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The Anti-inflammatory Effect of Selonsertib (GS-4997), an ASK1 Inhibitor, on LPS-Stimulated THP-1 Cells

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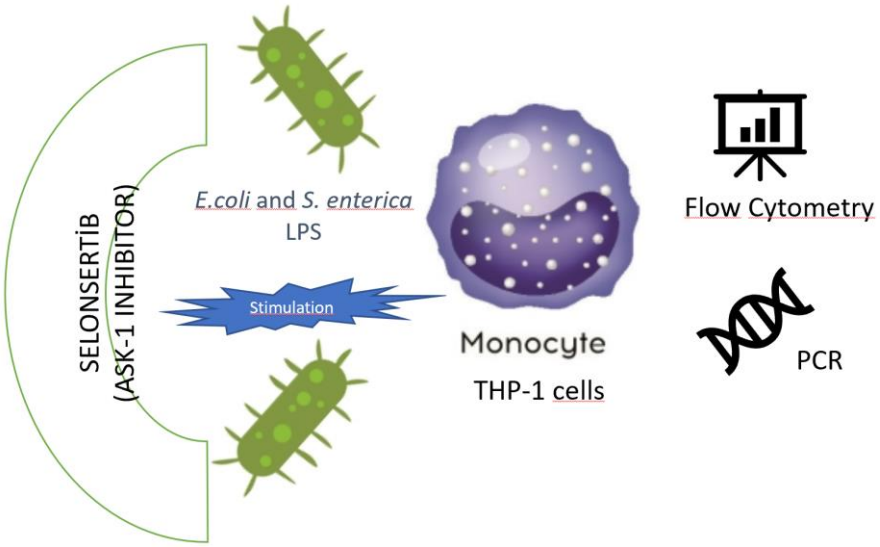
HIGHLIGHTS

- Selonsertib is an ASK-1 inhibitor.
- Selonsertib had a significantly anti-inflammatory effect like diclofenac sodium.
- Selonsertib has been shown to have a pro-inflammatory effect rather than an anti-inflammatory effect.

Abstract: Selonsertib (GS-4997), a serine/threonine kinase inhibitor, targets apoptosis signal-regulating kinase 1 (ASK1) and is now in a phase III clinical trial for the treatment of non-alcoholic steatohepatitis (NASH). Inflammation is a defense against an effect that can damage the tissue or organ itself. Cytokines are the agents that play a key role in acute inflammation and one of the most important cytokine proteins, IL-1β. In this study, we investigated whether selonsertib and diclofenac sodium (positive control) could inhibit IL-1β caused by two different LPS (*Salmonella enterica* serotype *enteritidis* and *Escherichia coli* O111:B4) stimulated on THP-1 cell line. As a result of the WST-1 assay, the inhibitory concentration of diclofenac sodium at doses of IC_{50} of 32.5 µM/mL and selonsertib was calculated to be 120 µM/mL. The immunological responses of diclofenac sodium and selonsertib (IL-1β) in different LPS-stimulated THP-1 cells were assessed cytometrically: diclofenac sodium and Selonsertib were found to be effective in stimulation from *E. coli*. The results showed that selonsertib had a significantly anti-inflammatory effect like diclofenac sodium.

Keywords: Antiinflammation, ASK1; IL-1β; Selonsertib (GS-4997).

GRAPHICAL ABSTRACT



INTRODUCTION

Apoptosis Signal Regulatory Kinase 1 (ASK-1) was first identified by Ichjio and coauthors as one of the mitogen-activated protein (MAPK) kinases [1]. The ASK-1 class belongs to the MAPK family [2]. These proteins in the cytoplasm are important in transferring information from the cell membrane to the nucleus. These cytoplasmic proteins can regulate their activities by transferring phosphate groups to the serine (Ser)/threonine (Thr) amino acids of other proteins in the cell. MAPK family; they constitute signal transduction pathways that control processes associated with gene expression, cell division, apoptosis, metabolism, and differentiation [3,4].

Selonsertib (GS-4997) is an inhibitor of ASK-1 with potential anti-inflammatory, antineoplastic and anti-fibrotic activities [5]. Targets bind to the catalytic kinase domain of ASK-1, thereby preventing its phosphorylation and activation. This prevents the phosphorylation of downstream kinases, similar c-Jun N-terminal kinases (JNKs) and p38 mitogen-activated protein kinase (p38 MAPK) [6]. Inhibits phosphorylation of downstream kinases such as JNKs and p38 MAPK. By precluding activation of ASK-1-dependent signal transduction pathways, selonsertib inhibits the production of inflammatory cytokines, down-regulates the expression of genes involved in fibrosis, suppresses excessive apoptosis, and inhibits cell proliferation [7]. Endotoxins, also called lipopolysaccharides (LPS), are external to Gram (-) bacteria is the main component of the membrane. Endotoxins, monocytes and macrophages activation of the immune system, especially the immune system, and therefore the immune response effect by increasing. Activated immune especially TNF, interleukins, prostaglandins, colony stimulated from system cells oxidizing factors, platelet activating factors, and mediators such as free radicals cause the release. TNF and IL-1β synergistically, increasing inflammation they cause the majority of clinical findings to occur [8]. Aim of the study, which, we investigated the proinflammatory/anti-inflammatory effects of selonsertib, which is an ASK-1 inhibitor, examined the stimulated different LPS (*Salmonella enterica* serotype *enteritidis* and *Escherichia coli* O111:B4) THP-1 cell-induced inflammation cell culture model by WST-1, Flow cytometry and Real Time PCR.

MATERIAL AND METHODS

Cell Culture

THP-1 human monocyte (ATCC® TIB-202™) cell line was obtained from American Type Culture Collection. The cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 1% penicillin-streptomycin and 2-mercaptoethanol to a final concentration of 0.05 mM at a temperature of 37 ^{o}C in a humidified incubator with a 5% CO_2 atmosphere. Cells were counted by staining with Trypan Blue dye on a Cedex-Roche counter and made ready for experiments after this stage.

LPS stimulation of THP-1 cell line

LPS from *Salmonella enterica serotype enteritidis* (Sigma, L4774, Germany), LPS from *Escherichia coli* O111:B4 (Sigma, L4391, Germany) were used to stimulated the THP-1 cells.

Determination of Cytotoxicity by WST-1 Method

Since THP-1 cell is a suspended cell, WST-1 dye was used in the cytotoxicity test. The WST-1 (4- [3- (4-Iodophenyl) -2- (4-nitrophenyl) -2H-5-tetrazolio] -1, 3-benzene disulfonate)((Roche, 11644807001, Germany)) assay protocol is based on the cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenase. The amount of the dye generated by the activity of dehydrogenase is directly proportional to the number of living cells. Selonsertib (Selleckchem, S8292, USA) and Diclofenac Sodium (DFS-Anti-Drugs, 16090267, India) concentrations (1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 µM) were prepared. Diclofenac Sodium and Selonsertib concentrations, which were freshly prepared in culture medium, were applied to the 11th wells as a chemical control group. Medium containing 0.1% DMSO was applied to the cells in the control group (for Selonsertib). The plates were then allowed to incubate for 24 hours. At the end of the incubation periods, 20 µL of WST-1 reagent was added to the cells in each 96 well, according to the instructions of the WST-1 kit (Catalog no: ab155902) procedure, and the cells were incubated for 3 hours in the incubator, and at the end of the incubation, the absorbances were measured using an Cytation 3 Cell Imaging Multi-Mode Reader at a wavelength of 420 nm. device, each group was read as 7 wells. Experiments were run as 3 independent repetitions [9].

Determination of IL-1β Levels in Flow Cytometry

THP-1 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin and 2-mercaptoethanol in a 5% CO_2 incubator at 37 °C. After the cells were allowed to proliferate sufficiently, the cells were counted and seeded into 6-well plates, each with $1x10^5$ cells in medium. Cells stimulated with LPS (100 ng *E. coli* LPS and 10 ng *S. enteritidis* LPS) were incubated for 4 hours. Then selonsertib and diclofenac sodium were added at the concentrations determined according to WST-1 results (IC_{50} values:120 µM/mL for selonsertib, 32 µM/mL for diclofenac Sodium) and incubated for 24 hours. In the control group well the THP-1 cells contained only fresh medium. The first four wells were then left in the plates for 4 hours of incubation for three different LPS stimulation analyses, and the other wells were left in the incubator for 24 hours analysis. Cells were centrifuged at 1200 rpm for 5 minutes, then washed twice with cold PBS (2000 µL in the first wash, 1000 µL in the second wash). 500 µL of cytofix/cytoperm was kept on ice for 20 minutes. At the end of this period, it was centrifuged at 1200 rpm for 5 minutes and the pellet was washed twice with 500 µL perm wash. After the last washing by perm wash 10 µL IL-1β Ab (Biolegend, USA) was added on the cells pellet and incubated for 30 min at room temperature. At the end of the incubation period, 1000 µL perm wash was added to each tube and the tubes were then centrifuged. At the end of centrifugation, the pellet was transferred to an eppendorf tube with 350 µL perm wash and analyzed using flow cytometry (Accuri C6, BD) [10, 11].

IL-1β and ASK-1 mRNA gene expression by qRT-PCR analysis

Expression levels of IL-1β and ASK-1 genes on selonsertib THP-1 were performed by real-time PCR. The method used by Engür and coauthors was applied [12].

RESULTS and DISCUSSION

Evaluation of Cytotoxic Effects of Diclofenac Sodium and Selonsertib in THP-1 Cells by WST-1 Method

In order to determine the IC_{50} concentration of diclofenac sodium and selonsertib, first the THP-1 cells were cultured in 96-well culture plates at $5x10^3$ cells per well. Because the THP-1 cells are a suspended cell line, the drugs were administered on the same day. The concentrations determined for diclofenac sodium and selonsertib were between 1.56-400 µM/mL. After these concentrations had been added, the plates were allowed to incubate for 24 hours. At the end of the period, WST-1 staining was allowed to determine the IC_{50} concentration in diclofenac sodium and selonsertib THP-1 cells (20 µL) and they were allowed to incubate for 3 hours. The values were transferred to Microsoft Excel and the IC_{50} level required by GraphPad Software was calculated to find the concentrations in which the drugs were most effective. As a result of the experiment, the concentration of diclofenac sodium determined as the IC_{50} concentrations was 32.5 µM/mL. The inhibition

concentration of selonsertib was calculated as 120 µM/mL (Figure 1). In a study conducted by Sima and coauthors the concentration of inhibition was calculated as 47.04 µM/mL for diclofenac sodium [13]. In another experiment using different cell lines, various concentrations of diclofenac sodium were determined by WST-1 method. A concentration of 38 µM/mL constituted the determined IC_{50} level and the lowest dose was 2.7 µM/mL. Diclofenac sodium caused the proliferation of the used cell line [14]. In a study to investigate the anti-cancer effect of diclofenac sodium on prostate cancer, the IC_{50} of diclofenac sodium determined in cytotoxicity experiments were calculated using two different inhibitors (LNCaP-Neo and LNCap-COX-2). These were, respectively, 42.2 µM/mL and 91.6 µM/mL [15]. In a study on tendon cells the IC_{50} of diclofenac sodium was a concentration of around 6.7 µM/mL [16]. A review of the literature on selonsertib, which is an ASK-1 inhibitor, revealed no findings for a cytotoxicity test with the WST-1 method, but the non-alcoholic effect of selonsertib was emphasized.

IL-1β levels by Flow Cytometry

When THP-1 cells were stimulated with 100 ng/mL *E. coli* LPS, the percentage of IL-1β was found to be 36.7%, and this value decreased to 1.7 after selonsertib administration. With the same method, the IL-1β in THP-1 cells stimulated with 10 ng/mL of *S. enteritidis* LPS was 34.8%, whereas after selonsertib administration it was detected at 2.0% using flow cytometry (Table 1 and Figure 2**).** In a study by Dudas and coauthors cytokine levels in cervical cancer using different inflammatory drugs were measured with flow cytometry, and there was a decrease in the IL-1β level [17]. In a study on the anti-inflammatory efficacy of diclofenac sodium in endotoxemia-induced rats injected with LPS, diclofenac sodium was reported to inhibit gastrointestinal toxicity for 3 days with the administration of daily doses. In the same study, it was reported that diclofenac sodium inhibited the increase in TNF-α after LPS administration, potentiating serum IL-10 concentration, and decreased iNOS enzyme activity and plasma IL-1β. Doğan and coauthors' study showed that nimesulide and diclofenac sodium prevented the increase of TNF-α in rats [18]. These and other articles support the results of the present study and explain the reduction of IL-1β.

Evaluation of mRNA Expression with Real Time PCR

For mRNA isolation, IC_{50} inhibition concentrations of diclofenac sodium and selonsertib were applied to THP-1 cells after LPS was given. After 4 hours of stimulation, RNA isolation of two different LPSs given to THP-1 cells was performed. The amount of each RNA sample was determined on the nanodrop device and 100 ngRNA was used for cDNA synthesis. The obtained cDNAs were analyzed for PCR using a LightCycler 480.

Evaluation of IL-1β Results

The result of 4-hour LPS stimulation was compared to the control: *E. coli* LPS-administered THP-1 cells had 71.2 fold the level of gene expression of IL-1β, while *S. enteritidis* LPS stimulation had a 26.6 fold increase. After incubation with diclofenac sodium for 24 hours, the level of gene expression of IL-1β in THP-1 cells given *E .coli* LPS increased by 2.08 fold compared to the control. After incubation with selonsertib for 24 hours, the level of gene expression of IL-1β in THP-1 cells administered *E. coli* LPS increased 3.6-fold and S. enteritidis LPS increased 7.3 fold compared to the control (Table 2). In terms of mRNA levels, diclofenac sodium was found to lead to better inhibition of IL-1β gene expression than selonsertib. Selonsertib has been shown to have a proinflammatory effect rather than the anti-inflammatory effect that diclofenac sodium has.

Evaluation of ASK-1 Results

The 4-hour LPS stimulation results were compared to the control: in the THP-1 cells given *E. coli* LPS, the level of gene expression of ASK-1 was 3.4 fold greater, and after *S. enteritidis* LPS stimulation a 0.5-fold increase was observed. After incubation with diclofenac sodium for 24 hours, the level of gene expression of ASK-1 in the THP-1 cells given *E. coli* LPS was 3.98 fold and 2.14 fold greater than the *S. enteritidis* LPS control. After incubation with selonsertib for 24 hours, the level of gene expression of ASK-1 in the THP-1 cells given *E. coli* LPS decreased by 7 fold and *S. enteritidis* LPS increased by fold times compared to the control. In terms of mRNA levels, it was found that selonsertib had better inhibition in terms of ASK-1 gene expression compared to diclofenac sodium (Table 2**).** The use of selonsertib, which is reported to have been used as an ASK-1 inhibitor in Phase II drug research, is also supported by our experimental findings. Shah and coauthors, in a study performed using MEAI instead of selonsertib and using diclofenac sodium as a positive control, found that TNF-α, a proinflammatory cytokine was more effective at the gene level than other

proinflammatory agents used [19]. In a study conducted by Joshi and coauthors these cytokines were found to increase when using purified peptide sequences instead of non-steroidal agents to increase the IL-1β and TNF-α cytokine levels in THP-1 cells stimulated with different LPSs [20]. In a study by Bonaterra and coauthors the anti-inflammatory effects of an STW agent in LPS stimulated THP-1 cells were investigated, and in the study, in which diclofenac sodium was used in the experiments, STW was observed to induce cytokines such as IL-1 and TNF-α [21].

In a study by Glaser and coauthors, stimulation of a different cell line with 100 ng of *E. coli* LPS resulted in a significant increase in IL-1β, TNF-α and IL-8 mRNA expression levels [22]. In a study by Fredriksson and coauthors, the expression of various genes on fibroblast cells was measured in a co-culture assay using genes such as IL-1β, IL-6, and COX-2. COX-2 and IL-6 expression levels were found, while IL-1β was upregulated [16]. As demonstrated in this and other studies, IL-1β increased the level of mRNA by producing cytokines against stimulations in the body. Against different microbial origin LPS stimulation, especially in flow cytometry, IL-1β measured as intracellular cytokine showed a significant inhibition of selonsertib. However, this effect could not be seen in the gene expression study at the mRNA level. This is thought to be the result of inadequate phosphorylation of intracellular cytokines at the gene level. Targets are attached to the catalytic kinase domain of ASK-1, thus preventing phosphorylation and activation. This prevents of phosphorylation of downstream kinases such as c-Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinase (p38 MAPK). This study, in which, we investigated the proinflammatory/anti-inflammatory effects of selonsertib, which is an ASK-1 inhibitor, examined the stimulated different LPS THP-1 cell-induced inflammation cell culture model.

Table 1. Effects of diclofenac sodium (IC_{50}: 32.5 µM/mL) and selonsertib concentrations (IC_{50}: 120 µM/mL) on IL-1β, % value in *E. coli* LPS and *S. enteritidis* LPS stimulated on THP-1 cells using flow cytometry.

LPS	IL-1β (-) %	IL-1β (+) %
E. coli LPS	59.5	36.7
E. coli Selonsertib	73.0	1.7
S. enteritidis LPS	61.8	34.8
S. enteritidis Selonsertib	65.1	2.0
E. coli LPS	59.5	36.7
E. coli Diclofenac sodium	73.6	12.1
S. enteritidis LPS	61.8	34.8
S. enteritidis Diclofenac sodium	83.2	7.1

Table 2. Effects of diclofenac sodium and selonsertib concentrations on IL-1β and ASK-1 gene expression fold levels on *E. coli* LPS and *S. enteritidis* LPS-stimulated THP-1 cells by Real Time PCR (Reference gene: Aktin- β).

	IL-1β	ASK-1	AKTIN-β
THP-1 Control	1.000	1.000	Reference gene
E. coli LPS	71.26	3.496	Reference gene
S. enteritidis LPS	26.60	0.557	Reference gene
E.coli LPS+Diclofenac sodium	148.3	-7.47	Reference gene
S.enteritidis LPS+Diclofenac sodium	90.97	0.264	Reference gene
E.coli LPS+Selonsertib	257.1	0.493	Reference gene
S.enteritidis LPS+Selonsertib	195.7	1.985	Reference gene

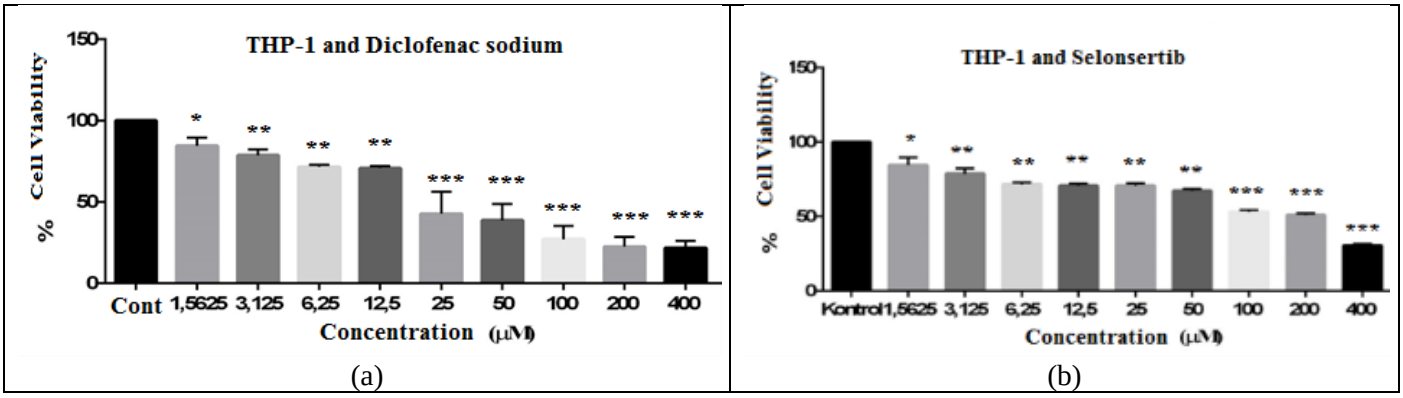
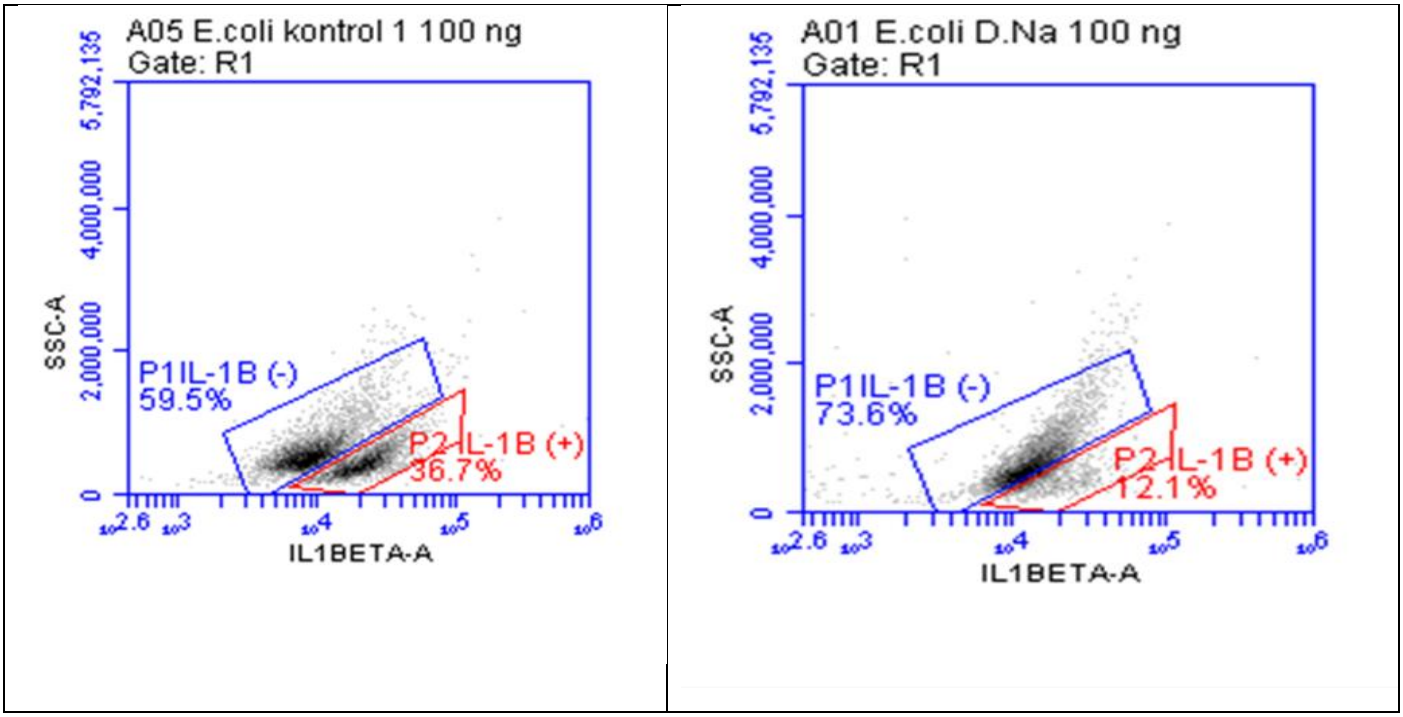


Figure 1. (a) The cells were treated with 1.5625-400 µM/mL concentrations of diclofenac sodium for 24 h and percentage cell viability was determined from the WST-1 results. (b) The cells were treated with 1.5635-400 µM/mL concentrations of selonsertib for 24 h and percentage cell viability was determined from WST-1 results. Results are expressed as mean±standard deviation (n=8) and the means of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 were considered to be significant compared to the control (0.1 % DMSO) group.



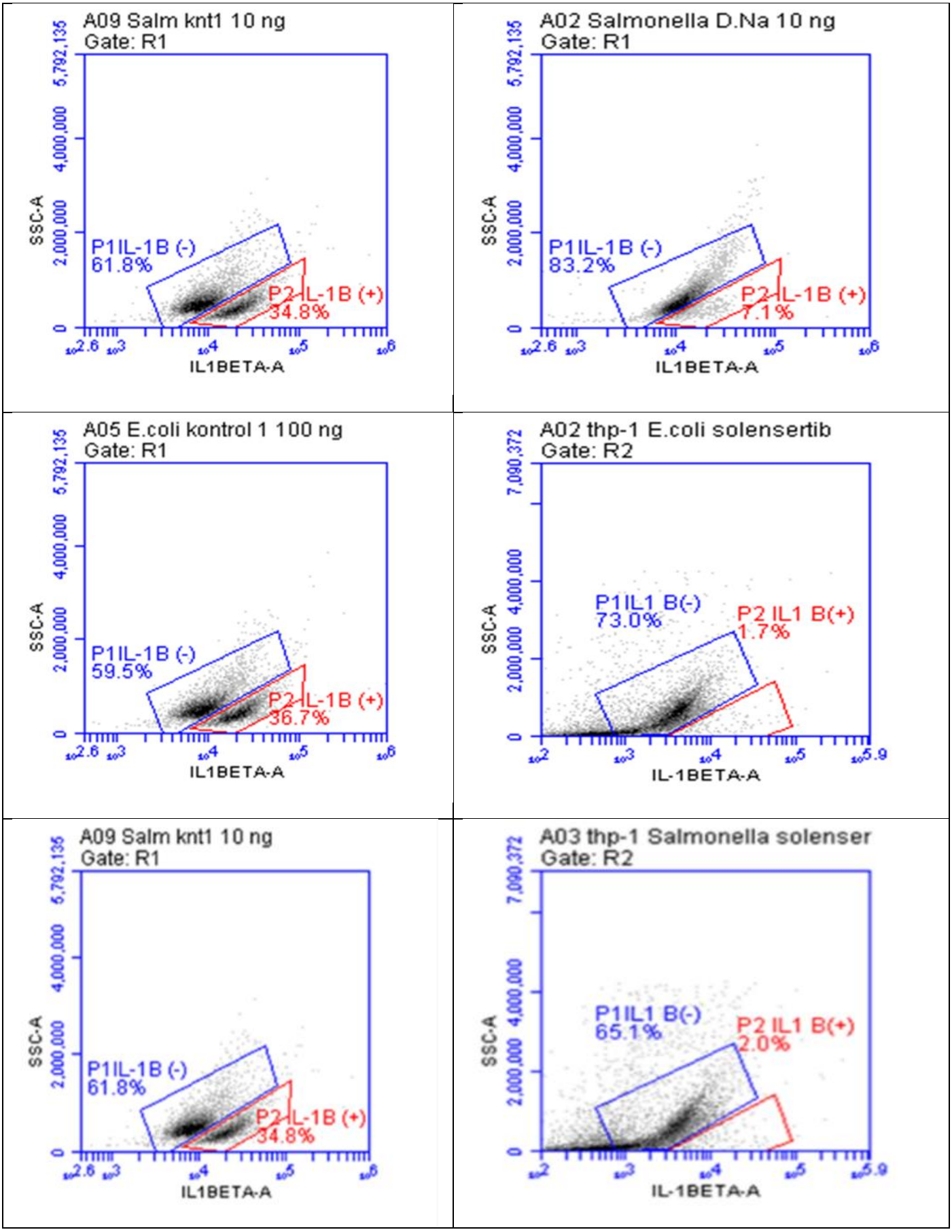


Figure 2. Effects of diclofenac sodium and selonsertib concentrations on IL-1β levels on *E. coli* LPS and *S. enteritidis* LPS stimulated on THP-1 cells by flow cytometry (Accuri C6).

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REFERENCES

1. Ichijo H, Nishida E, Irie K, Ten Dijke P, Saitoh M, Moriguchi T, et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science. 1997 Jan 3;275(5296):90-4.
2. Obara Y, Nakahata N. The signaling pathway leading to extracellular signal-regulated kinase 5 (ERK5) activation via G-proteins and ERK5-dependent neurotrophic effects. Mol Pharmacol. 2010 Jan;77(1):10-6.
3. Hayakawa R, Hayakawa T, Takeda K, Ichijo H. Therapeutic targets in the ASK1-dependent stress signaling pathways. Proc Jpn Acad Ser B Phys Biol Sci. 2012;88(8):434-53.
4. Nishida T, Hattori K, Watanabe K. The regulatory and signaling mechanisms of the ASK family. Adv Biol Regul. 2017 Dec;66:2-22.
5. Ryuno H, Naguro I, Kamiyama M. ASK family and cancer. Adv Biol Regul. 2017 Dec;66:72-84.
6. Akeda K, Noguchi T, Naguro I, Ichijo H. Apoptosis signal-regulating kinase 1 in stress and immune response. Annu Rev Pharmacol Toxicol. 2008;48:199-225.
7. Fujisawa T. Therapeutic application of apoptosis signal-regulating kinase 1 inhibitors. Adv Biol Regul. 2017 Dec;66:85-90.
8. Buer JK. Origins and impact of the term 'NSAID'. Inflammopharmacology. 2014 Oct;22(5):263-7.
9. Koyanagi M, Kawakabe S, Arimura Y. A comparative study of colorimetric cell proliferation assays in immune cells. Cytotechnology. 2016 Aug;68(4):1489-98.
10. Dunphy CH. Applications of flow cytometry and immunohistochemistry to diagnostic hematopathology. Arch Pathol Lab Med. 2004 Sep;128(9):1004-22.
11. Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, Moore PK. Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. Free Radic Biol Med. 2007 Mar 1;42(5):706-19.
12. Engür S, Dikmen M. The evaluation of the anti-cancer activity of ixazomib on Caco2 colon solid tumor cells, comparison with bortezomib. Acta Clinica Belgica. 2017 72:6, 391-398.
13. Sima S, Lal UR. Evaluation of in-vitro Anti-Inflammatory Activity of Chebulinic Acid From Terminalia Chebula Linn. Against the Denaturation of Protein, in Proceedings of the 18th International Electronic Conference on Synthetic Organic Chemistry, 1–30 November 2014, MDPI: Basel, Switzerland,
14. Will OM, Purcz N, Chalaris A, Heneweer C, Boretius S, Purcz L, et al. Increased survival rate by local release of diclofenac in a murine model of recurrent oral carcinoma. Int J Nanomedicine. 2016 Oct 12;11:5311-21.
15. Inoue T, Anai S, Onishi S, Miyake M, Tanaka N, Hirayama A, Fujimoto K, Hirao Y. Inhibition of COX-2 expression by topical diclofenac enhanced radiation sensitivity via enhancement of TRAIL in human prostate adenocarcinoma xenograft model. BMC Urol. 2013 Jan 5;13:1.
16. Fredriksson M, Li Y, Stålman A, Haldosén LA, Felländer-Tsai L. Diclofenac and triamcinolone acetonide impair tenocytic differentiation and promote adipocytic differentiation of mesenchymal stem cells. J Orthop Surg Res. 2013 Sep 2;8:30.
17. Dudás J, Fullár A, Bitsche M, Schartinger V, Kovalszky I, Sprinzl GM, Riechelmann H. Tumor-produced, active interleukin-1β regulates gene expression in carcinoma-associated fibroblasts. Exp Cell Res. 2011 Sep 10;317(15):2222-9.
18. Dogan MD, Ataoglu H, Akarsu ES. Nimesulide and diclofenac inhibit lipopolysaccharide-induced hypothermia and tumour necrosis factor-alpha elevation in rats. Fundam Clin Pharmacol. 2002 Aug;16(4):303-9.
19. Shah JH, Anand IS, Shah GB, Shah KK. Inhibition of Pro-Inflammatory Cytokine TNF-α by Azadirachta indica in LPS Stimulated Human THP-1 Cells and Evaluation of its Effect on Experimental Model of Asthma in Mice. J Immunol Clin Res. 2017;4(2):1042.
20. Joshi I, Sudhakar S, Nazeer RA. Anti-inflammatory Properties of Bioactive Peptide Derived from Gastropod Influenced by Enzymatic Hydrolysis. Appl Biochem Biotechnol. 2016 Nov;180(6):1128-40.
21. Bonaterra GA, Heinrich EU, Kelber O, Weiser D, Metz J, Kinscherf R. Anti-inflammatory effects of the willow bark extract STW 33-I (Proaktiv(®)) in LPS-activated human monocytes and differentiated macrophages. Phytomedicine. 2010 Dec 1;17(14):1106-13.
22. Glaser K, Fehrholz M, Curstedt T, Kunzmann S, Speer CP. Effects of the New Generation Synthetic Reconstituted Surfactant CHF5633 on Pro- and Anti-Inflammatory Cytokine Expression in Native and LPS-Stimulated Adult CD14+ Monocytes. PLoS One. 2016 Jan 20;11(1):e0146898.