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## Effects of Aspirin and Indomethacin on Galectin-3\*

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Galectin-3, a  $\beta$ -galactoside binding lectin, acts as a strong pro-inflammatory signal. Many immunomodulatory drugs affect signaling pathways that comprise transcription factors involved in regulation of galectin-3 gene (*LGALS3*) expression. The aim of this study was to investigate the effects of non-steroidal anti-inflammatory drugs aspirin and indomethacin on the expression of galectin-3, both on mRNA and protein levels. The human monocytic cell line THP-1 was exposed to various therapeutic concentrations of aspirin and indomethacin for 72 hours.

**Keywords** aspirin, galectin-3, indomethacin, *LGALS3*. Relative RT-PCR method and the GeneScan analysis software were used for assessing the galectin-3 mRNA level and chemiluminescent-western blot analysis was used for measuring the galectin-3 level. The results showed that galectin-3 is a new target molecule of NSAIDs, which cause reduction of both gene and protein expression of galectin-3, but the intensity and time-course of the changes strongly depend on the kind and concentration of the drugs.

### INTRODUCTION

Galectin-3 is a  $\approx 32$  kDa  $\beta$ -galactoside-binding lectin found widely in epithelial,<sup>1</sup> immune<sup>2</sup> and tumor cells.<sup>3</sup> Galectin-3 can be found in the cytoplasm, nucleus, as well as on the cell surface. Inside the cell, galectin-3 can act as a factor in pre-mRNA splicing<sup>4</sup> and as a regulator of the cell cycle<sup>5</sup> and apoptosis.<sup>6</sup> However, the precise mechanisms of these actions are still elusive. Galectin-3, secreted in the extracellular space by an unknown mechanism, can bind numerous cell-surface or extracellular matrix ligands and consequently mediate important biological processes such as cell-cell and cell-matrix adhesion<sup>7</sup> and cell signaling.<sup>2</sup> Upon the binding to the specific structures of surface glycoconjugates, galectin-3 molecules are able to oligomerize through their N-terminal domains and cause cross-

linking of their surface ligands.<sup>8</sup> It has been suggested that through such cross-linking galectin-3 initiates signal transduction cascades (involved in several immune responses), thereby triggering i) an oxidative burst in monocytes and neutrophils;<sup>9,10</sup> ii) augmentation of LPS-induced release of IL-1 in monocytes;<sup>11</sup> iii) initiation of degranulation in mast cells;<sup>12</sup> iv) chemoattraction of monocytes and macrophages;<sup>13</sup> v) induction of leukocyte recruitment;<sup>14</sup> vi) selective down-regulation of IL-5 expression;<sup>15</sup> vii) modulation of lymphocyte T activation.<sup>16</sup>

Galectin-3 is coded by a single gene (*LGALS3*). The promoter region contains five Sp1 binding sites (GC boxes), which is a common feature of housekeeping genes. However, *LGALS3* is considered to be an immediate early gene due to the presence of binding sites for several other

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transcription factors, such as AP-1, AP-4, NF- $\kappa$ B and CREB.<sup>17</sup> Galectin-3 expression seems to be affected by various stimuli,<sup>18,19</sup> but the signaling pathways ruling these reactions have not been well studied.

The immune response can be modulated by the action of non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs, along with their analgesic, anti-pyretic and anti-inflammatory uses, are now being also considered for prevention of cardiovascular diseases, cancer and treatment of human immunodeficiency virus.<sup>20,21</sup> Although many of the NSAIDs' pharmacological actions are related to their ability to inhibit prostaglandin biosynthesis, some of their beneficial therapeutic effects are not completely understood. These effects are mediated through inhibition of certain transcription factors (such as NF- $\kappa$ B and AP-1) and modulation of the transcriptional activation.<sup>22,23</sup> Differences in the cyclooxygenase-independent mechanism may result in a specific use of these drugs in individual therapy, because additional effects may either enhance the efficacy or reduce the toxicity of particular compounds. Since NSAIDs may affect transcription factors NF- $\kappa$ B and AP-1, which were shown to be involved in the regulation of galectin-3, the important factor in many immune reactions, in this study we investigated the effects of two most frequently used NSAIDs (aspirin and indomethacin), applied in different therapeutic ranges, on the expression of *LGALS3* and galectin-3 in the monocytic cell line THP-1.

## EXPERIMENTAL

### Materials

All chemicals were of analytical grade and, if not stated otherwise, were purchased from Sigma (St. Louis, MO, USA). Indomethacin was obtained from Fluka (St. Louis, MO, USA). Aspirin and indomethacin were initially dissolved in DMSO and subsequently diluted with culture medium to the final concentration prior to use.

Immobilon PVDF-membranes were purchased from Millipore Corp. (Bedford, MA, USA), CellTiter 96<sup>®</sup>AQ<sub>aqueous</sub> One Solution Cell Proliferation Assay from Promega (Madison, WI, USA), ECL Western blot detection kit from Amersham Biosciences (Cardiff, UK).

Murine anti- $\beta$ -actin antibodies and HRP-labeled goat anti-mouse IgM antibodies were from Oncogene Research Products (Darmstadt, Germany), hybridoma cell line TIB-166 that produces rat monoclonal anti-human-galectin-3 antibodies (M3/38) was from ATCC (Manassas, VA, USA) while HRP-labeled goat anti-rat IgG antibodies were from Sigma (St. Louis, MO, USA).

### Cell Culture

Human acute monocytic leukemia suspension cell line THP-1 was obtained from ATCC (Manassas, VA, USA). Cells were asynchronously grown (37 °C, 5 % CO<sub>2</sub>, relative humidity

95 %) in RPMI 1640 medium supplemented with fetal bovine serum (10 %), penicillin G (100 U/mL,  $\approx$ 60  $\mu$ g/mL), streptomycin-sulphate (100  $\mu$ g/mL), amphotericin B (250 ng/mL) and plasmocin (2.5  $\mu$ g/mL) (all purchased from Gibco, Grand Island, NY, USA) and subcultured every 2–3 days to maintain a cell concentration between  $1 \times 10^5$  and  $1 \times 10^6$  cells/mL. Viable cells and the cell count were assessed with trypan blue (0.04 %) exclusion dye using a hemocytometer.

### Treatment of THP-1 Cells with Aspirin and Indomethacin

Aspirin (Sigma) and indomethacin (Fluka) were initially dissolved in dimethyl-sulphoxide (DMSO) and subsequently added to the culture medium to the final concentration (0.1, 0.5, 1 and 2 mmol dm<sup>-3</sup> aspirin; 0.1, 1, 5 and 10  $\mu$ mol dm<sup>-3</sup> indomethacin; DMSO  $\leq$  0.2 %) prior to use. After addition of the medium containing test compounds, cells ( $2 \times 10^5$ /mL) were cultivated in 175 mm<sup>2</sup> tissue culture flasks (Nunclon, Roskilde, Denmark) for the next 1, 3, 5, 24, 48 and 72 hours.

The cells cultivated in 0.2 % DMSO during the same period of time served as controls.

### Cell Viability MTS Test

A cell viability and potential cytotoxic effect of test compounds was investigated using the CellTiter 96<sup>®</sup>AQ<sub>aqueous</sub> One Solution Cell Proliferation Assay. Fifty microliters of the cell culture ( $1 \times 10^6$  cells/mL) or fifty microliters of RPMI 1640 medium were transferred into the wells of a 96-microwell plate. The test compounds were serially diluted and 50  $\mu$ L of 2-fold dilution was added into each well to obtain the final concentration of aspirin of 0.1, 0.5, 1 and 2 mmol dm<sup>-3</sup>, indomethacin 0.1, 1, 5 and 10  $\mu$ mol dm<sup>-3</sup> and DMSO 0.2 %. The controls contained test model cells, culture medium but no test compounds. The cells were incubated for 3 days before 20  $\mu$ L MTS was added and were additionally incubated in the dark at 37 °C for the following 2 hours.<sup>24</sup> The absorbance was read at the wavelength of 490 nm using the microplate reader Victor<sup>2</sup>-1420 Multilabel Counter (Perkin-Elmer, Wellesley, MA, USA).

### Sample Collection

After the pre-determined incubation time, aliquots of the cell culture containing  $4 \times 10^6$  cells (for protein analysis) and  $1 \times 10^6$  cells (for RNA analysis) were centrifuged at 600 g for 10 min. The cell pellet ( $4 \times 10^6$  cells) was washed twice with ice-cold PBS, resuspended in 0.15 mL lysis buffer (1 % Triton X-100 in 5 mmol dm<sup>-3</sup> Tris-HCl, pH = 8.0, 15 mmol dm<sup>-3</sup> NaCl, 2 mmol dm<sup>-3</sup> PMSF) and additionally homogenized by sonication with an ultrasound disintegrator (MSE, 100 W, London, England) for 2 min (amplitude 5  $\mu$ m,  $\nu$  = 22 kHz).

Protein concentrations in cell homogenates were determined by the method of BCA.<sup>25</sup> Total RNA was extracted from  $1 \times 10^6$  cells as described afterwards.

### Western-blot Analysis

SDS-polyacrylamide gel electrophoresis was performed in 12 % polyacrylamide gels according to Laemmli.<sup>26</sup> Separated proteins were transferred onto PVDF-membrane using the semi-dry transfer technique. The part of the membrane containing proteins smaller than  $\approx 40$  kDa was incubated overnight with rat M3/38 anti-galectin-3 antibodies (culture supernatant from hybridoma cell line TIB 166) diluted 1:200 in TBS. The part of the membrane containing proteins bigger than  $\approx 40$  kDa was incubated overnight with murine anti- $\beta$ -actin antibodies diluted 1:2500 in TBS. After one hour of incubation with HRP-labeled secondary antibodies (goat anti-rat IgG diluted 1:10000 in TBS or goat anti-mouse IgM diluted 1:10000 in TBS) galectin-3 and  $\beta$ -actin were visualized with the ECL Western blot detection kit (Amersham, Cardiff, UK). Quantification of the band intensity was performed using the Scion Image Analysis software, Microsoft®. The level of galectin-3 expression in each sample was normalized to the level of  $\beta$ -actin.

### RNA Isolation and RT-PCR

Total RNA was extracted from  $10^6$  cells with the RNeasy® Mini Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's protocol.

Using the 6000 Nano LabChip®Kit, analysis of RNA quality and measurement of RNA concentration were performed on an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), according to the manufacturer's instructions.

Total RNA ( $\approx 0.8$   $\mu\text{g}$ ) was reverse transcribed using oligo-dT primers ( $2.5$   $\mu\text{mol dm}^{-3}$ ) and murine leukemia virus (MuLV) reverse transcriptase ( $2.5$  U/ $\mu\text{L}$ ), in a final volume of  $20$   $\mu\text{l}$  containing  $1$   $\text{mmol dm}^{-3}$  dNTPs,  $5$   $\text{mmol dm}^{-3}$   $\text{MgCl}_2$  and PCR buffer ( $50$   $\text{mmol dm}^{-3}$  KCl,  $10$   $\text{mmol dm}^{-3}$  Tris-HCl, pH = 8.3) during 1-hour incubation at  $37$  °C. The samples were heated for  $5$  min at  $95$  °C and cooled down to  $4$  °C.

The PCR using Platinum *Taq* polymerase ( $0.5$  U;  $1$  U of *Taq* DNA polymerase is the amount of enzyme required to incorporate  $10$  nmol of deoxyribonucleotide into acid-precipitable material in  $30$  min at  $74$  °C in the specified reaction mixture) was performed in a final volume of  $10$   $\mu\text{l}$  containing  $2$   $\mu\text{l}$  of cDNA in a GeneAmp®PCR System 2700 (Applied Biosystems, Foster City, CA, USA). Other reaction constituents included  $\text{MgCl}_2$  ( $1.5$   $\text{mmol dm}^{-3}$ ), dNTPs ( $0.2$   $\text{mmol dm}^{-3}$ ), PCR buffer and 3' and 5' primers ( $0.5$   $\mu\text{mol dm}^{-3}$  each). The PCR was conducted in 21 cycles for galectin-3 and 14 cycles for  $\beta$ -actin under the following conditions: 1 min of denaturation at  $94$  °C, 1 min of annealing at  $58$  °C for galectin-3 or at  $63$  °C for  $\beta$ -actin and 1 min of extension at  $72$  °C, followed by the final extension for  $5$  min at  $72$  °C and cooling down to  $4$  °C. The following primer pairs were used: for galectin-3: TGCTGATAACAATTCTGGGC (sense) and TAACCCGATGATTGTAAGTGC (antisense), for  $\beta$ -actin: CATGTGCAAGGCCGCTTCG (sense) and GAAGGTGTGGTGCCAGATTT (antisense). Sense primers were labeled with ABI fluorescence dyes 6-FAM on the 5' end.

### Sequencing Analysis

To confirm that the PCR products were derived from targeted mRNA, the products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and the nucleotide sequences were analyzed with the DNA Sequencing Big Dye Terminator v3.0 kit on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions.

### GeneScan® Method

The same volume of RT-PCR amplicons for galectin-3 and  $\beta$ -actin ( $0.5$   $\mu\text{l}$  each) were pooled together in one tube. GS 500 TAMRA-labeled size marker ( $0.5$   $\mu\text{l}$ ) and formamide ( $11.5$   $\mu\text{l}$ ) were added to the same tube. RT-PCR amplicons were resolved by capillary electrophoresis, and fluorescence detection with a virtual A filter was performed on an ABI Prism 310 Genetic Analyzer. The runs were executed with the module GS STR POP 4 (1 ml) A with  $3$  s and  $15$  kV injection and run voltage,  $60$  °C constant temperature,  $24$  min running time, using polymer POP 4 and the running buffer Genetic Analyzer. GeneScan 2.1 software was then used to analyze the RT-PCR amplicons, with accurate sizing and quantification of the peak areas.

### Statistical Analysis

Results are shown as means  $\pm$  SD. Differences between various treatments were tested with unpaired Student's *t*-tests; *p* values  $< 0.05$  were considered significant.

## RESULTS

To investigate the effects of NSAIDs on the expression of galectin-3 on both the mRNA and protein levels, we exposed the monocytic cell line THP-1 to the various therapeutic concentrations of aspirin ( $0.1$ ,  $0.5$ ,  $1$  and  $2$   $\text{mmol dm}^{-3}$ ) and indomethacin ( $0.1$ ,  $1$ ,  $5$  and  $10$   $\mu\text{mol dm}^{-3}$ ). Measurements of cell viability and metabolic activity with trypan exclusion dye and the MTS test showed that none of the applied concentrations of aspirin or indomethacin during  $72$  hours of cultivation affected the cellular metabolism either in a cytotoxic or a proliferative manner. Cultivation of THP-1 cells in the presence of  $0.2$  % DMSO, which was the final DMSO concentration after dilution of the test compound dissolved in DMSO, did not change the metabolic status of cells either (data not shown).

The relative amount of mRNA for galectin-3 (*i.e.*, relative expression of *LGALS3*) was determined by the GeneScan Analysis of RT-PCR amplicons for galectin-3 and  $\beta$ -actin. The optimized method includes amplification of galectin-3 cDNA during 21 cycles of PCR and amplification of  $\beta$ -actin cDNA during 14 cycles of PCR. Subsequent quantification was feasible under these conditions because polymerase chain reactions were still in

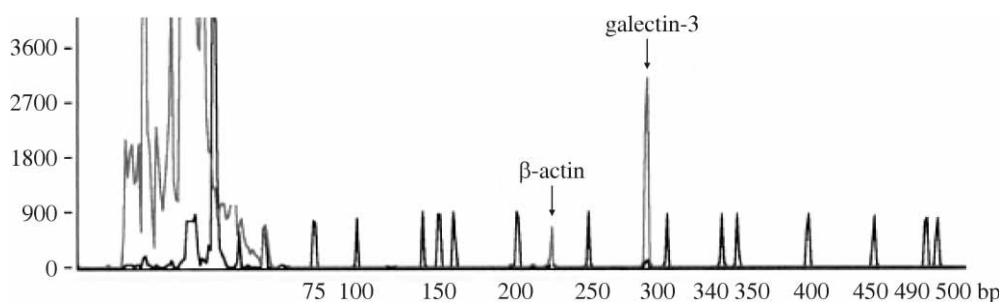


Figure 1. GeneScan® electrophoretogram display of RT-PCR amplicons. Total RNA was isolated from THP-1 cells. After reverse transcription using oligo-dT primers, 21 cycles of PCR were performed using fluorescent primers for galectin-3 and 14 cycles of PCR using fluorescent primers for  $\beta$ -actin. RT-PCR amplicons were pooled and separated by capillary electrophoresis on an AbiPrism 310 Genetic Analyzer. The y-axis is the relative intensity of the fluorescence and the x-axis represents the designate size of the fragments in the base pair (bp), as shown at the bottom of the electrophoretogram. Black peaks are GeneScan 500 TAMRA internal line size standards. Grey peaks are  $\beta$ -actin (226 bp) and galectin-3 (288 bp).

the exponential phase when the reactions were stopped (Figure 1). Sequencing analysis of the PCR products showed the high specificity and accuracy of the PCR reactions; the synthesized PCR products were the only products of the PCR reactions and their sequences corresponded entirely to the parts of galectin-3 or  $\beta$ -actin mRNA.

Cultivation of THP-1 cells for 72 hours did not affect the expression of *LGALS3* or the expression of the corresponding protein, galectin-3. The basal levels of the expression of both *LGALS3* and galectin-3 were also maintained during 72 hours of cultivation in 0.2 % DMSO (data not shown).

The lowest applied concentration of aspirin (0.1 mmol dm<sup>-3</sup>) did not affect the expression level of *LGALS3* during the first three hours of cultivation. A decrease was observed after 5 hours of cultivation, though it became statistically significant 24 hours after the beginning of the treatment. The level of mRNA continued to fall and it reached (31.1 ± 1.4) % of the initial value after 72 hours of cultivation (Figure 2). When THP-1 cells were exposed to medium (0.5 and 1 mmol dm<sup>-3</sup>) and high (2 mmol dm<sup>-3</sup>) therapeutic concentrations of ASA, the level of mRNA

for galectin-3 dropped sharply already after the first hour of cultivation (to (59.6 ± 7.6) %, (52.2 ± 1.7) % and (45 ± 3.4) % of the initial value, respectively, Figure 2). Prolonged cultivation caused a further decrease of the galectin-3 mRNA level in a dose dependent manner. At the end of the treatment (after 72 hours) the expression of *LGALS3* dropped to (25.6 ± 4.8) %, (15.5 ± 0.5) % and (12.3 ± 0.04) % of the expression observed in the control cells, respectively (Figure 2).

Expression of galectin-3 was measured by the chemiluminescent-western blot analysis. Incubation of the cells for 24 hours with aspirin in all applied concentrations did not affect the level of galectin-3 expression, except in the case of the highest ASA concentration applied (2 mmol dm<sup>-3</sup>), which caused a 20 % decrease of galectin-3 expression, compared to the control cells. The same intensity of decrease was observed when the cells were cultivated for 48 hours with 1 mmol dm<sup>-3</sup> ASA, while at the same time 2 mmol dm<sup>-3</sup> ASA caused a further decline of galectin-3 expression (to (71 ± 3) % of the initial value). The inhibitory effect of ASA on galectin-3 expression observed after 72 hours of cultivation correlates with the applied ASA concentration, varying

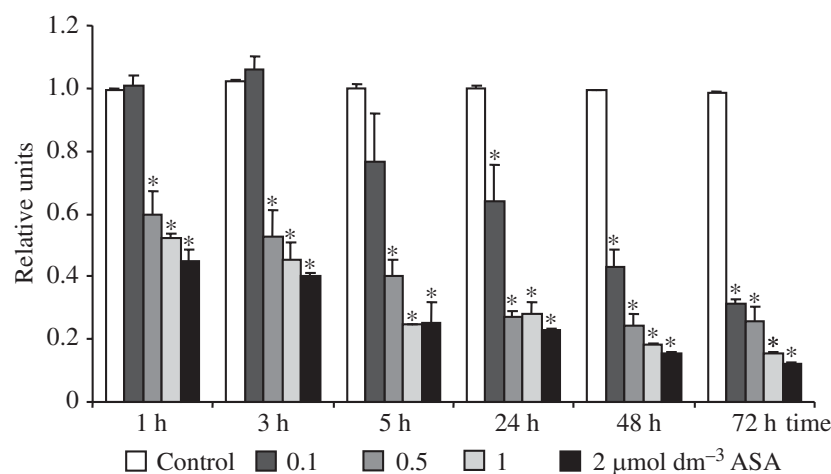
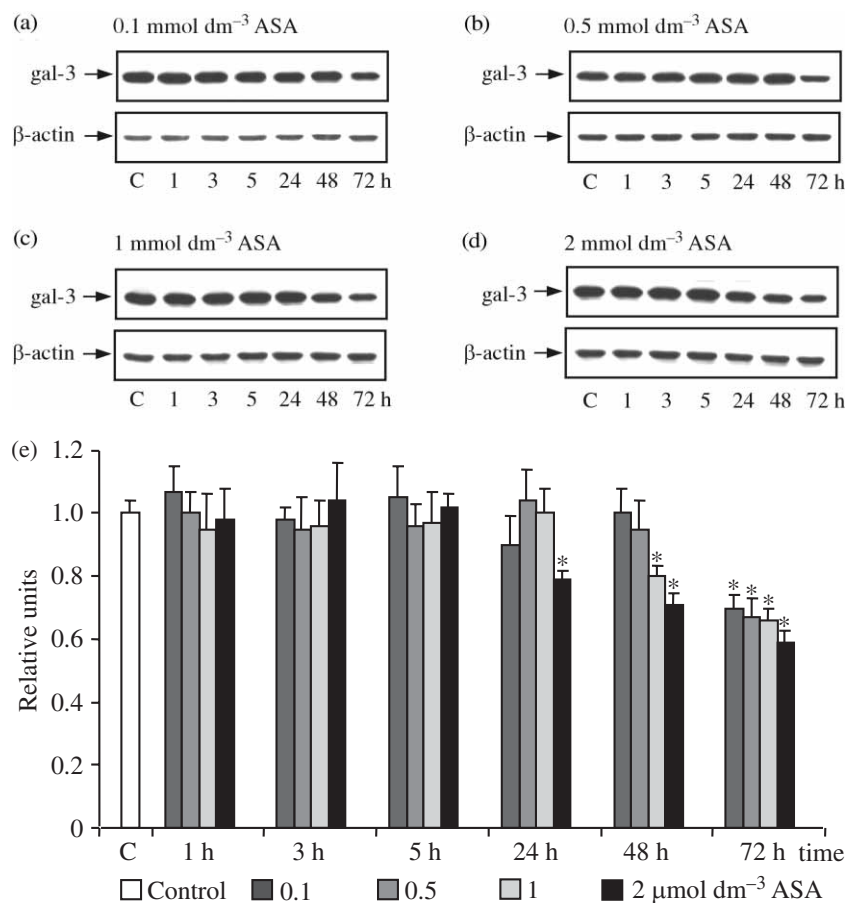


Figure 2. Aspirin inhibits expression of *LGALS3* in THP-1 cells. THP-1 cells were cultivated for 1, 3, 5, 24, 48 and 72 hours with 0.1, 0.5, 1 and 2 mmol dm<sup>-3</sup> aspirin. Following the total RNA isolation and reverse transcription, 21 cycles of PCR were performed using fluorescent primers for galectin-3 and 14 cycles of PCR using fluorescent primers for  $\beta$ -actin. RT-PCR amplicons were pooled and separated by capillary electrophoresis on an AbiPrism 310 Genetic Analyzer. Results of the two independent experiments performed in duplicate were calculated using the GeneScan 2.1 Analysis software and expressed as galectin-3/ $\beta$ -actin RT-PCR amplicon peak areas compared to the control ( $\bar{x} \pm SD$ ). Statistically significant differences ( $p < 0.05$  compared to the controls) are marked with asterisks.

Figure 3. Aspirin affects galectin-3 expression in THP-1 cells. Immunoblot analysis of galectin-3 in THP-1 cell homogenates cultivated for 1, 3, 5, 24, 48 and 72 hours in a) 0.1 mmol dm<sup>-3</sup> ASA, b) 0.5 mmol dm<sup>-3</sup> ASA, c) 1 mmol dm<sup>-3</sup> ASA, d) 2 mmol dm<sup>-3</sup> ASA (5 mg of proteins/well). e) Results of the densitometric analysis of the two independent experiments performed in duplicate are expressed as the relative level of galectin-3/ $\beta$ -actin compared to the control ( $\bar{x} \pm$  SD). Statistically significant differences ( $p < 0.05$  compared to the control) are marked with asterisks.



from approximately 30 % (0.1 mmol dm<sup>-3</sup> ASA) to 40 % of inhibition (2 mmol dm<sup>-3</sup> ASA) (Figure 3).

Expression of *LGALS3* was also attenuated during the cultivation of THP-1 cells in the presence of therapeutic doses of indomethacin, though the pattern of inhibition was somewhat different from the pattern observed when cells were treated with aspirin. All the applied indomethacin concentrations (0.1, 1, 5 and 10  $\mu$ mol dm<sup>-3</sup>) caused a decrease of galectin-3 mRNA level already after the first hour of cultivation (ranging from  $(66.7 \pm 4.2)$  to  $(34.7 \pm 1.8)$  % of the initial value, depending on the

applied concentration). The intensity of inhibition continued to increase with time, and it depended more on time than on the applied dose. After 72 hours of cultivation, the level of *LGALS3* expression ranged from 28 to 8 % of the initial value, depending on the applied indomethacin concentration (Figure 4).

The basal level of galectin-3 did not change significantly after the first 24 hours of cultivation of THP-1 cells with indomethacin (concentration range 0.1 to 10  $\mu$ mol dm<sup>-3</sup>). Although 48 hours of cultivation with 0.1  $\mu$ mol dm<sup>-3</sup> Ind caused a reduction of the galectin-3 level

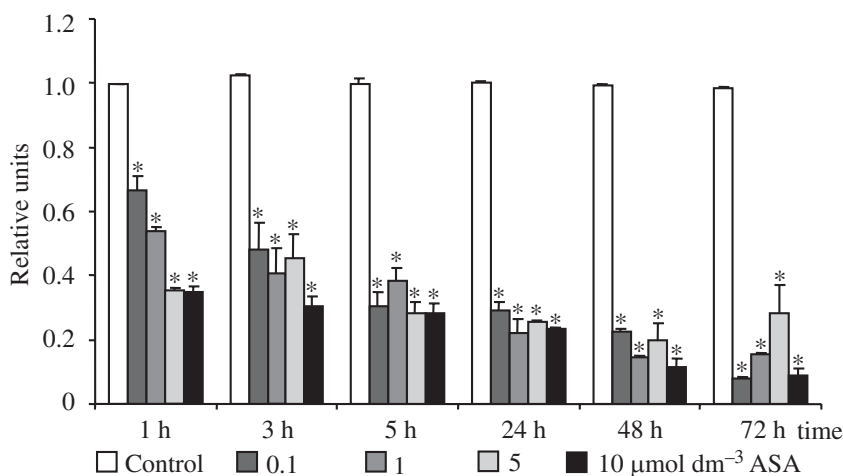
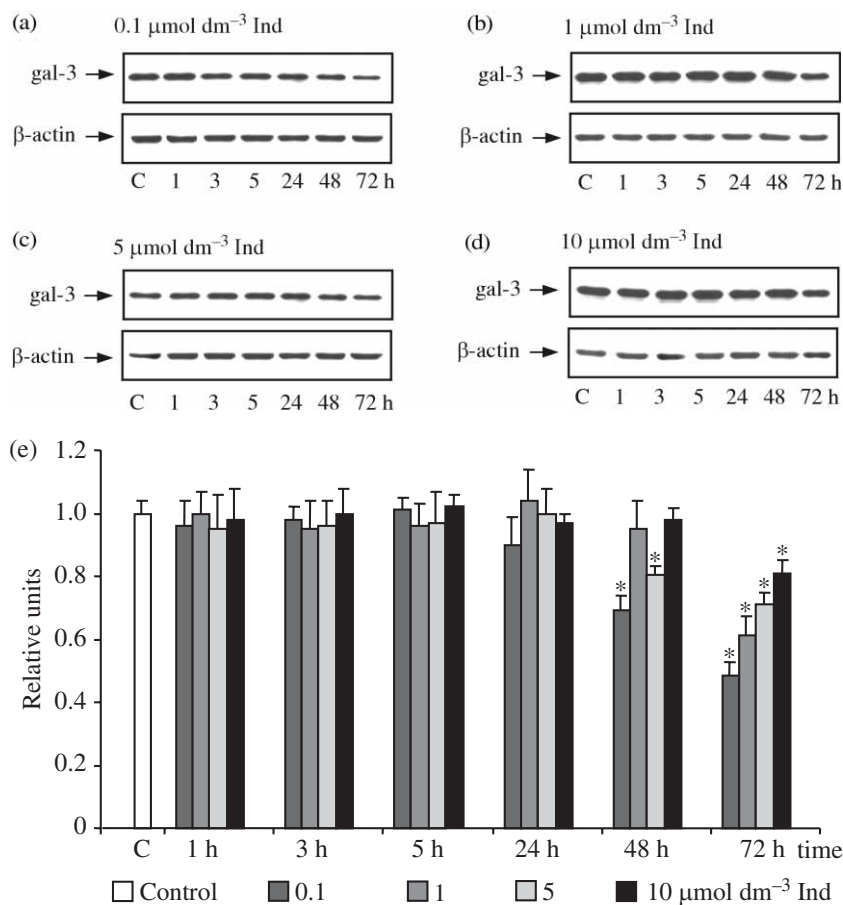


Figure 4. Indomethacin inhibits expression of *LGALS3* in THP-1 cells. THP-1 cells were cultivated for 1, 3, 5, 24, 48 and 72 hours with 0.1, 1, 5 and 10  $\mu$ mol dm<sup>-3</sup> indomethacin. Following the total RNA isolation and reverse transcription, 21 cycles of PCR were performed using fluorescent primers for galectin-3 and 14 cycles of PCR using fluorescent primers for  $\beta$ -actin. RT-PCR amplicons were pooled and separated by capillary electrophoresis on an AbiPrism 310 Genetic Analyzer. Results of the two independent experiments performed in duplicate were calculated using the GeneScan 2.1 Analysis software and expressed as galectin-3/ $\beta$ -actin RT-PCR amplicon peak areas compared to the control ( $\bar{x} \pm$  SD). Statistically significant differences ( $p < 0.05$  compared to the controls) are marked with asterisks.

Figure 5. Indomethacin affects galectin-3 expression in THP-1 cells. Immunoblot analysis of galectin-3 in THP-1 cell homogenates cultivated for 1, 3, 5, 24, 48 and 72 hours in a)  $0.1 \mu\text{mol dm}^{-3}$  Ind, b)  $1 \mu\text{mol dm}^{-3}$  Ind, c)  $5 \mu\text{mol dm}^{-3}$  Ind, d)  $10 \mu\text{mol dm}^{-3}$  Ind ( $5 \mu\text{g}$  of proteins/well). e) Results of the densitometric analysis of the two independent experiments performed in duplicate are expressed as the relative level of galectin-3/ $\beta$ -actin compared to the control ( $\bar{x} \pm \text{SD}$ ). Statistically significant differences ( $p < 0.05$  compared to the control) are marked with asterisks.



to  $(69.0 \pm 5.1) \%$ , and cultivation with  $5 \mu\text{mol dm}^{-3}$  Ind caused a reduction to  $(80.5 \pm 2.7) \%$  of the initial value,  $1 \mu\text{mol dm}^{-3}$  Ind and  $10 \mu\text{mol dm}^{-3}$  Ind did not affect the expression of the lectin. Surprisingly, after 72 h of incubation, the lower concentration of indomethacin had a stronger inhibitory effect on galectin-3 than higher doses – the inhibition varied from approximately 50 to 20 %, respectively (Figure 5).

## DISCUSSION

The importance of non-steroidal anti-inflammatory drugs in modern medicine and pharmacy has stimulated an impressive amount of scientific studies on the effects of NSAIDs on their cellular and molecular targets in the last few decades. Recent findings indicating that NSAIDs affect the activity of cellular transcription factors and modulate transcriptional activation prompted an enormous interest in gene expression as a new target of NSAIDs.

Galectin-3, a  $\beta$ -galactoside binding lectin, has recently emerged as a new powerful cellular modulator at inflammatory foci. Yet, until now there were no data about the effects of NSAIDs on the expression of either galectin-3 or any other lectin. In the presented study, we have demonstrated for the first time that non-steroidal anti-inflammatory drugs aspirin and indomethacin, applied in clinically relevant doses, effectively inhibited galectin-3 ex-

pression at the mRNA level, and to a lesser extent at its protein level in the monocytic cell line THP-1.

The promoter region of *LGALS3* gene, whose sequence was determined by Kadrofske *et al.*<sup>17</sup> in 1998, contains binding sites for several transcription factors. Previously, we showed that galectin-3 expression is influenced by various stimuli,<sup>18,19</sup> and that the involvement of transcription factors AP-1 and NF- $\kappa$ B depends on the type of the stimuli.<sup>27</sup> NSAIDs comprise a large class of diverse drugs, classified in two main groups – weak and strong NSAIDs. Mechanisms of action of these drugs, especially as far as their influence on the signal transduction pathway is concerned, are somewhat different. The observed time- and dose-dependent suppression of *LGALS3* expression caused by aspirin, the most frequently used weak NSAID, might be a consequence of aspirin's ability to inhibit either the transcription factor AP-1<sup>28</sup> or NF- $\kappa$ B<sup>23</sup>. According to the literature data, indomethacin, the most commonly applied strong NSAID, does not affect directly the activity of AP-1 or NF- $\kappa$ B, but activates the transcription factor PPAR $\gamma$ . The activation of PPAR $\gamma$  may, on the other hand, cause inhibition of NF- $\kappa$ B activity through the process of transrepression.<sup>29</sup> The other possibility is that the inhibitory effect of NSAIDs on *LGALS3* expression is mediated through some other molecules whose expression is affected by NSAIDs (such as TNF $\alpha$ ,

IL-4 and IL-8) through the complex network of signal transduction pathways.

Under the influence of NSAIDs, changes in galectin-3 expression did not follow the pattern of changes of the mRNA level. Discrepancy between the changes in mRNA and protein expression levels is not rare and was previously reported by many other authors.<sup>30–32</sup> As far as this study is concerned, they could be a result of an enhanced translation rate and/or reduced degradation rate (turnover). On the other hand, enhanced binding of previously secreted galectin-3 on its membrane receptors, whose expression might also be modulated as a result of NSAIDs application, could also be the cause of a sustained protein level.

Maintenance of an unchanged level of galectin-3 during the first 24 hours of exposure to NSAIDs compared to the strong decrease of mRNA expression observed in that period suggests the necessity and the importance of galectin-3 for a proper monocyte function. It seems that these cells require a particular amount of galectin-3 and somehow they succeed in preserving it despite the strong reduction of its mRNA. However, after the prolonged exposure to NSAIDs (48 and 72 hours), the galectin-3 level significantly decreased. The observed changes in *LGALS3* and galectin-3 expression should be considered in the sense of multiple roles of galectin-3 that were shown to be involved in transcription control, in pre-mRNA splicing and to be an anti-apoptotic factor. Additionally, galectin-3, secreted in extracellular space affects the physiology of many various cells. A prolonged therapy with NSAIDs is frequently used for the prevention of cardiovascular diseases and since recently as a supportive cancer treatment. Thus, the observed reduction of galectin-3 expression due to the prolonged NSAIDs' application can modulate numerous biological processes in which galectin-3 is involved. For instance, according to very recent data,<sup>33,34</sup> extracellular galectin-3 was shown to be one of the key molecules engaged in the control of apoptotic signaling pathways, so the changes in its expression can modulate apoptosis, an extremely important process especially for tumor and immune cells.

Taken together, the obtained data suggest that besides the numerous already known effects of NSAIDs therapy, the effects of NSAIDs on galectin-3 expression and the consequences that can be provoked should be inevitably considered in terms of a better understanding of the mechanisms of NSAIDs' action not only in monocytes but rather in all cells expressing galectin-3. In summary, the results obtained in this study have established galectin-3 as a new target molecule of NSAIDs and at the same time they represent a valuable contribution for the understanding of the effects of immunomodulatory drugs.

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## SAŽETAK

### Utjecaj aspirina i indometacina na galektin-3

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Galektin-3, lektin koji veže  $\beta$ -galaktozidne jedinice, djeluje kao jak proinflamatorni signal. Mnoge imunomodulatorne tvari utječu na signalne putove i transkripcijske faktore uključene i u regulaciju ekspresije gena za galektin-3 (*LGALS3*). Cilj je ovoga rada bio ispitati učinke nesteroidnih tvari s imunomodulatornim djelovanjem – aspirina i indometacina – na ekspresiju galektina-3, kako na razini mRNA tako i na razini proteina. Stanice humane stanične linije THP-1 izložene su različitim terapijskim koncentracijama aspirina i indometacina tijekom 72 sata. Količina mRNA za galektin-3 određena je primjenom metode RT-PCR i analize programom GeneScan, a razina galektina-3 kemiluminescentnom western-blot analizom. Rezultati su pokazali da galektin-3 predstavlja novu ciljnu molekulu djelovanja nesteroidnih imunomodulatornih tvari, koje uzrokuju smanjenje ekspresije gena za galektin-3 i samoga proteina, premda jakost promjena i vrijeme njihove pojave jako ovise o vrsti i koncentraciji primijenjene tvari.