For evaluation of diene conjugates [11] and Schiff bases [12] 5ml of mixture of heptane and isopropyl alcohol (1:1) were added to sample containing 100 µg of protein, homogenized (10 min) and centrifugated (1000 g, 15 min). Water (0.5 ml) was added and two phases were separated. Ethanol (1.5 ml) was added to an upper heptane phase (0.3 ml) and this mixture was subjected to spectrophotometric analysis at 233 nm for detecting of diene conjugates. The DC level was expressed as nmol/mg of protein. Schiff bases were detected in heptane phase with fluorophotometer RF-510, Shimadzu (Japan) at λ=360 nm (excitation) and λ=420 nm (emission) and expressed as absorbance units (a.u.)/mg of protein.

The TBA-active products were detected by formation of the colored complex with TBA. In brief, sample with 0.5 mg of protein in TRIS centrifugated with 0.2 ml of 17% trichloroacetic acid. 0.25 ml of 0.8% TBA was added to 0.5 ml of supernatant and the obtained solution was heated in a boiling water bath for 10 min. The optical density was evaluated by spectrophotometry at 532 nm. The concentration of TBA-active products was presented as nmol/mg of protein.

Aldehyde and ketone groups in oxidized proteins products were measured by the reaction with 2,4-dinitrophenylhydrazine [13]. Aliquot of a sample contained 500 µg of protein was placed in phosphate buffer (pH 7.4) and incubated at 37º C for 15 min. After incubation, 1 ml of 2,4-dinitrophenylhydrazine (in 1-2 M HCl) was diluted and placed again in an incubator at 37º C for 45 min. After incubation the sample was filled up with 1.5 ml of 20% trichloroacetic acid and centrifuged at 1000 g for 15 min. The precipitate was washed in ethanol-ethyl acetate (1:1) mixture and dried. The residue was dissolved in 8M urea and kept for 5 min in a boiling water bath. The neutral products of oxidative modification were measured at 356 nm (aldehyde products) and 370 nm (ketone products). The products of oxidative modification with basic character were determined at 430 nm (aldehyde products) and 530 nm (ketone products). Their content was expressed in a.u./mg protein.

Evaluation of antioxidant systems activity

Rats GM superoxide dismutase (SOD), catalase, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione transferase (GT), glutathione reductase (GR), glutathione peroxidase (GP) activities were estimated by standard biochemical methods. SOD activity was determined by Chevari's method [14] based on the ability of SOD to compete with nitroblue tetrazolium for superoxide anions, which are formed during the interaction of NADH with phenazine methosulfate. In the presence of SOD percentage of superoxide anions recovery by nitroblue tetrazolium is decreased.

Catalase activity was detected by the reaction of H_2O_2 cleavage [15]. H_2O_2 forms a colored complex with molybdenum salts. The sample contained 100 µg of protein was added to 2 ml of 0.03% H_2O_2. After 10 min the reaction was stopped by adding of 1 ml of 4% ammonium molybdate. The optical density was detected spectrophotometrically at 410 nm against the blank sample.

The measurement of reduced glutathione was based on the reaction with orthophthalic aldehyde [16,17]. The GM sample was incubated during 45 min with orthophthalic aldehyde and fluorescence intensity was measured by fluorophotometer RF-510, Shimadzu (Japan) at λ=350 nm (excitation) and λ=420 nm (emission).

The activity of glutathione peroxidase was determined by the accumulation of GSSG [18]. The reaction mixture consisted of 1 ml of 0.3 M phosphate buffer containing 12 mM sodium azide, 6 mM EDTA (pH 7.4), 0.5 ml of 2.5 mM GSSG and 0.2 ml of the sample. The reaction was activated by adding of 0.5 ml of 1.8 mM H_2O_2 and stopped by adding of 0.5 ml of 10% trichloroacetic acid after 2 min. After 15 min centrifugation the optical density of GSSG was estimated at 260 nm and expressed as micromoles of GSSG per 1 mg of protein per min.
Glutathione transferase activity was determined by the rate of formation of conjugate with 1-chloro-2,4-dinitrobenzene, which has a maximum absorption at 346 nm [18,19]. The mixture (1.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.2 ml of 10 mM GSH, 0.1 ml of the tested sample supernatant) was filled up with 0.02 ml of 0.1 M 1-chloro-2,4-dinitrobenzene. Optical density increasing was recorded for 5 minutes at 340 nm spectrophotometrically and expressed as nanomoles of glutathione conjugate (GSR) per 1 mg of protein per min.

The activity of glutathione reductase was evaluated by reduction of NADPH [18]. The reaction mixture consisted of 350 µl of phosphate buffer (0.05 M, pH 8.0), 35 µl of 1 mM EDTA, 50 µl of 7.5 mM GSSG, 50 µl of sample, 50 µl of 1.2 mM NADPH. The enzyme activity was determined by decreasing a NADPH level at 37 °C for 8 min, at a wavelength of 340 nm by a spectrophotometer. Activity was expressed in nanomoles of NADPH per 1 mg of protein per min.

Measurement of the products of the protective protein catabolism in the gastric mucosa

In the GM we measured the content of oxyproline, fucose and hexuronic acid. In brief, oxyproline was evaluated by the oxyproline oxidation with *p*-dimethylaminobenzaldehyde [20]. Formation of pyrrole-2-carboxylic acid takes place as a reaction result and optical density of probes was measured at 540 nm. The fucose level was estimated by the adding of 85% sulfuric acid and cysteine [21]. The concentration of the product in this reaction depends proportionally from fucose content in probes. The optical density of the obtained solutions was measured at 396 nm and 430 nm.

The hexuronic acid level was measured by oxidation with sulfuric acid [22]. Furfural formed under these conditions interacts with aromatic phenols. The product of condensation was determined photometrically using a green optical filter. The content of the protective protein catabolism products was expressed as µmol/g of tissue.

ELISA method for cytokine evaluation

The contents of interleukins (ILs) 1β, 4, 10, 12B p40, interferon (INF) γ, tumor necrosis factor (TNF) α, tumor growth factor (TGF) β, leukotrienes (LTs) B4 and C4 and prostaglandin (PG) E2 in rats serum were determined by ELISA using specific mono- and polyclonal antibodies (Sigma) to these proteins. Antibodies to ILs 4, 10 and TGF-β were obtained from monoclonal mice. Antibodies to IL 12B p40, TNF α, PGE2, LTs B4 and

C4 were produced in rabbits. Antibodies against IL-1β and INF γ were produced in goats. Studied molecules were immobilized in 96-well plates with sorption surface. These substances were attached with primary antibodies which were added with a secondary antibody labeled with enzyme. The optical density of the solution in each well after the addition of substrate showed the protein amount in the well, and therefore the content of immobilized molecules. The content was expressed as absorbance units of optical density.

Statistical analysis

Statistical analysis of all data was performed by the "Statistica 8.0" software package. Shapiro-Wilk W criterion was used for the analysis of the data distribution type. Student test was used for the comparison of independent samples if results were normally distributed. Mean of value (M) and standard error of the mean (SEM) were calculated. Significant differences were considered at $p \leq 0.05$.

RESULTS

Characterization of compound KUD869.

The purity of small-molecule compound KUD869 was confirmed by TLC and elemental analysis data. Structural assignments were made by NMR spectra analysis and correlation of spectral characteristics with earlier established parameters for analogs [7].

Evaluation of Gastric Lesions

Figure 2: The ulcer area in the rats GM under conditions of KUD869 treatment of indomethacin-induced gastric ulcer: 1 – indomethacin group, 2 – indomethacin+KUD869 group, [a]p<0.05 compared to indomethacin group (M±SEM).

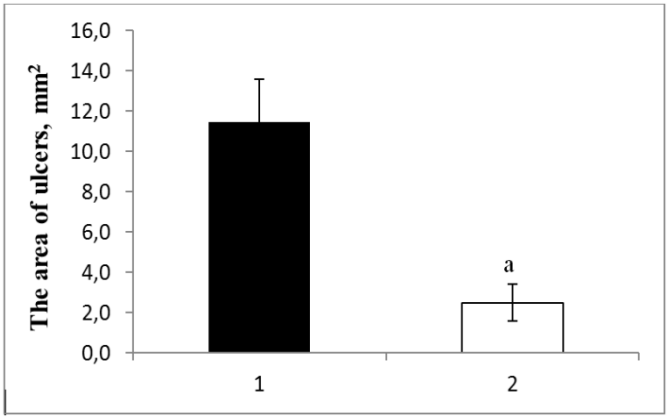
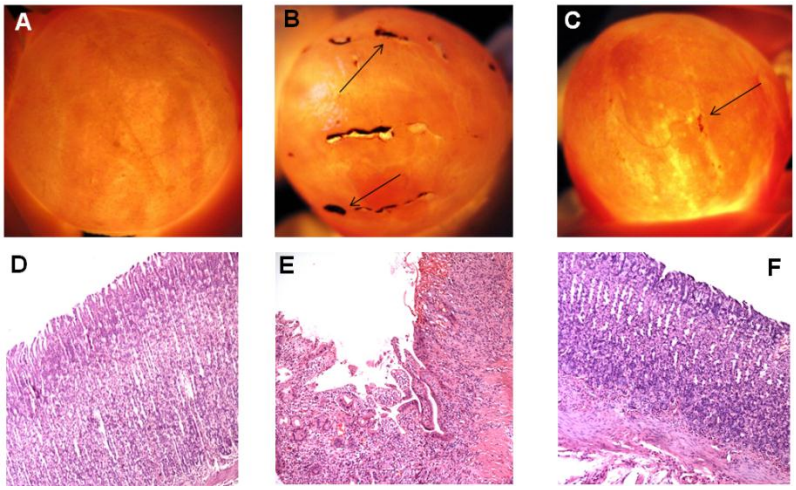


Figure 3: Images of transilluminated stomachs and light microscopic microphotographs of rats GM stained with haematoxylin and eosin. A, D – the intact group; B, E – the indomethacin group; C, F – the indomethacin+KUD869 group.



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Essential lesions in the rats GM were observed three day after administration of indomethacin. The area of ulcers was equal to 11.4±2.1 mm^2. Therapeutic administration of KUD869 significantly decreased ulcers area to 2.5±0.9 mm^2, that is lower by 78.1% ($p<0.05$) in comparison with the indomethacin group (Figure 2).

The original photos under transillumination and microphotographs of the GM are given on Figure 3. These images demonstrate deep ulcerative lesions caused by the indomethacin treatment. Inflammation of GM and necrotic tissues in the bottom of ulcer were also detected in the indomethacin group. Small-molecule agent KUD869 prevented the ulcer development and partially restored the state of the GM.

Lipid peroxidation and enzymatic activity of antioxidant system under conditions of indomethacin-induced gastric ulceration and KUD869 treatment

The intensity of lipid peroxidation (LP) in the rats GM increased significantly after the administration of indomethacin. The content of DC raised by 6.3 times ($p<0.001$), TBA-active products content increased by 3.3 times ($p<0.001$), Schiff bases (SB) content increased by 7.2 times ($p<0.001$) compared to the intact group (Table 1). Elevation of the hydrogen peroxide content in the rats GM by approximately 4 times ($p<0.001$) was registered. All these data indicate an accumulation of reactive oxygen species (ROS) in GM caused by indomethacin. As a result of LP intensification activities of SOD and catalase have grown by 4.4 times ($p<0.001$) and 2.1 times ($p<0.001$) respectively compared to the intact group (Table 2).

Table 1: Lipid peroxidation in the rats GM under conditions of KUD869 treatment of indomethacin-induced gastric ulcer

	Intact group	Indomethacin group	Indomethacin+ **KUD869** group
H_2O_2, µmol/mg of protein	2.24 ± 0.19	8.91 ± 0.73***	5.66 ± 0.46***/#
Diene conjugates, nmol/mg of protein	80.33 ± 7.36	509.04 ± 40.01***	431.68 ± 37.23***/#
TBA-active products, nmol/mg of protein	49.24 ± 3.77	161.31 ± 12.42***	136.08 ± 9.62***/#
Schiff bases, a.u./mg of protein	1.18 ± 0.14	8.46 ± 0.57***	7.03 ± 0.54***/##

All values are expressed as M±SEM, n=10 in each group. a.u. – absorbance units. *** – $p<0.001$ compared to the intact group; #,## – $p<0.05$, $p<0.01$ compared to the indomethacin group.

Table 2: Activities of the antioxidant system enzymes in the rats GM under conditions of KUD869 treatment of indomethacin-induced gastric ulcer

	Intact group	Indomethacin group	Indomethacin+ **KUD869** group
Superoxide dismutase activity, a.u./(min × mg of protein)	0.174 ± 0.012	0.773 ± 0.071***	0.657 ± 0.047***/#
Catalase activity, nmol of H_2O_2 /(min × mg of protein)	307.49 ± 29.23	632.51 ± 41.85***	438.56 ± 32.53***/#

All values are expressed as M±SEM, n=10 in each group. *** – $p<0.01$ compared to the intact group; # – $p<0.05$ compared to the indomethacin group.

Administration of KUD869 decreased the contents of DC by 15.2% ($p<0.05$), TBA-active products by 15.6% ($p<0.05$), SB by 16.9% ($p<0.05$) in the rats GM as compared with the indomethacin group (Table 1). SOD and catalase activities in the indomethacin+KUD869 group were partially restored against the corresponding levels of indomethacin group by 15.1% ($p<0.05$), catalase activities by 30.7% ($p<0.01$) (Table 2). Such effect correlates with the decrease of lipid peroxidation.

Significant decrease of GSH content in the indomethacin group of animals was observed (Table 3). It was also found reduced activity of GP by 53.6% ($p<0.001$) compared to the intact group. That can be explained the depletion of its substrate GSH. GR and GT activity did not differ from the group of intact rats. The glutathione content in rats GM treated with KUD869 decreased by 10.6% ($p<0.05$) as compared with the intact control but was significantly elevated by 36.9% ($p<0.05$) compared to the indomethacin group. The GT activity decreased by 20.3% ($p<0.05$) as compared with intact rats, that may indicate GT participation in the

consumption of TBA-active products. Indeed, the level of TBA-active products in the indomethacin+KUD869 group was lower compared to the indomethacin group. The activity of GP in the indomethacin+KUD869 group did not match values of the intact group but increased by 23.3% ($p<0.05$) compared to the indomethacin group (Table 3). These data indicate a restoration of normal GP activity under conditions of therapeutic administration of KUD869.

Table 3: Glutathione content and enzymatic activity of glutathione antioxidant system in the gastric mucosa of rats under conditions of KUD869 treatment of indomethacin-induced gastric ulceration

	Intact group	Indomethacin group	Indomethacin+ **KUD869** group
GSH, nmol/mg of protein	33.26±0.82	21.71±0.38***	29.72±0.31*/##
Glutathione transferase activity, nmol of conjugate of GSH with 1-chloro-2,4-dinitrobenzene/(min × mg of protein)	195.55 ± 24.1	173.78 ± 11.41	155.88 ± 2.11*
Glutathione reductase activity, µmol of NADPH/(min × mg of protein)	1.13 ± 0.23	1.05 ± 0.07	0.9 ± 0.1
Glutathione peroxidase activity, nmol of GSH/(min × mg of protein)	2.22 ± 0.13	1.03 ± 0.01*	1.27 ± 0.06*#

All values are expressed as M±SEM, n=10 in each group. GSH – reduced glutathione, GSSG − oxidized glutathione. *, *** – $p<0.05$, $p<0.001$ compared to the intact group; #,## – $p<0.05$, $p<0.01$ compared to the indomethacin group.

Indomethacin increased significantly the content of GM proteins oxidative modifications (Table 4). The level of neutral aldehyde and ketone 2,4-dinitrophenylhydrazones increased by 17 times ($p<0.001$) and 10.9 times ($p<0.001$) and basic aldehyde and ketone 2,4-dinitrophenylhydrazones by 4.7 times ($p<0.001$) and 11.7 times ($p<0.001$) respectively compared with intact rats (Table 4). The level of neutral and basic aldehyde and ketone 2,4-dinitrophenylhydrazones in rats treated with KUD869 was not restored to the control values. KUD869 reduced the content of neutral aldehyde and ketone 2,4-dinitrophenylhydrazones by 1.2 times ($p<0.05$) and 1.3 times ($p<0.01$), respectively, and the level of basic aldehyde and ketone 2,4-dinitrophenylhydrazones by 1.3 ($p<0.05$) and 2.3 times ($p<0.05$), respectively, as compared with the indomethacin group (Table 4).

Table 4: Content of proteins oxidative modification products in the gastric mucosa of rats under conditions of KUD869 treatment of indomethacin-induced gastric ulceration, (a.u./mg of protein)

Groups	Neutral products		Basic products	
	Aldehyde products	Ketone products	Aldehyde products	Ketone products
	λ =356 нм	λ =370 нм	λ =430 нм	λ =530 нм
Intact group	21.66 ± 1.32	34.62 ± 2.85	24.62 ± 2.12	17.63 ± 0.97
Indomethacin group	368.0 ± 28.0*	377.8 ± 27.8*	116.2 ± 10.4*	205.6 ± 16.4*
Indomethacin+ KUD869 group	302.7 ± 22.6*#	288.2 ± 28.5*##	88.7 ± 8.8*	89.4 ± 8.7 */#

All values are expressed as M±SEM, n=10 in each group. a.u. – absorbance units. * – $p<0.001$ compared to the intact group; #, ## – $p<0.05$, $p<0.01$ compared to the indomethacin group.

Effects of KUD869 on degradation of protective proteins in the gastric mucosa of rats under indomethacin-induced gastric ulceration

The development of erosive and ulcerative lesions in the GM caused by indomethacin was accompanied with elevated degradation of collagen fibers and other mucous epithelial barrier protective proteins, that is supported by the growth of free oxyproline, fucose and hexuronic acid contents by 53.6% ($p<0.001$), 254,3% ($p<0.001$) and 75% ($p<0.01$) respectively compared to the intact group (Table 5). The therapeutic administration of KUD869 didn't influence on the oxyproline content in the mucosal barrier. However, small-molecule agent KUD869 prevented considerably the degradation of fucoproteins by 44.8% ($p<0.01$) against the indomethacin group. The level of hexuronic acids decreased only by 10.4% ($p<0.05$) in the GM of animals treated with KUD869 (Table 5). Thus, KUD869 prevented the degradation of rats GM protective proteins but not collagen in conditions of indomethacin ulcer.

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Table 5: Content of protective proteins catabolism products in the gastric mucosa of rats under conditions of KUD869 treatment of indomethacin-induced gastric ulceration

	Intact group	Indomethacin group	Indomethacin+KUD869 group
Oxyproline	1.23±0.04	1.89±0.03***	1.88±0.03**
Fucose	1.38±0.03	4.89±0.04***	2.70±0.05**/##
Hexuronic acids	0.44±0.01	0.77±0.03**	0.69±0.01*/#

All values are expressed as M±SEM, n=10 in each group. *, **, *** – p<0.05, p<0.01, p<0.001 compared to the intact group; #, ## – p<0.05, p<0.01 compared to the indomethacin group.

Effects of KUD869 on leukotrienes B4 and C4 and prostaglandin E2 level in rats serum under indomethacin-induced gastric ulceration

The erosive and ulcerative lesions in the indomethacin-treated rats GM were supplemented by increase of LT B4 and C4 and decrease of PGE2 in rats serum. The augment of LT B4 and C4 by 17.3% (p<0.01) and 22.7% (p<0.05) respectively was observed compared to intact animals (Figures 4, 5). Therapeutic administration of KUD869 decreased content of both LT B4 and C4 by 18.3% (p<0.05) and 13.4% (p<0.05) compared to the indomethacin group. Reduction of serum PGE2 by 18.4% (p<0.05) versus the intact group was induced by indomethacin (Figure 6). Treated with KUD869 rats restored PGE2 level that exceeded by 18.6% (p<0.05) value of the indomethacin group. Thus, KUD869 gastroprotective effect includes normalization of leukotrienes and prostaglandin levels.

Figure 4: Leukotriene B4 level in rats serum under conditions of indomethacin-induced gastric ulceration and KUD869 treatment: 1 – intact group, 2 – indomethacin group, 3 – indomethacin+KUD869 group; * – p<0.05 compared to the intact group, # – p<0.05 compared to the indomethacin group (M±SEM).

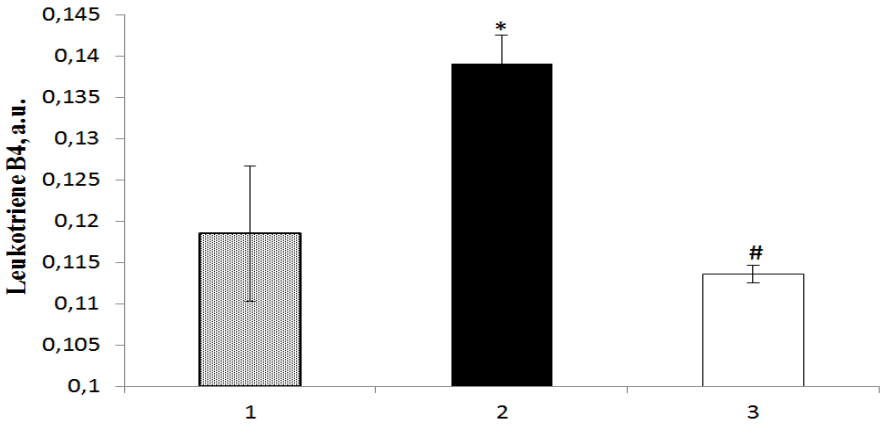

Figure 5: Leukotriene C4 level in rats serum under conditions of indomethacin-induced gastric ulceration and KUD869 treatment: 1 – intact group, 2 – indomethacin group, 3 – indomethacin+KUD869 group; * – p<0.05 compared to the intact group, # – p<0.05 compared to the indomethacin group (M±SEM).

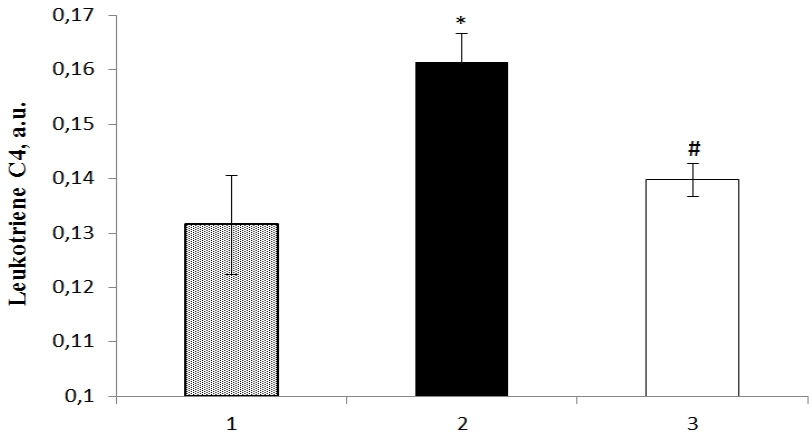

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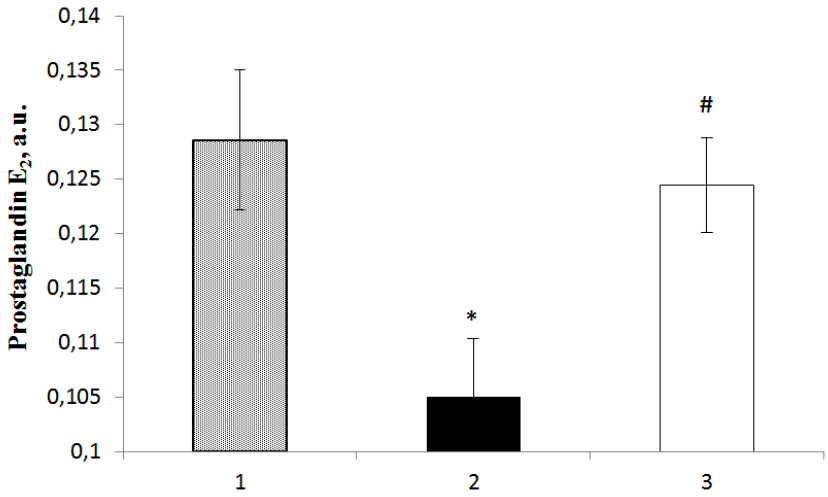
Figure 6: Prostaglandin E2 level in rats serum under conditions of indomethacin-induced gastric ulceration and KUD869 treatment: 1 – intact group, 2 – indomethacin group, 3 – indomethacin+KUD869 group; * – p<0.05 compared to the intact group, # – p<0.05 compared to the indomethacin group (M±SEM).



Rats serum cytokines system under indomethacin-induced gastric ulceration and treatment with KUD869

Indomethacin induced inflammation in rats serum that provoked as proinflammatory cytokine synthesis. Increase of IL-1β and IL-12B p40 levels in the indomethacin group by 118.1% (p<0.001) and 36.7% (p<0.001) was observed against the intact control (Table 6). Therapeutic administration of KUD869 decreased IL-1β by 24.1% (p<0.05) and IL-12B p40 by 13.6% (p<0.05) levels respectively compared to the indomethacin group (Table 6). The content of INF-γ in rats serum with indomethacin-induced lesions was higher by 31.5% (p<0.01) versus intact rats. KUD869 decreased INF-γ level by 14.5% (p<0.05) (Table 6). Indomethacin treatment was also accompanied with rising of the serum TNF-α content by 79.2% (p<0.05) compared to the intact group. KUD869 reduced the TNF-α content by 13.1% (p<0.05) as compared with the indomethacin group. In condition of indomethacin ulcer we registered the elevated content of anti-inflammatory cytokines IL-4 and IL-10 in rats serum by 26.2% (p<0.01) and 10.7% (p<0.05) accordingly compared with intact rats. That can be compensatory answer to increase of inflammation. Therapeutic administration of KUD869 decreased only the level of IL-4 by 8.5% (p<0.05). And there was no significant effect of small-molecule agent on the content of IL-10 and TGF-β compared to the indomethacin group. Thus, the obtained data suggest attenuation of inflammatory processes but little effect on anti-inflammatory defense under the KUD869 treatment of indomethacin-induced gastric ulceration.

Table 6: Proinflammatory cytokines in rats serum under conditions of KUD869 treatment of indomethacin-induced gastric ulceration

	Intact group	Indomethacin group	Indomethacin+ **KUD869** group
IL-1β, a.u.	0.160±0.023	0.349±0.021***	0.265 ±0.016*#
IL-12B p40, a.u.	0.215±0.014	0.294±0.012***	0.254±0.013*/#
TNF-α, a.u.	0.212±0.020	0.380±0.023***	0.330 ±0.014*#
INF-γ, a.u.	0.184±0.016	0.242±0.015**	0.207±0.012#

All values are expressed M±SEM, n=10 in each group. *, *** – p<0.05, p<0.001 compared to the intact group; # – p<0.05 compared to the indomethacin group.

Table 7: Anti-inflammatory cytokines in rats serum under conditions of KUD869 treatment of indomethacin-induced gastric ulceration

	Intact group	Indomethacin group	Indomethacin+ **KUD869** group
IL-4, a.u.	0.187±0.012	0.236±0.012**	0.216±0.008*#
IL-10, a.u.	0.242±0.011	0.268±0.011*	0.279±0.011*
TGF-β, a.u.	0.310±0.006	0.309±0.010	0.324±0.009

All values are expressed M±SEM, n=10 in each group. *, ** – p<0.05, p<0.01 compared to the intact group; # – p<0.05 compared to the indomethacin group.

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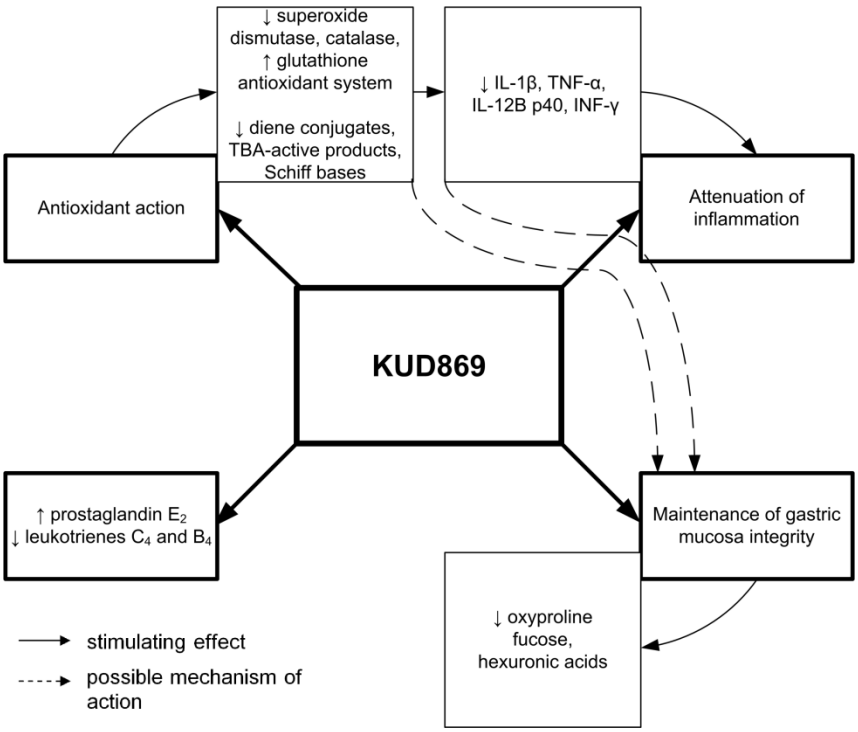
DISCUSSION

The reported study was designed to examine the drug effects of the novel water-soluble, small-molecule agent KUD869 on the indomethacin-induced gastropathy. The studied compound was obtained by rational structural modification of the earlier discovered proline-based antiulcerative agent [7]. We established the gastroprotective properties of KUD869, which decreases twice ulcerative lesions areas in animal model experiments at 1 mg/kg dose. The histological analysis of rats GM revealed ulcer depth diminishing under KUD869 influence. Antiulcer action of the tested compound is realized by multiple mechanisms (Figure 7).

It was established a significant increase of the intensity of lipid peroxidation in GM under indomethacin administration [23,24]. It is known that indomethacin breaks oxidative phosphorylation in mitochondria and inhibits the electron transport chain, resulting in formation of superoxide and hydroxyl radicals. These ROS oxidize and destroy the cell membrane lipids of GM that leads to cells apoptosis [25]. In our study a significant increase of LPO products (diene conjugates, TBA-active products and Schiff bases) was observed in GM in a day after indomethacin administration. Prominent antioxidative properties of KUD869 were demonstrated. It reduces the levels of rats GM DC, TBA-active products and SB that were elevated under the indomethacin-induced gastric ulceration. The level of neutral and basic ketone products of proteins oxidative modifications is also decreased by KUD869.

Figure 7: Mechanisms of gastroprotective effects of KUD869 under indomethacin-induced gastric ulceration in gastric mucosa of rats. KUD869 stimulated the antioxidant systems in gastric mucosa of rats that resulted in significant diminishing of lipid peroxidation product (diene conjugates, TBA-active products and Schiff bases) content. Scavenging of reactive oxygen species led to decrease in pro-inflammatory cytokine release and consequent attenuation of inflammation. In terms of KUD869 treatment it was observed reduced content of oxyproline, fucose and hexuronic acids which are the indicators of indomethacin-induced depolymerization of collagen and non-collagen protective proteins of mucin and connective tissue of gastric mucosa. One of the possible mechanisms of this effect is decrease of lipid peroxidation and attenuation of inflammation mentioned above. Also it was shown the raise of gastroprotective prostaglandin E_2 content and fall of leukotrienes B_4 and C_4 content in rats serum under treatment of indomethacin lesions with tested compound.



Although superoxide radical is increased under indometacin ulcer [26], studies have shown that SOD activity decrease in GM after indomethacin introduction [27-29]. One of the explanations of that is inhibition of the enzyme by excessive hydrogen peroxide [30,31]. In our work, despite of the high level of hydrogen peroxide in the two groups of rats treated with indomethacin SOD activity was significant, suggesting a high

content of superoxide as SOD substrate. SOD activity was slightly reduced compared to the indomethacin group in conditions of KUD869 injection. The same effect of KUD869 was on the activity of catalase. Therefore, we can conclude the decrease of the antioxidant enzymes activation due to the attenuation of lipid peroxidation under the influence of the test substance.

Three days after the administration of indomethacin it was found the decreased glutathione levels compared to intact rats, which coincides with the data of other authors [23,29]. Reduction of glutathione is associated with its more intensive use of enzymes of glutathione antioxidant system (GP and GT) [32-34]. Under KUD869 administration glutathione level was restored compared to indomethacin+saline group. Also we observed normalization of GP activity in indomethacin+KUD869 group that suggests its favorable effect on glutathione antioxidant system. So faster scavenging of reactive oxygen species and lipid peroxidation products by tested compound creates favorable conditions for indometacin ulcers healing, and prevents the formation of new ones.

According to Yadav et al (2012) a key role in GM lesions caused by indomethacin is played by pro-inflammatory cytokine TNF-α [35]. It triggers a cascade of intracellular cytokine reactions leading to increased synthesis of cell adhesion proteins (selectins, ICAM-1, VCAM-1) [36]. This leads to an increase of neutrophil infiltration into the GM, followed by release of myeloperoxidase and free radicals, leading to oxidative stress and enhances mucosal injury [37,38]. In our experiments, it was shown that the test substance prevented of proinflammatory TNF-α, IL1, IL12 increase and decreased lipid peroxidation in the GM in conditions of indomethacin introduction. It was shown that ROS may increase TNF-α content in the alveolar macrophages of the mice under conditions of intoxication with silica that further enhances the oxidative stress [39]. So one of the possible mechanisms of inflammation reduce and fall of proinflammatory cytokines content is the antioxidant properties of KUD869.

The therapeutic administration of KUD869 prevented the degradation of collagen fibers and other protective proteins of mucous epithelial barrier that led to decreased concentrations of free oxyproline, fucose and hexuronic acids. The most significant drop was registered for the fucose level suggesting preferential protection of fucoproteins under KUD869 treatment.

It is known that one of the main mechanisms of the development of NSAIDs lesions in the GM is the inhibition of cyclooxygenase (COX), with a subsequent decrease in the content of prostaglandins (PG) [2]. PGE_2 has been shown to increase the secretion of mucus and bicarbonate in the GM, and the expression of proangiogenic factor VEGF, which helps to maintain mucosal homeostasis [1,40,41]. Yadav et al (2013) found that in the conditions of indomethacin-induced ulceration COX1 activity in mice GM was significantly reduced. Despite of the COX2 activity elevation PGE_2 level was reduced significantly [42]. In our study we also found a decrease in PGE_2 in rat serum and the ability to increase its content by KUD869. The mechanism of this effect remains unclear.

The results indicated that the test compound KUD 869 exhibit antiulcer action in conditions of indomethacin introduction. This effect was accompanied by a decrease in LPO products content, reduce of gastric mucosal barrier destruction, inflammation attenuation and normalization of the eicosanoids level.

CONCLUSION

Methods of gastrointestinal diseases healing may depend on etiology of damage events. Drugs modulating different biological targets provide valuable alternatives or additions to the existing therapeutics. Marked gastroprotective activity of proline derivative KUD869 and characterization of its influence on different organism systems provide basis for the development of novel chemotypes of antiulcer agents.

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