



## INFLUENCE OF GAMMA-BUTYROLACTONE DERIVATIVES WITH ANALGESIC PROPERTIES ON THE PROSTAGLANDIN E<sub>2</sub> LEVEL AND GASTRIC MUCOSA IN RODENTS

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Our recent studies indicate that some derivatives of gamma-butyrolactone (GBL) possess analgesic properties in rodent models of pain and inflammation. Despite this pronounced biological activity, the mechanism of action of these GBL still remains unclear. Searching for the plausible mechanism of their action we investigated whether they were (similarly to nonsteroidal anti-inflammatory drugs) capable of influencing the PGE<sub>2</sub> level in mice subjected experimentally to zymosan-induced peritonitis. The PGE<sub>2</sub> level was evaluated *in vitro* by means of the ELISA assay. We also investigated the influence of orally administered GBL on the rat gastric mucosa in *post mortem* studies. We have demonstrated that neither of the GBL derivatives influences the PGE<sub>2</sub> level in zymosan-induced inflammation of the peritoneal cavity in mice. The investigated compounds are also devoid of adverse effects within the stomach, typical of cyclooxygenase inhibitors, which also indirectly suggests that their analgesic and anti-inflammatory activities result from mechanisms other than cyclooxygenase inhibition.

**Key words:** gamma-butyrolactone, peritonitis, prostaglandin E<sub>2</sub>, gastric mucosa, enzyme-linked immunosorbent assay

**Abbreviations:** AA – arachidonic acid, ASA – acetylsalicylic acid, COX – cyclooxygenase, GBL – gamma-butyrolactone, GCS – glucocorticosteroids, NSAIDs – nonsteroidal antiinflammatory drugs, PGE<sub>2</sub> – prostaglandin E<sub>2</sub>, PGI<sub>2</sub> – prostaglandin I<sub>2</sub>

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## INTRODUCTION

Inflammation is an important defensive process in the living organisms. It involves many complex mechanisms that induce cellular and vascular responses and different mediators, including eicosanoids, histamine, free oxygen radicals and pro-inflammatory cytokines.

Inflammatory acute or chronic pain is one of the main features of inflammation (CELOTTI and LAUFER, 2001; GUAY et al., 2004; KHANAPURE et al., 2007; KULKARNI and SINGH, 2007; NAM et al., 2010; RAINSFORD, 2007). The inflammatory nociception results from the peripheral sensitization of the primary nociceptive neurons. It is caused by hyperalgesic mediators, such as arachidonic acid (AA) metabolites (e.g. prostaglandins) and catecholamines (GUAY et al., 2004; LIMA et al., 2010). The currently used pharmacotherapy of inflammatory pain comprises non-opioid analgesics, namely nonsteroidal anti-inflammatory drugs (NSAIDs) often used as first-line medications effective under a wide range of inflammatory and pain conditions (HAMZA and DIONNE, 2009; LIMA et al., 2010) and several groups of analgesic adjuvants, of which glucocorticosteroids (GCS) – potent anti-inflammatory agents – play a pivotal role in the therapy of inflammation. The most important limitation of the therapy using both NSAIDs and GCS is their ulcerogenic effect within the gastrointestinal tract (POLAĆ et al., 2011).

The pharmacological mechanism of analgesic and anti-inflammatory actions of NSAIDs is very well established. The inhibition of proinflammatory and hyperalgesic prostaglandin synthesis (mainly prostaglandin  $E_2$ ,  $PGE_2$ ) results in the attenuation of inflammatory symptoms and diminishes the peripheral sensitization of pain. A great body of evidence indicates that cyclooxygenase (COX; prostaglandin H synthase) is a bi-functional enzyme with cyclooxygenase and peroxidase properties, so drugs that inhibit its function are thought to regulate and control the oxidative/antioxidative balance and may promote antioxidant or pro-oxidant processes (KOPFF et al., 2007). Free oxygen radicals, highly reactive forms of partially reduced oxygen, are capable of attacking various cellular components impairing their stability and proper functions. Peroxidation of lipids disrupts their fluidity and may cause excessive cell membrane permeability, and oxidative stress plays an

important role in the pathogenesis and progression of many diseases, including chronic pain and inflammation (SALVEMINI et al., 2011).

Our recent studies indicate that some derivatives of dihydro-furan-2-one, i.e. gamma-butyrolactone (GBL), possess significant analgesic properties in rodent models of pain and inflammation (SALAT et al., 2009; SALAT et al., 2010a; SALAT et al., 2010b; SALAT et al., 2012a; SALAT et al., 2012b; WIECKOWSKI et al., 2012). Despite this pronounced biological activity, the mechanism of action of these GBL remains unclear. Some hypotheses are taken into consideration: the influence on voltage-gated ion channels (anticonvulsant activity in electrical models of seizures and local anesthetic properties in mice and guinea pigs) (SALAT et al., 2009; SALAT et al., 2012a), anti-inflammatory activity (SALAT et al., 2012b) and antioxidant properties (SALAT et al., 2012a; and our unpublished data).

It is well known that the anti-inflammatory, antipyretic and analgesic activities of NSAIDs result from the inhibition of COX. This, in turn, reduces the tissue level of AA metabolites, such as  $PGE_2$ . Unfortunately, the same effect underlies their irritating activity on the gastric mucosa. Searching for the plausible mechanism of action of GBL derivatives we investigated whether they were (similarly to NSAIDs) capable of influencing the  $PGE_2$  level in mice subjected to experimental model of zymosan-induced peritonitis. We also investigated the influence of GBL on the rat gastric mucosa in *post mortem* studies.

## MATERIALS AND METHODS

### Drugs

Two GBL derivatives were investigated: LPP and LPP1. They were synthesized at the Department of Physicochemical Drug Analysis, Chair of Pharmaceutical Chemistry, Jagiellonian University, Medical College in Cracow. Their synthesis was described earlier (SALAT et al., 2009). For the experiment evaluating their influence on the  $PGE_2$  level they were suspended in a 0.5% methylcellulose solution (Loba Chemie, Germany) and administered at a dose of 30 mg/kg by the intraperitoneal route 30 min before the induction of peritoneal inflammation by means of zymosan. The reference compound – acetylsalicylic acid (ASA; Polfa Ku-

tro, Poland) was administered orally at a dose of 100 mg/kg 1 h before zymosan (Sigma Aldrich, Poland). Ketoprofen, used as a reference to investigate the ulcerogenic activity of GBL derivatives, was purchased from Sigma Aldrich (Poland).

#### Animals

Male albino Wistar rats (200-250 g) and male albino Swiss (CD-1) mice (18 - 26 g) were used in the tests. The animals were housed and fed in a laboratory kept at a constant temperature of 22°C under standard conditions (12:12 h light-dark cycle, standard pellet diet, tap water). The experimental groups consisted of 6-8 animals/dose and each animal was used only once. The treatment of laboratory animals was in full accordance with the respective Polish and European regulations and was approved by the Local Ethics Committee of the Jagiellonian University in Cracow (ZI/329/2006).

#### Influence on the prostaglandin E<sub>2</sub> level using the ELISA assay

The PGE<sub>2</sub> level was determined by the enzyme-linked immunosorbent assay (ELISA) in mice. The PGE<sub>2</sub> level was evaluated in control animals that received only zymosan and in the test group (the group that received either LPP or LPP1 or ASA which was the reference compound). The ability of the compounds to decrease the PGE<sub>2</sub> level was evaluated in the fluid isolated from the peritoneal cavity of mice. The experiment was divided into two steps: induction of acute inflammation (phase I) and *in vitro* evaluation of the PGE<sub>2</sub> level (phase II).

#### *Phase I: Triggering of acute inflammation in the mouse peritoneal cavity*

To induce acute peritonitis, the animals were treated intraperitoneally with 1 mg of zymosan suspended in 1 ml of sterile phosphate buffer (PBS). After 4 h the mice were sacrificed. The peritoneal cavity was washed with 1 ml of PBS. The fluid from the peritoneal cavity was centrifuged at 800xg for 5 min. After that time the supernatant was collected and frozen by storing at -80 ° C until the determination of PGE<sub>2</sub>.

#### *Phase II: In vitro evaluation of the PGE<sub>2</sub> level*

The ELISA method is based on the competition between PGE<sub>2</sub> and acetylcholinesterase conjugate of PGE<sub>2</sub> (PGE<sub>2</sub>-tracer, = PGET) for a limited amount of monoclonal antibodies to PGE<sub>2</sub>. As the concentration of PGET is fixed and the concentration of PGE<sub>2</sub> is variable, the PGET level which can bind to the PGE<sub>2</sub> antibody is inversely proportional to the concentration of PGE<sub>2</sub> in the sample. The antibody - PGE<sub>2</sub> complex binds to immunoglobulin G present in the plate of the ELISA kit. Then the plate is washed and the Ellman reagent containing the substrate for acetylcholinesterase is added. The product of the enzymatic reaction is distinctly yellow and absorbs light strongly at 412 nm. The intensity of the color, as measured spectrophotometrically, is proportional to the amount of bound PGET, and inversely proportional to the amount of free PGE<sub>2</sub>.

#### *Preparation of the standard curve*

The ELISA kit (Cayman Chemicals, USA) was applied according to the manufacturer's instructions. The absorbance of the plate was read at 412 nm. To read the results, at the right time the absorbance of B0 sample was checked periodically. The proper reading of the plate was made when B0 absorbance reached 0.3 AU. The standard curve is a plot of % B/B0 values (Y axis) against the concentration of PGE<sub>2</sub> (in pg/ml) in the samples S1-S8 (X-axis).

#### *Evaluation of the irritant action on the rat gastric mucosa*

The ulcerogenic effect was determined according to the method described by KOMATSU et al. (1973). Twenty-four hours after oral administration of the compounds, the rats were sacrificed and the stomach was removed. After incision along the lesser curvature, the stomach was rinsed with a tap soaked in warm (37°C) saline, spread on a cork board and pinned down. The mucosa of the glandular part of the stomach was inspected using a binocular microscope (10-fold magnification).

### Data analysis results

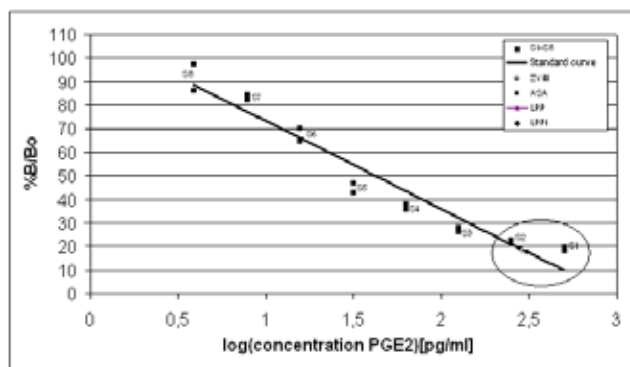
In the ELISA test the absorbance values obtained for the standards S1-S8 and the samples containing the investigated GBL derivatives, zymosan or ASA were converted by the computer program into PGE<sub>2</sub> concentration values.

The mucosal lesions were evaluated using a five-point scale (0 – no lesions, 1 – erythema, 2 – punctiform ulcers, 3 – small ulcers, 4 – large ulcers, 5 – perforation).

## RESULTS

### *Influence of LPP, LPP1 and ASA on the PGE<sub>2</sub> level determined by ELISA*

For each sample (S1-S8 and the test samples) the % B/B<sub>0</sub> (expressed as the percentage binding of the sample or the standard per maximal binding) were calculated. On the basis of these values obtained for standards (S1-S8), the standard curve for the dependence between % B/B<sub>0</sub> (Y axis) and the concentration of PGE<sub>2</sub> (in pg/ml) in S1-S8 (X-axis) was plotted using Matlab software (ver. 5) (Fig. 1A, 1B).



**Fig. 1A.** PGE<sub>2</sub> concentration (in pg/ml) in the peritoneal fluid of mice subjected to zymosan-induced inflammation.

The equation for this curve is:

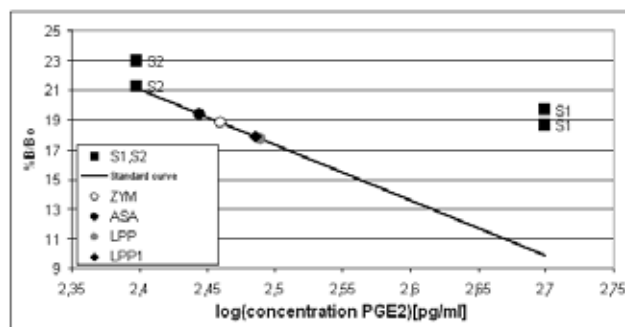
$$y = a + b$$

Where:

$$a = -37.414$$

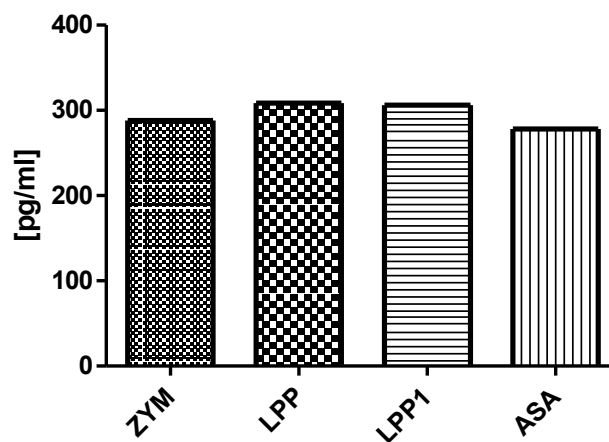
$$b = 110.859$$

From this curve, the % B/B<sub>0</sub> values were read for the test samples (Fig. 1A, 1B). Average concen-



**Fig. 1B.** PGE<sub>2</sub> concentration (in pg/ml) in the peritoneal fluid of mice subjected to zymosan-induced inflammation.

trations of PGE<sub>2</sub> in the tested samples are shown in Fig. 2.



**Fig. 2.** Mean PGE<sub>2</sub> concentrations in the peritoneal cavity fluid.

As demonstrated above, the tested compounds did not decrease the PGE<sub>2</sub> level in zymosan-induced inflammation.

### Irritant action on the gastric mucosa

The influence of the investigated LPP, LPP1, ASA and ketoprofen on the stomach mucosa is demonstrated in Table 1 and Fig. 3. In contrast to ASA (at doses of 100 and 200 mg/kg) and ketoprofen (50, 100 and 200 mg/kg), which caused erythema, the GBL derivatives did not damage the stomach mucous membrane at any dose tested. The investigated compounds did not possess ulcerogenic activity and they did not cause microbleeding from the stomach mucous membrane. ASA at a

**TABLE 1.** Influence of the compounds on the rat gastric mucosa.

Compound	Dose [mg/kg]	Mucosal lesions (point scale)				
		Erythrema (1)	Punctiform ulcers (2)	Small ulcers (3)	Large ulcers (4)	Perforation (5)
LPP	50	-	-	-	-	-
	100	-	-	-	-	-
	200	-	-	-	-	-
LPP1	50	-	-	-	-	-
	100	-	-	-	-	-
	200	-	-	-	-	-
ASA	50	-	-	-	-	-
	100	x	-	-	-	-
	200	x	-	-	-	-
Ketoprofen	50	x	-	-	-	-
	100	x	x	x	-	-
	200	x	x	x	x	x

The mucosa of the glandular part of the rat stomach was inspected using a binocular microscope (10-fold magnification). The mucosal lesions were evaluated using the 0-5 scale.

(- = no lesions; x = presence of the effect).

Route of administration: oral

dose of 50 mg/kg did not show ulcerogenic activity but higher doses of it caused visible erythema of the mucous membrane of the rat stomach (1 point on the scale). All doses of ketoprofen strongly damaged the gastric mucosa (1-5 points on the scale for 50, 100 and 200 mg/kg, respectively).

## DISCUSSION

The main aim of the present research was to investigate the mechanism of action of two GBL derivatives with strong analgesic and anti-inflammatory activities in rodents. Since among known analgesic drugs NSAIDs have a similar profile of pharmacological activity, we first evaluated whether LPP and LPP1 were capable of reducing the tissue concentration of pro-inflammatory arachidonic acid derivative formed by COX, namely PGE<sub>2</sub>. NSAIDs act by inhibiting the activity of the COX enzyme. Two isoforms of COX exist. COX-1 is constitutively expressed, whereas COX-2 is an inducible isoform. The demonstration that the test compounds lower PGE<sub>2</sub> concentration in the fluid from the peritoneal cavity would indirectly confirm that GBL derivatives inhibit the enzyme involved in the synthesis of this autacoid, namely COX.

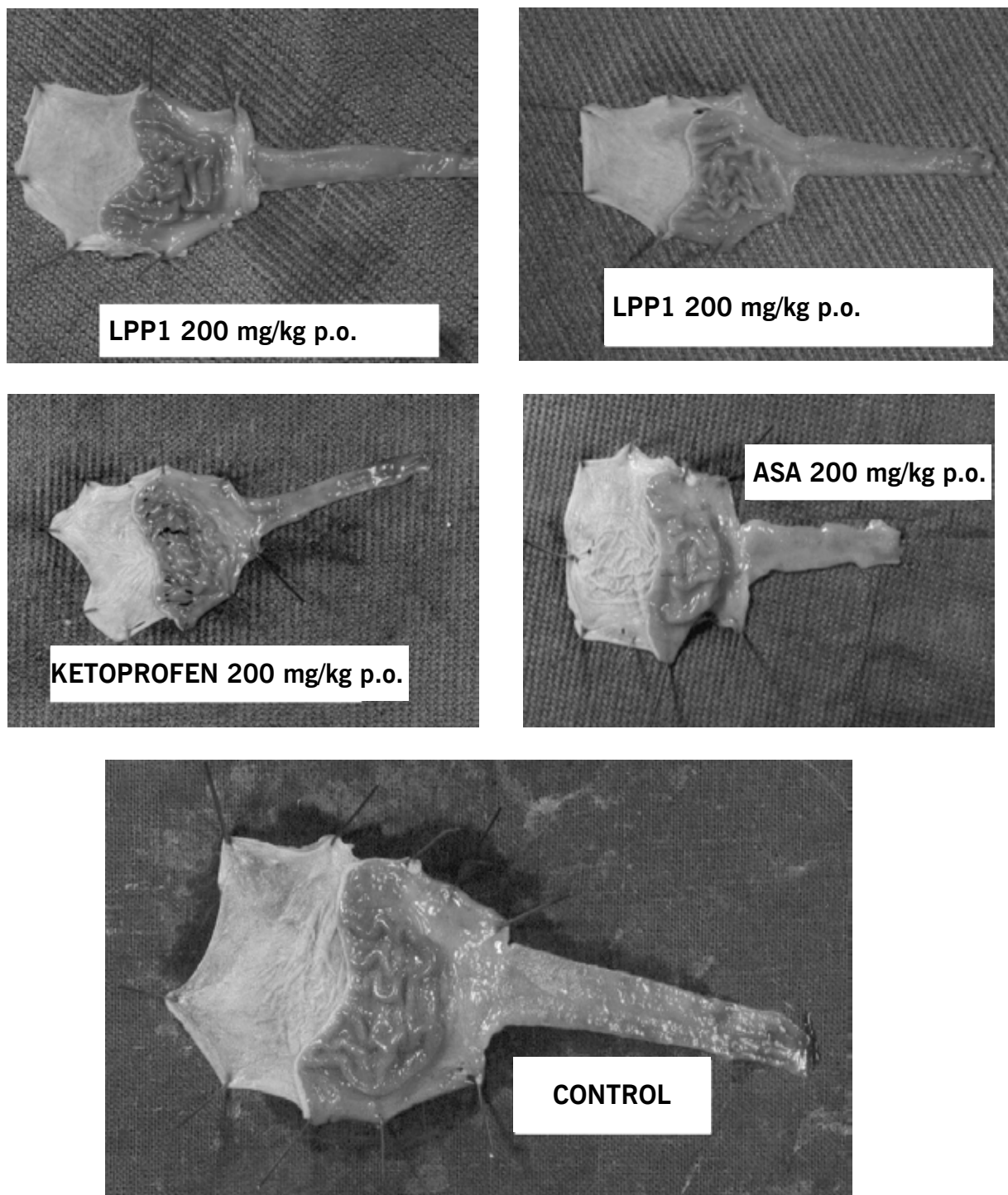
PGE<sub>2</sub>, as a product of metabolism of AA, is present in many cells. Like most eicosanoids it is not stored but it is formed *de novo* during the inflammatory activation of cells and then released into the extracellular space. Apart from strong gastroprotective properties (HOSHINO et al., 2003), PGE<sub>2</sub> is also implicated in pain sensitization and it has potent pro-inflammatory properties (WILLIAMS, 1979). *In vivo* PGE<sub>2</sub> is rapidly metabolized to an inactive derivative (13,14-dihydro-15-keto PGE<sub>2</sub>) and its biological half-life in the systemic circulation is about 30 s. Noteworthy, its normal level in mammalian tissues ranges between 3-12 pg/ml.

In order to evaluate the influence of GBL derivatives on the PGE<sub>2</sub> level, we selected two compounds which possessed potent analgesic properties in rodent models of inflammatory pain. In our recent studies (SALAT et al., 2009; SALAT et al., 2012a; SALAT et al. 2012b) we showed that LPP and LPP1 diminished nocifensive reactions under inflammatory conditions and their ED<sub>50</sub> values established in the writhing test (i.e. inflammatory pain model) were 2.4 mg/kg and 0.79 mg/kg, respectively. Those values were lower than the ED<sub>50</sub> value for the reference compound, ASA (39.15 mg/kg) (SALAT et al., 2009). Despite this activity profile,

the immunoenzymatic ELISA assay did not confirm the influence of two GBL derivatives on the  $\text{PGE}_2$  level in our study. Therefore we may conclude that the mechanism of the analgesic activity of

LPP and LPP1 does not result from the inhibition of the COX enzyme.

Prostaglandins synthesized by the constitutively expressed COX-1 are implicated in the mainte-



**Fig. 3.** The influence of GBL derivatives and reference drugs (200 mg/kg; *per os*) on the rat gastric mucosa.

nance of normal physiological functions and show a cytoprotective action in the stomach. COX-2 expression is normally low but it is induced by inflammatory stimuli and cytokines. It is thought that the anti-inflammatory actions of NSAIDs are caused by the inhibition of COX-2, whereas the unwanted side effects, such as gastrointestinal and renal toxicities, are caused by the inhibition of the constitutively expressed COX-1. Since the integral action of NSAIDs (and GCs) is their ulcerogenic adverse effect due to the reduced level of gastroprotective mediators, such as PGE<sub>2</sub>, we also evaluated *post mortem* the irritating effect of LPP, LPP1, ASA and ketoprofen on the rat gastric mucosa 24 h after the administration of the test compounds. Only ASA and ketoprofen damaged the gastric mucosa, whereas GBL derivatives did not. This finding can be linked to the influence or lack of influence of these substances on the synthesis of PGE<sub>2</sub>.

It remains to be elucidated why ASA only weakly decreased the PGE<sub>2</sub> level as measured by the ELISA assay. Several explanations should be considered, including very high levels of PGE<sub>2</sub> in the zymosan model of inflammation and relatively low anti-inflammatory activity of ASA under these very severe inflammatory conditions. The dose of 100 mg/kg could be insufficient to inhibit COX activity and decrease PGE<sub>2</sub> generation, but it was high enough to exert an undesirable effect within the rat gastric mucosa. Available literature also suggests a possible involvement of metabolites other than PGE<sub>2</sub> metabolites of AA (e.g. PGI<sub>2</sub>) in zymosan-induced inflammation models (BERKENKOPF and WEICHMAN, 1988; DOHERTY et al., 1990; YUHKI et al., 2008). It has to be emphasized that the level of PGI<sub>2</sub> was not determined in the present study.

The fact that GBL derivatives do not decrease the PGE<sub>2</sub> level is also in agreement with the observation that these compounds do not irritate the gastric mucosa. Neither LPP1 nor LPP showed irritancy on a five-point scale, while ASA was moderately irritating (erythema at doses of 100 and 200 mg/kg) and ketoprofen showed a strong irritating effect within the gastric mucosa at all three doses tested. Various NSAIDs show different selectivity against the COX-1 and COX-2 isoforms. NSAIDs that are relatively selective towards COX-2, such as meloxicam, may have an improved side-effect profile over non-selective NSAIDs. The potency of anti-inflammatory activity of ketoprofen is several-

fold higher as compared with ASA. Unfortunately, the irritating effect of ketoprofen on the stomach mucosa is also more pronounced.

Concluding, in the present study we have demonstrated that neither of the tested GBL derivatives reduces the PGE<sub>2</sub> level in zymosan-induced peritonitis in mice. These compounds are also devoid of adverse effects within the stomach, that are typical of COX inhibitors, which confirms that their analgesic and anti-inflammatory activities result from other mechanisms of action. Further studies are necessary to investigate this issue more thoroughly.

#### ACKNOWLEDGMENTS

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