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Anti-inflammatory activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in LPS-induced rat model

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Anti-inflammatory activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in LPS-induced rat model

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The evaluation of your manuscript is complete. The reviewers recommend reconsideration of your manuscript following minor revision and modification. I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by May 01, 2021.

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MICHAL SCHWARTZMAN

Editor-in-Chief

Prostaglandins and Other Lipid Mediators

Editor and Reviewer comments:

Reviewer #1: Abstract section:
Please correct the grammar error present in the first sentence.
The acronyms should explained at their first appearance in the text.
It is not clear the use of T120 and T360: please explain.
In abstract section, the citation of in silico studies is misleading. In the text it is reported that they have been already published. I suggest to eliminate the citation of these studies in the abstract.

Experimental section:
The approval number of the study should be provided.
Authors have to include the total number of animals used. Additionally, the statistical approach to calculate the animal number per experimental condition should be included.
It is not clear the statistical analysis. Authors declared they used ANOVA. However, the indication of asteriks in figures and figure captions suggests a post hoc analysis. Please give elucidation about.
In some figures the asteriks are missing. Please check and eventually correct.

Reviewer #2: In the reviewed manuscript, the authors presented the results of several tests that prove that the 3-CH2Cl derivative has stronger anti-inflammatory properties than acetylsalicylic acid. I appreciate the great contribution of the authors, but I have the following comments:
1. You need to proofread the English language
2. The quality and attractiveness of the figures should be improved
3. The 3-CH2Cl derivative is compared to ASA. Why were test compound cyclooxygenase inhibition tests not performed?
4. Why was this compound selected for research?

*****

Data in Brief (optional):
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Anti-inflammatory activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in LPS-induced rat model

Dear Ms. Caroline,

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Manuscript Number: POLM-D-20-00105R1

Anti-inflammatory activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in LPS-induced rat model

Dear Ms. Caroline,

Thank you for submitting your manuscript to Prostaglandins and Other Lipid Mediators.

I am pleased to inform you that your manuscript has been accepted for publication.

My comments, and any reviewer comments, are below.
Your accepted manuscript will now be transferred to our production department. We will create a proof which you will be asked to check, and you will also be asked to complete a number of online forms required for publication. If we need additional information from you during the production process, we will contact you directly.

We appreciate you submitting your manuscript to Prostaglandins and Other Lipid Mediators and hope you will consider us again for future submissions.

Kind regards,
MICHAL SCHWARTZMAN
Editor-in-Chief

Prostaglandins and Other Lipid Mediators

Editor and Reviewer comments:

Reviewer #2: Thank you for your response to my suggestions. I accept manuskryp for publication in present form.

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corrections.esch@elsevier.thomsondigital.com Thu, Apr 8, 2021, 1:29 PM

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Prostaglandins and Other Lipid Mediators

Anti-inflammatory activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in LPS-induced rat model

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full Length Article
Keywords:	Anti-inflammatory activity; 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid; LPS; rat
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	Süleyman Ergün
	Ratna Megawati Widharna, Dr.
	Caroline Caroline, S.Si., M.Si., Apt
Abstract:	Introduction Salicylic acid derivatives drug is very famous for its activity to suppress pain, fever, and inflammation. Acetylsalicylic acid is one of the examples. It has been reported repeatedly that, as a non-steroidal anti-inflammatory drug (NSAID), acetylsalicylic acid (ASA) has also a cardioprotective effect. Although ASA has various advantages, several studies have reported that it may induce severe peptic ulcer disease. We recently synthesized a new compound derived from salicylic acid, namely 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH 2 Cl) which still has the benefit of acetylsalicylic acid as an analgesic and antiplatelet, but lacks its harmful side effects (Caroline et al., 2019). In addition, in silico studies of 3-CH 2 Cl showed higher affinity towards protein receptor cyclooxygenase-2 (COX-2; PDB: 5F1A) than ASA (Caroline et al., 2019). We hypothesized that 3-CH 2 Cl inhibits the COX-2 activity, therefore could presumably decrease the inflammatory responses. However, no knowledge is available on the anti-inflammatory response and molecular signaling of this new compound. Hence, in this study, we investigated the potential functional relevance of 3-CH 2 Cl in regulating the inflammatory response in lipopolysaccharide (LPS)-induced rats. Indeed, our results demonstrated that this compound could significantly reduce the inflammatory parameter in LPS-induced rats. Material and Methods Rats were induced with LPS 0.5mg/kg bw intravenously, prior oral administration with vehicle (3% Pulvis Gummi Arabicum / PGA), 500 mg/60kg body weight (bw; rat dosage converted to human) of 3-CH 2 Cl and ASA. The inflammatory parameters

	such as changes in the temperature of septic shock, cardiac blood plasma concentrations of IL-1β and TNF-α (ELISA), blood inflammation parameters, white blood cell concentrations, and lung histopathology were observed. Meanwhile, the stability of 3-CH 2 Cl powder was evaluated. Result After the administration of 500mg/60kg bw of 3-CH 2 Cl (rat dosage converted to human) to LPS-induced rats, we observed a significant reduction of both TNF-α (5.70+/-1.04 x 10 3 pg/mL, p=<0.001) and IL-1β (2.32+/-0.28 x 10 3 pg/mL, p=<0.001) cardiac blood plasma concentrations. Further, we found the reduction of white blood cell concentration and the severity of lung injury in the 3-CH 2 Cl group compared to the LPS-induced rat group. Additionally, this compound maintained the rat body temperature within normal limits during inflammation, preventing the rats to undergo septic shock, characterized by hypothermic (t=120) or hyperthermic (t=360) condition. Furthermore, 3-CH 2 Cl was found to be stable until 3 years at 25°C with a relative humidity of 75 ± 5%. Conclusion 3-CH 2 Cl compound inhibits inflammation in the LPS-induced inflammation response model in rats, hypothetically through binding to COX-2, and presumably inhibited LPS-induced NF-κβ signaling pathways. This study could be used as a preliminary hint to investigate the target molecular pathways of 3-CH 2 Cl as a novel and less toxic therapeutical agent in alleviating the COX-related inflammatory diseases, and most importantly to support the planning and development of clinical trial.

17 October 2020

To Editor in Chief of Journal Prostaglandin and Other Lipid Mediators

Dr. Michal Laniado Schwartzman,

Dear Dr. Schwartzman,

Please find the enclosed manuscript entitled "**Anti-inflammatory activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in LPS-induced rat model**". It is our hope for this original research which has never been published before or submitted to other journals to be considered for publication in Prostaglandin and Other Lipid Mediators.

In this manuscript, we successfully demonstrate the ability of our previously published 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH_2Cl) compound (Caroline et al., Prostaglandins and Other Lipid Mediators, 2019) to inhibit inflammation in LPS-induced inflammation response model in rats. Besides, this manuscript supports our general hypothesis regarding the anti-inflammation property of this compound by binding to COX-2 and presumably inhibited LPS-induced NF-κβ signaling pathways. The data on this manuscript is very critical to further investigate the target molecular pathways of 3-CH_2Cl as a novel and less toxic therapeutical agent in alleviating the COX-related inflammatory diseases, and most importantly to support the planning of clinical trial proposal of 3-CH_2Cl.

We hope that this original research fits the journal scope and its standard quality to be considered for publication in Prostaglandins and Lipid Mediators.

Sincerely yours,

Yudy Tjahjono, B.Sc., M.Sc.Biol.

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ABSTRACT

Introduction: Salicylic acid derivatives drug is very famous for its activity to suppress pain, fever, and inflammation. Acetylsalicylic acid is one of the examples. It has been reported repeatedly that, as a non-steroidal anti-inflammatory drug (NSAID), acetylsalicylic acid (ASA) has also a cardioprotective effect. Although ASA has various advantages, several studies have reported that it may induce severe peptic ulcer disease. We recently synthesized a new compound derived from salicylic acid, namely 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH_2Cl) which still has the benefit of acetylsalicylic acid as an analgesic and antiplatelet, but lacks its harmful side effects (Caroline et al., 2019). In addition, *in silico* studies of 3-CH_2Cl showed higher affinity towards protein receptor cyclooxygenase-2 (COX-2; PDB: 5F1A) than ASA (Caroline et al., 2019). We hypothesized that 3-CH_2Cl inhibits the COX-2 activity, therefore could presumably decrease the inflammatory responses. However, no knowledge is available on the anti-inflammatory response and molecular signaling of this new compound. Hence, in this study, we investigated the potential functional relevance of 3-CH_2Cl in regulating the inflammatory response in lipopolysaccharide (LPS)-induced rats. Indeed, our results demonstrated that this compound could significantly reduce the inflammatory parameter in LPS-induced rats.

Material and Methods: Rats were induced with LPS 0.5mg/kg bw intravenously, prior oral administration with vehicle (3% *Pulvis Gummi Arabicum* / PGA), 500 mg/60kg body weight (bw; rat dosage converted to human) of 3-CH_2Cl and ASA. The inflammatory parameters such as changes in the temperature of septic shock, cardiac blood plasma concentrations of IL-1β and TNF-α (ELISA), blood inflammation parameters, white blood cell concentrations, and lung histopathology were observed. Meanwhile, the stability of 3-CH_2Cl powder was evaluated.

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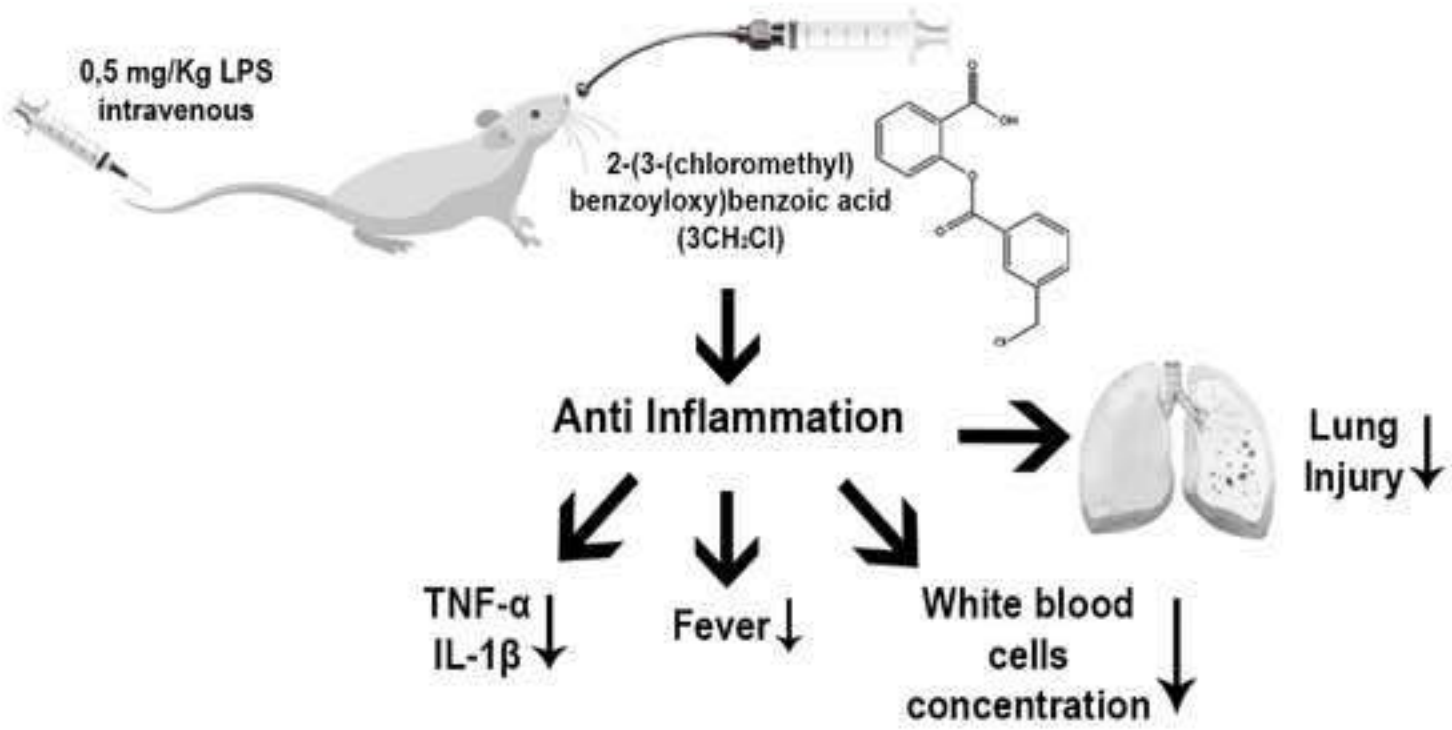
Graphical Abstract



Anti-inflammatory activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in LPS-induced rat model

Yudy Tjahjono[1†], Srikanth Karnati[2], Kuncoro Foe[1], Efendi Anggara[1], Yongky Novandi Gunawan[1], Hendy Wijaya[1], Steven[3], Handi Suyono[3], Senny Yesery Esar[1], Wuryanto Hadinugroho[1], Hevi Wihadmadyatami[4], Süleyman Ergün[2], Ratna Megawati Widharna[1], Caroline[1†*]

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ABSTRACT

Introduction: Salicylic acid derivatives drug is very famous for its activity to suppress pain, fever, and inflammation. Acetylsalicylic acid is one of the examples. It has been reported repeatedly that, as a non-steroidal anti-inflammatory drug (NSAID), acetylsalicylic acid (ASA) has also a cardioprotective effect. Although ASA has various advantages, several studies have reported that it may induce severe peptic ulcer disease. We recently synthesized a new compound derived from salicylic acid, namely 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH_2Cl) which still has the benefit of acetylsalicylic acid as an analgesic and antiplatelet, but lacks its harmful side effects (Caroline et al., 2019). In addition, *in silico* studies of 3-CH_2Cl showed higher affinity towards protein receptor cyclooxygenase-2 (COX-2; PDB: 5F1A) than ASA (Caroline et al., 2019). We hypothesized that 3-CH_2Cl inhibits the COX-2 activity, therefore could presumably decrease the inflammatory responses. However, no knowledge is available on the anti-inflammatory response and molecular signaling of this new compound. Hence, in this study, we investigated the potential functional relevance of 3-CH_2Cl in regulating the inflammatory response in lipopolysaccharide (LPS)-induced rats. Indeed, our results demonstrated that this compound could significantly reduce the inflammatory parameter in LPS-induced rats.

Material and Methods: Rats were induced with LPS 0.5mg/kg bw intravenously, prior oral administration with vehicle (3% *Pulvis Gummi Arabicum* / PGA), 500 mg/60kg body weight (bw; rat dosage converted to human) of 3-CH_2Cl and ASA. The inflammatory parameters such as changes in the temperature of septic shock, cardiac blood plasma concentrations of IL-1β and TNF-α (ELISA), blood inflammation parameters, white blood cell concentrations,

and lung histopathology were observed. Meanwhile, the stability of 3-CH_2Cl powder was evaluated.

Result: After the administration of 500mg/60kg bw of 3-CH_2Cl (rat dosage converted to human) to LPS-induced rats, we observed a significant reduction of both TNF-α (5.70+/-1.04 x 10^3 pg/mL, p=<0.001) and IL-1β (2.32+/-0.28 x 10^3 pg/mL, p=<0.001) cardiac blood plasma concentrations. Further, we found the reduction of white blood cell concentration and the severity of lung injury in the 3-CH_2Cl group compared to the LPS-induced rat group. Additionally, this compound maintained the rat body temperature within normal limits during inflammation, preventing the rats to undergo septic shock, characterized by hypothermic (t=120) or hyperthermic (t=360) condition. Furthermore, 3-CH_2Cl was found to be stable until 3 years at 25°C with a relative humidity of 75 ± 5%.

Conclusion: 3-CH_2Cl compound inhibits inflammation in the LPS-induced inflammation response model in rats, hypothetically through binding to COX-2, and presumably inhibited LPS-induced NF-κβ signaling pathways. This study could be used as a preliminary hint to investigate the target molecular pathways of 3-CH_2Cl as a novel and less toxic therapeutical agent in alleviating the COX-related inflammatory diseases, and most importantly to support the planning and development of clinical trial.

INTRODUCTION

Salicylic acid derivatives drug is widely known for its activity to suppress pain, fever, and inflammation. Acetylsalicylic acid (ASA) is one of those derivatives, which has been widely distributed commercially as a non-steroidal anti-inflammatory drug (NSAID) and thoroughly investigated (Vane et al., 2003). In addition to the anti-inflammatory functions, it has been also reported to mediate anti-platelet function or cardioprotective properties (Lanas et al., 2006), and improve bone regeneration particularly in osteoporotic conditions (Liu et

al., 2017; Yue et al., 2020). Furthermore, recent studies reported peroxisome proliferator-activated receptor alpha (PPARα) served as a specific ASA-receptor mediating neuroprotective effect (Patel et al., 2019). Although, ASA has various advantages, however, several studies have reported its harmful impact on the gastrointestinal tract, ranging from mild upper gastrointestinal problems to severe peptic ulcer disease (Cryer & Mahaffey, 2014; Valkhoff et al., 2012; Vane et al., 1998).

Our group recently synthesized a new compound derived from salicylic acid, namely 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH_2Cl) that has the benefits of acetylsalicylic acid but lacks its harmful side effects (Caroline et al. 2019). The analgesic and anti-platelet activity of 3-CH_2Cl was administered in normal Wistar rats. The data indicated that 3-CH_2Cl has a longer elimination half-life ($t_{1/2}$ = 39.4 ± 3.9 minutes) and higher C max (0.57 ± 0.02 μg/mL) compared to ASA. These pharmacokinetic parameters showed that 3-CH_2Cl is widely and deeply distributed in all body tissues, yielding a slower onset of action and longer elimination time compared to ASA (Caroline et al., 2019). Moreover, our studies on *in vitro* human platelet aggregation studies postulated the mechanism of antiplatelet activity of 3-CH_2Cl, by inhibiting COX-1. Furthermore, *in silico* studies demonstrated a better affinity of 3-CH_2Cl for the COX2 receptor (PDB: 5F1A) than ASA (Caroline et al., 2019). Indeed, the COX-2 protein is predominantly induced the main sources of prostaglandins (PGs) during inflammation (Vane et al., 1998). Many researchers investigated the specific COX-2 mediated inflammatory responses, by administering endotoxin lipopolysaccharide (LPS) in various organs and tissues (Waage et al., 1987; Nikoui et al., 2020; Shen et al., 2020).

LPS is the outer membrane component of Gram-negative bacteria as the main pathogenic stimulator for severe infections (sepsis and acute lung injury) by inducing local and systemic inflammatory responses. The administration of LPS in healthy mice can activate

LPS/TLR4 signal, inducing NF-κβ activation and the production of pro-inflammatory cytokines (IL-1β and TNF-α) (Wang et al., 2014; Barner and Karin, 1997). The observation of drug effects with animal models given LPS emphasizes changes in specific inflammatory parameters such as febrile septic shock and pulmonary edema (Dogan et al., 2000; Vaez et al., 2016).

Despite the novelty of 3-CH_2Cl, no data is available to explain the anti-inflammatory response, molecular signaling, and the stability study of this compound. Therefore as part of our continuous efforts to develop better anti-inflammatory agents and based on the above observations, in this study, we investigate the anti-inflammatory ability of 3-CH_2Cl by comparing the changes in LPS-induced specific inflammatory parameters, such as changes in temperature of septic shock, cardiac blood plasma IL-1β and TNF-α concentrations, blood inflammation, cell concentration, and lung histopathology. The data generated in this study could be used as a preliminary guideline to investigate the target molecular pathways of 3-CH_2Cl as a therapeutical agent in alleviating the COX-related inflammatory diseases, and most importantly to support the planning and development of this compound as a new drug candidate in the clinical trial. Meanwhile, the stability of material was evaluated to provide basic information for further material design. Finally, we confirmed the anti-inflammatory response of 3-CH_2Cl particularly by observing the significant reduction of cardiac blood plasma IL-1β and TNF-α concentrations as well as other supporting parameters in LPS-induced rats treated with 3-CH_2Cl compared with untreated LPS-induced rats and proposed the COX-2 and NF-κβ signaling pathways for the next level of studies.

MATERIALS AND METHODS

Chemical synthesis, characterization, and stability study

2-((3-(Chloromethyl)benzoyl)oxy)benzoic acid was synthesized in our laboratory as previously reported (Caroline et al., 2019). To observe the changes in chemical properties, we used Infrared (IR) Spectra Perkin Elmer System 60825 ranged from 4000 to 400 cm^{-1} (Perkin Elmer, Devon, UK) and High-Performance Liquid Chromatography (HPLC) Agilent 1220 Infinity LC G4288C HPLC systems (Agilent Technologies, California, USA). Additionally, we used Rheodyne 7725 100-µL injector and Shimadzu Shim-pack VP-ODS 150x4.6 mm (Shimadzu Corporation, Tokyo, Japan) as a stationary phase. Sample analysis was conducted isocratically using a mixture of methanol: phosphate buffer pH 4.0 (1:1, v/v) as a mobile phase with a flow rate of 1.0 mL/min. The KBF 720 climatic chamber binder (Binder GmbH, Tuttlingen, Germany) was used to store compounds at a constant condition.

Stability study

The stability study of $3CH_2Cl$ was conducted at a constant temperature (40° ± 2°C) and 75% ± 5% relative humidity for six months, according to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) protocols: Q1A (R2) Stability Testing Method of New Material and Drug Product. The compound was observed before and after six months of storage. Samples collected at 13 different time points were analyzed using HPLC to determine whether there are physicochemical changes observed during storage. The shelf-life of this compound was then determined using the previously reported validation method analysis (Caroline et al., 2019), as % recovery (resulted in weight / theoretical weight x 100%).

Animal model

The experimental animals used in this study were male *Rattus norvegicus* rats (3-month old, 150-200 grams (Pusvetma, Surabaya, Indonesia). Animals were housed in a temperature-controlled (21-25ºC) room, with a 12-h light/dark cycle and they were allowed

to consume food and drink *ad libitum* for 7 days. This study was approved by the University of Gadjah Mada Committee on the Use and Care of Animals. Healthy rats were measured at body temperatures between 37.2-38.5ºC (Briese, 1998) using a digital thermometer (Omron Healthcare, Singapore). In general, the rats were divided into four groups, consisting of the vehicle/untreated control, LPS treated, LPS+ASA treated, and LPS+3-CH_2Cl treated groups. For cytokine concentration experiments, the animal groups were divided into more than four groups, animals, due to different ASA and 3-CH_2Cl dosage applied (see below). Each experimental group consisted of six animals (n=6).

Lipopolysaccharide (LPS) treatment

Lyophilized powder of Lipopolysaccharide (LPS) isolated from Gram-negative of *Escherichia coli* type O111:B4 was diluted, yielding 0.5 mg/mL stock solution in 15 mM NaCl according to manufacturer's instruction (Sigma Aldrich, Saint Louis, USA). A single dose of LPS stock solution (0.5 mg/kg bw) was injected intravenously through the tail vein for 30 minutes, subsequently followed by drug administered orally.

Acetylsalicylic acid (ASA) and 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH_2Cl) dosage administration

Pure acetylsalicylic acid (Labtech Citra Persada, Surabaya, Indonesia) was diluted by 3% *Pulvis Gummi Arabicum* (PGA) (Pharmalab, Bandung, Indonesia) suspension. 3-CH_2Cl was synthesized as previously described (Caroline et al., 2019), and diluted by 3% PGA suspension. Following the injection of LPS, each dose of diluted ASA (LPS+ASA) and 3-CH_2Cl (LPS+3-CH_2Cl) compounds was orally administered to the animals. The cytokine concentration assay was then performed (see below). The doses administered to rats were

10.33; 51.65; 93.00; 134.33; and 175.67 mg/kg body weight. The drug dosages were calculated according to the previously conversion method (Nair et al., 2016), representing 100; 500; 900; 1,300; and 1,700 mg/60 kg bw as the usual dose of drug treatment in human, respectively.

In another experimental setup, the white blood cells **(**WBC) cell blood count, temperature, and histological examinations were performed following the administration of ASA and 3-CH_2Cl at a single dose of 10.33 mg/200 kg bw (equivalent to 500 mg/60 kg bw) to a pre-determined group of rats that had been treated by LPS. The ASA and 3-CH_2Cl were administered orally at the first hour and the sixth hour after LPS injection. The control group (untreated rats) was injected with 15 mM NaCl (PT Widarta Bakti, Surabaya, Indonesia) intravenously and subsequently followed by the oral administration of 2 mL of 3% PGA.

Blood preparation for WBC count and cytokine TNF-α and IL-1β concentrations assay

Plasma for cytokine testing was obtained from the blood via intracardiac and collected on microtubes containing EDTA (500 mM). The microtube was centrifuged for 15 minutes at 1000 rpm. The supernatant containing blood plasma was collected in a tube and stored at -4^{o}C, for further cytokine testing.

In another experimental group, rat blood was collected (according to the previous method), 24 hours after the oral administration of ASA, 3-CH_2Cl, or control. A small amount of pure blood was taken to determine the concentrations of leukocytes, monocytes, granulocytes, and lymphocytes (Automatic Hematology Analyzer Horiba, USA). This analysis focused on the number of WBC cells in rats by administering LPS with ASA/3-CH_2Cl (10.33 mg/200 kg bw).

Cytokine TNF-α and IL-1β concentrations assay

TNF-α and IL-1β cytokines were tested using the Enzyme-linked immunosorbent assay kit (ELISA, Elabscience, Wuhan, China). Rat blood plasma was diluted with 500 mM EDTA (1:2, v/v) and incubated in a coated ELISA 96-er tube. The extracellular TNF-α and IL-1β concentrations were analyzed by color change (Multiskan GO microplate spectrophotometer, Thermo Scientific, Vantaa, Finland). Cytokine concentrations were determined using optical density (OD) regression and GraphPad Prism Software v.7 as a standard.

Temperature measurement assay

The rectal temperature of each animal in each group was measured periodically with a digital thermometer (Omron Healthcare, Singapore). The initial temperature (T0: 37.2-38.5^{o}C) was the temperature of rats following adaptation for 30 minutes at 24-26°C (Briese, 1998). The observation of temperature changes was carried out every hour for 10 hours. The temperature changes (△T) was calculated as changes in the body temperature of each animal at each measurement time interval against the initial temperature.

Histopathology study

The rats' lungs were dissected after drug administration, temperature measurements, and euthanasia. The lung organs were fixed with 10% formalin solution, dehydrated with alcohol-xylene, and immersed in paraffin before cutting the tissue. All tissue sections were stained with hematoxylin-eosin. Lung injury was observed microscopically at 10 randomly selected spots. The observation scores were determined according to the observation of pulmonary edema, as follows: normal = 0; perivascular edema = 1; peribronchial edema, interstitial edema, perivascular cell infiltration = 2; alveolar edema, interstitial cell infiltration = 3; and alveolar cell infiltration = 4 (Chian et al., 2012).

Statistical evaluation

Statistical analysis was performed using ANOVA and *p* values less than 0.05 were considered statistically significant. The data subsets were graphically presented using GraphPad Prism Software v.7. Unless others mentioned, all of the data related to 3-CH_2Cl animal groups were compared with the positive control (LPS+Vehicle groups).

RESULTS

3-CH_2Cl exerts anti-inflammatory activity by a significant reduction of cardiac blood plasma cytokine TNF-α and IL-1β concentrations in LPS-treated rat model

To assess the degree of inflammation through humoral components, we observed the cardiac blood plasma cytokines concentration of LPS-induced rats, particularly pro-inflammatory TNF-α and IL-1β (Figure 1). The TNF-α and IL-1β levels were significantly increased in LPS-treated rats, and decreased in LPS+ASA treated and LPS+3-CH_2Cl treated groups (see also supplementary figure 2). We observed significant reduction of both TNF-α and IL-1β cytokines, particularly following the treatment of LPS-induced rats with 500mg/60kg bw of ASA (TNF-α 4.97+/-1.07 x 10^3 pg/mL, p=<0.001; IL-1β 2.01+/-0.33 x 10^3 pg/mL, p=<0.001) or 3-CH_2Cl (TNF-α 5.70+/-1.04 x 10^3 pg/mL, p=<0.001); IL-1β 2.32+/-0.28 x 10^3 pg/mL, p=<0.001). We found no dose-dependent decrement of TNF-α and IL-1β levels in the LPS+3-CH_2Cl treated group. The cytokines level in LPS+ASA treated animal group decreased after treatment with 100mg/60 kg bw and 500mg/60kg bw of ASA and increased again at other dosages greater than 500mg/60kg bw. The highest cytokines levels were observed in the LPS treated animal group and LPS+ASA treated animal group (1,700mg/60kg bw of ASA). Although the concentration of cytokines in the LPS+3-CH_2Cl treated group positioned approximately at the same level, a subtle elevated cytokine level was

observed in 3-CH_2Cl treated animal group (1,300mg/60kg bw of 3-CH_2Cl). Taken together, we observed the significant reduction of cardiac blood plasma TNF-α and IL-1β in 3-CH_2Cl treated animal groups particularly following the treatment of LPS-induced rats with 500mg/60kg bw compound dosage.

3-CH_2Cl exerts an anti-pyrogenic effect by reducing the rectal temperature of LPS-treated rat hyperthermic model

To measure the anti-pyrogenic activity of the compounds in endotoxin LPS-treated animals, we observed the mean temperature difference (ΔT) for 10 hours in the 60-min interval, with T0 was defined as the starting point, when the animal was intravenously injected by a single dose of 0.5mg/kg bw of LPS. As shown in Figure 2, the rats displayed typical septic shock in response to a single dose of 0.5mg/kg bw of LPS (+LPS vehicle group), indicated by hypothermic condition (negative ΔT), particularly at T60 and T120. The rectal temperature difference began to rise 240-300 min after the LPS injection and reached its peak value at about 360 min, interpreted as a hyperthermic condition. The rectal temperature remained elevated until the end of the observation. In the LPS+3-CH_2Cl group, the rectal temperature was relatively stable from T0 until the first 3-CH_2Cl oral administration at T60. No significant differences were observed in comparison with its basal normal temperature until T180. It began to rise slightly at 240-360 minutes observation point, and gradually decreased again following the second oral administration of 500mg/60kg bw of 3-CH_2Cl at T360, until reaching its basal temperature at T600. However, in LPS+ASA treated group, the animals showed no hypothermic response following LPS injection. It began to rise about 60 minutes after the first oral administration of 500mg/60kg bw of ASA. In contrast to the LPS treated group, the LPS+ASA group exhibited a slight hyperthermic condition with the maximum temperature difference (ΔT) reaching +0,55±0,22°C. The

temperature began to decrease gradually and fell rapidly after the second oral administration of ASA. It reached the basal normal temperature at T480 until the end of the observation. Taken together, animals treated with 3-CH_2Cl group shows relatively stable temperature from T0 until T600. Now significant hypothermic and hyperthermic conditions were observed within the 3-CH_2Cl treated group.

3-CH_2Cl suppresses the immune cells and therefore exerts anti-inflammatory property by decreasing the cardiac white blood cell concentration in LPS-induced rats.

To investigate whether 3-CH_2Cl could suppress the immune cells in LPS-treated rats, we measured the number of absolute leukocytes (figure 3A), lymphocytes (figure 3B), monocytes (figure 3C), and granulocytes (figure 3D) in isolated whole blood. Twenty-four hours after LPS treatment with additional repeated doses (two times) of oral administration of the salicylic acid compound in between, the animals were euthanized and the blood cells were counted immediately. We observed a significant increase of all WBCs differential counts, with the highest WBCs concentration observed in all LPS-injected rat group, followed by LPS+ASA and LPS+3-CH_2Cl groups. The untreated rats showed the lowest WBCs concentration. In leukocytes and lymphocytes parameters, a very low blood cell concentration was observed. Additionally, the leukocytes and lymphocytes parameters observed in the LPS+3-CH_2Cl group were slightly lower than those observed in the LPS+ASA group, suggesting the alleviated suppression of immune cells mediated by 3-CH_2Cl compared with ASA.

Administration of 3-CH_2Cl reduce LPS induced acute lung injury

To analyze the direct impact of salicylic acid derived compound on lessening the typical acute lung injury 24 hours after LPS administration, we performed the microscopic

histological analysis with a scoring system. Representative histological sections from all experimental groups were presented in figure 4A-D. The normal untreated rat group (Figure 4A) showed relatively clear alveolar spaces and indicated no infiltration of immune cells. In contrast, the lung of LPS-treated animal groups (figure 4B) exhibited intra-alveolar edema, massive cell infiltration, and hemorrhage. Following the administration of 500mg/60kg bw of ASA in the LPS-injected rat group (figure 4C), we observed a significant reduction of cell infiltrates and alveolar edema. Meanwhile, the administration of 500mg/60kg bw of 3-CH_2Cl (figure 4D) could reduce the degree of lung injury better than ASA, indicated visually by slightly bigger intra-alveolar space. To have a better analysis of the observation statistically, we converted the visual interpretation into the numeric score and presented the data in graphical lung injury scores (figure 4E). Indeed, the highest score indicating severely damaged lungs was significantly demonstrated in LPS treated animal groups (3.125±0.39). On the other hand, a slight lung injury score was demonstrated in LPS+ASA and 3-CH_2Cl group summary.

The 3-CH_2Cl powder is stable until 3 years at 25°C with a relative humidity of 75 ± 5%.

The Physico-chemical characteristics of 3-CH_2Cl were white powder and odorless. Following storage at 40° ± 2°C/75% ± 5% RH for 6 months, the recovery percentages of this compound at 13 different sampling points can be seen in Supplementary Table 1. This compound was found to be stable, as shown in the HPLC chromatogram and IR spectroscopy results (Figure 5), by comparing the results before and after storage for 6 months (Figure 5a). In addition, there was no additional peak attributed to salicylic acid in the HPLC chromatogram (Figure 5b), indicating no chemical degradation observed until the end of the stability study. Through theoretical conversion according to ICH guidelines, the 3-CH_2Cl powder is stable until 3 years at 25°C with a relative humidity of 75 ± 5%.

DISCUSSION

It has been previously reported that salicylic acid and its derivate ASA prevent inflammation in part by enzyme cyclooxygenase inhibition. Besides, salicylic acid and its derivate ASA could prevent inflammation by their specific inhibition of IKK-β, preventing the activation of NF-kβ and thereby significantly suppress genes involved in the pathogenesis of inflammatory response such as cytokines (Yin et al., 1998). Although, ASA as an anti-inflammatory agent has various advantages, however, its harmful impact on the gastrointestinal tract motivated our research group to investigate the anti-inflammatory property of a novel and less toxic salicylic acid derived 3-CH_2Cl as another therapeutic drug in LPS-induced rat model.

LPS-administration in rodents has been used frequently to study the inflammatory response, specific organ failure, and its typical physiological changes (Khedoe et al., 2017). LPS could bind to its receptor in Toll-like receptor 4 (TLR4)-dependent pathway, and stimulate the cytokine through the Mitogen-activated protein kinase (MAPK) and nuclear factor kappa beta (NF-κβ) routes, which may activate several immunological responses (Wang et al., 2014), particularly cytokines transcription, and therefore may cause severe inner organ injury, such as typical LPS-generated acute lung injury (ALI). Although the application of LPS-induced inflammation response model in rats exerts various differences compared with humans, there are several similarities which have been reported, in the inflammatory responses to LPS between rodents and human. Therefore this method is still reliable for preliminary investigation of the inflammation response (Foster et al., 1993), as carried out for the anti-inflammatory study of potential 3-CH_2Cl in the pre-clinical phase. In addition to our previously reported *in silico* docking results which showed that 3-CH_2Cl could act as a potential COX-2 ligand (Caroline et al., 2019; Tamayanti et al., 2016), the above-mentioned

signal transduction led us towards another hypothesis of the 3-CH_2Cl mechanism of action, which might have the similar pathway with ASA.

The pro-inflammatory cytokines concentration plays a pivotal role, particularly in the investigation of the drug's effectiveness to inhibit LPS-induced inflammation. TNF-α and IL-1β are widely known as a representative of pro-inflammatory cytokines and has been widely used as a peripheral marker, particularly because of the association with its transcription factor, NF-κβ. Indeed, our data in figure 1 did support this hypothesis by the specific reduction of rat TNF-α and IL-1β cardiac blood plasma concentrations following the oral administration of 500mg/60kg bw of 3-CH_2Cl in LPS-induced systemic inflammation rats. This may indicate that this compound might exert anti-inflammatory molecular pathways properties through NF-κβ signaling. To investigate the dose-dependent effect, we evaluated the cytokine concentration following the administration of salicylic acid derivate in five increment concentrations. Interestingly, we found the dose-dependent decrement of both plasma cytokine TNF-α and IL-1β concentrations, range from 100-500 mg/60kg bw with the nadir was reached by 500mg/60kg bw. The preliminary findings pointed towards the similar anti-inflammatory effect of 3-CH_2Cl compared with ASA, particularly at the dose of 500mg/60kg bw. Therefore to simplify the overall experimental design due to limited resources, we focused on the observation of other anti-inflammatory parameters following 500mg/60kg bw dosage administration only. Another physiological changes following the reduction of the pro-inflammatory cytokine, such as isothermic anti-pyretic effect, immune cell depletion, and the reduction of organ damage severity, is expected after 500mg/60kg bw dose of 3-CH_2Cl administration compared with vehicle-administered LPS-rats.

It is known that after LPS-administration, typical leukopenia is observed in the first 1-4 hours examination, followed by a rebound leukocytosis in a zenith of 12-24 h after LPS injection. This is indicated as IL-6 stimulated neutrophilia to increase the survival of

neutrophils during the acute inflammatory condition (Cox and Gauldie, 1997). Therefore to reproduce a contrast result of 3-CH_2Cl action in white blood cells concentration of LPS-treated animals group, we used the 24-h time point as our starting analysis. A significant increase of white blood cell concentration was observed in LPS-treated animals (figure 3), this is in agreement with Tavares et al., 2006. The administration of 500mg/60kg bw of 3-CH_2Cl in LPS-animals could reduce the white blood cell concentration. This phenomenon could also be seen in ASA treated animals group, indicating the anti-inflammatory action of 3-CH_2Cl and ASA to inhibit neutrophilia, particularly 24 hours post endotoxin LPS injection.

In terms of temperature changes, LPS generated fevers commonly polyphasic (Rudaya et al., 2005), and may vary depending on multiple methodological factors such as dose and laboratory ambient. During the initial phase of intravenous LPS injection, the animals show typical septic shock hypothermia and subsequently followed by the hyperthermia phase (Dogan et al., 2000). Those typical polyphasic temperatures during systemic inflammation are triggered mainly by cyclooxygenase isoforms and maintained particularly in the brain (Blomqvist & Engblom, 2018). As expected, our results in figure 2 support hypothetical arguments of 3-CH_2Cl potential inhibitory ligands namely COX-2. In comparison to LPS-treated rats, the dose administration of 500mg/60kg bw of 3-CH_2Cl could stabilize the rat's body temperature, preventing them to undergo polyphasic hypothermic and hyperthermic conditions. The hyperthermic prevention of 3-CH_2Cl may indicate that this compound could have anti-pyretic properties. Following the administration of 3-CH_2Cl, this compound may block the COX-2 activity and thus inhibit hyperthermia during systemic inflammation.

As mentioned before, beside the polyphasic thermal character, intravenous LPS administration could induce severe acute lung injury (ALI) through histologically observed massive infiltration of the inflammatory cell causing pulmonary edema, which is triggered by

the generation of reactive oxygen species (ROS), increased cytokine responses, MAPK activation, NF-κβ expression, and its associated molecules (Vaez et al., 2016). As expected, severe ALI was demonstrated in the typical histological section of LPS-treated rat lungs (Figure 4A), as well as its associated scoring data (Figure 4B) 24 hours post LPS injection. Additionally, we demonstrated that 3-CH_2Cl treatment could reduce partially the severity of ALI, better than ASA. Even though the effect of 3-CH_2Cl has not been well studied at the molecular level in the context of inflammatory cascades, these histological findings supported our general observational study, namely the anti-inflammatory of 3-CH_2Cl which may cause the inhibition of immunological signal during inflammation, as well as decrease the immune cells concentration and its cytokine response. For the next aim of studies, investigation of reactive oxygen species (ROS) production in the presence of 3-CH_2Cl would strengthen the preclinical observation of this compound in inhibiting inflammation.

Meanwhile, to complete the previously reported physicochemical characterization of 3-CH_2Cl (Caroline et al., 2019), in this study we observed the stability of this compound based on storage time and humidity parameters. Our data as shown in Figure 5A with additional HPLC pattern (figure 5B) indicated that 3-CH_2Cl was still stable after 6 months of storage in 40° ± 2°C with a relative humidity of 75 ± 5%. No chemical degradation was observed. In other words, according to Q1A(R2) Stability Testing Method of New Material and Drug Product guidelines, this compound could be stored and used for various testing until 3 years at 25°C with a relative humidity of 75 ± 5%, without making an extra effort to synthesize a new 3-CH_2Cl compound.

In summary (Figure 6), our results showed that 3-CH_2Cl oral administration in intravenous LPS-treated rat model exhibited anti-inflammatory activity, particularly through decreased TNF-α and IL-1β pro-inflammatory cytokines, decreased white blood cells concentration, and reduced severity of lung injury. These results led to a better

characterization of 3-CH_2Cl as a potential anti-inflammatory drug, particularly focusing on investigating the cyclooxygenases and NF-κβ signaling pathways. The compound, 3-CH_2Cl could also stabilize the rat's body temperature during the inflammatory condition, preventing the rats to undergo hyperthermic condition, and thus, it exhibited anti-pyretic activity. Additionally, 3-CH_2Cl was found to be stable until 3 years at 25°C with a relative humidity of 75 ± 5%. Taken together, this paper pointed towards the hypothetical mechanism of 3-CH_2Cl as a therapeutical agent in alleviating the COX-related inflammatory diseases. The results could support the planning and development of 3-CH_2Cl in the preclinical and clinical trials.

FIGURES AND TABLE LEGENDS

Figure 1

Administration of 3-CH_2Cl lowered cytokine TNF-α and IL-1β concentrations in the LPS-treated rat model.

Effect of 500 mg/60kg bw of 3-CH_2Cl as well as ASA on plasma TNF-α (A) and IL-1β (B) levels in LPS-induced rat (n=6), showing a significant reduction of both cytokines concentration compared with the control group (+LPS+vehicle). Blood samples were collected after LPS+ASA and LPS+3-CH_2Cl administration as presented in the method section. Results were expressed as mean ± standard deviation (SD), and statistical significance was shown as ***$P<0.001$.

Figure 2

Administration of 3-CH_2Cl reduced rectal temperature of LPS-treated rat hyperthermic model.

LPS-treated rat group displayed a typical septic shock as a response to 0.5 mg/kg bw of LPS, indicated by the hypothermic condition at the nadir of T120 and hyperthermic condition at T360. The changes of rectal temperature following the administration of ASA (500mg/60kg bw) and 3-CH_2Cl (500mg/60kg bw) in LPS-treated rats are shown in the graphic. Results were expressed as mean ± standard deviation (SD), and statistical significance was shown as *P<0.05 or **P<0.01.

Figure 3.

Administration of 3-CH_2Cl reduced differential white blood cells count in the LPS-treated rat model.

The number of leukocytes (A) as well as monocytes (B), lymphocytes (C), and granulocytes (D) increased in LPS-treated rats groups (n=10) compared with the control group - LPS+vehicle. After administration of LPS+ASA (500mg/60kg bw) and 3-CH_2Cl (500mg/60kg bw), the blood cells count decreased and it was shown that 3-CH_2Cl had a good effect as ASA. Results were expressed as mean ± standard deviation (SD), and statistical significance was shown as NS P>0.05, *P<0.05, **P<0.01, ***P<0.001, or ****P<0.0001.

Figure 4.

Administration of 3-CH_2Cl reduced lung edema in the LPS-treated rat model.

Representative histological sections from the experimental group (n=10) showed acute lung injury, characterized by lung edema, intra-alveolar hemorrhage, and interstitial cell infiltration in the +LPS+vehicle group (B) compared with the control group -LPS+vehicle (A). The administration of the dose of 500mg/60kg bw of ASA (C) and particularly with 500mg/60kg bw of 3-CH_2Cl in intravenous LPS-injected rats exhibited lesser lung injury (D). Lung injury score was shown (E) as the mean ± standard deviation (SD).

Figure 5.

A fingerprint of the 3-CH_2Cl compound.

(A) Patterns of 3-CH_2Cl infrared spectroscopy on day-0 (red) and day-185 (black) showed an identical pattern. (B) HPLC pattern of a typical 3-CH_2Cl compound. Small graphic indicates a salicylic acid pattern as a precursor compound. No impurities were detected.

Figure 6.

Oral administration of 3-CH_2Cl in intravenous LPS-treated rat model exhibited anti-inflammatory activity.

(A) Classical LPS-induced inflammatory pathways at the cellular level: TLR-4 signal transduction. (B) 3-CH_2Cl decreased the production of TNF-α and IL-1β pro-inflammatory cytokines, decreased the white blood cell concentration, and reduced the severity of lung injury, presumably through IKK and NF-κβ signaling pathways. 3-CH_2Cl had an anti-pyretic property due to binding on its hypothetical receptor, COX-2 (C).

Supplementary Table 1.

Stability measurement protocols.

Day	Mean % recovery (n = 3)
1	99.85 ± 0.85
2	99.13 ± 0.52
3	98.95 ± 0.67
4	99.07 ± 0.57
5	98.61 ± 0.37

6	99.40 ± 0.61
7	99.76 ± 0.45
14	98.52 ± 0.29
21	98.35 ± 0.90
35	97.90 ± 0.67
49	98.87 ± 0.50
84	98.66 ± 0.59
110	99.35 ± 0.23
114	99.42 ± 1.05
127	98.66 ± 0.31
163	98.79 ± 0.74
185	99.35 ± 0.68

Supplementary Figure 1.

Histological scoring interpretations for lung edema in the LPS-treated rat model.

Acute lung injury was characterized by: (A) normal = 0; (B) low grade perivascular edema = score 1; (C) milder severity edema with more cell infiltration = score 2; (D) alveolar edema, interstitial cell infiltration = score 3; and (E) severe edema with alveolar cell infiltration covering about 90% areale = score 4.

Supplementary Figure 2.

Administration of 3-CH_2Cl lowered cytokines TNF-α and IL-1β concentrations in the LPS-treated rat model.

Administration of ASA and 3-CH_2Cl with 5 variant doses (100; 500; 900; 1300; 1700 mg/60kg bw) lowered cytokines TNF-α (a) and IL-1β (b) expression, compared with the control group +LPS+vehicle. In LPS+3-CH_2Cl treated group, there was no dose-dependent reduction of TNF-α and IL-1β. Results were expressed as mean ± standard deviation (SD), and statistical significance was shown as NS $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$, or ****$P<0.0001$.

AUTHORS CONTRIBUTION.

Yudy Tjahjono and Caroline designed the experiments, carried out experiments, analyzed the data, and prepared the manuscript. Efendi Anggara and Yongky Novandi carried out the experiments and analyzed the data. Srikanth Karnati, Kuncoro Foe, Hendy Wijaya, Steven, Handi Suyono, Senny Yesery Esar, Wuryanto Hadinugroho, Hevi Wihadmadyatami, Süleyman Ergün, and Ratna Megawati Widharna assisted the experiments and analyzed the data.

GRANT

The work was supported by the following grants: Research and Community Service Institute of Widya Mandala Catholic University, Surabaya, Indonesia (954a/WM01.5/N/2018 to Yudy Tjahjono) and Ministry of Research, Technology and Higher Education of the Republic of Indonesia (115w/WM01.5/N/2018 to Kuncoro Foe).

ACKNOWLEDGEMENT

We would like to thank Ms. Siti Surdijati, Mr. Arnold Sulistyo Cionander, Mr. Ignasius Agyo Palmado, –Ms. Helen Kristiana Budi Agung, and Ms. Wiska Stephani Tjiali (Faculty of Pharmacy, Widya Mandala Catholic University, Surabaya) for fruitful discussion.

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Figure 1

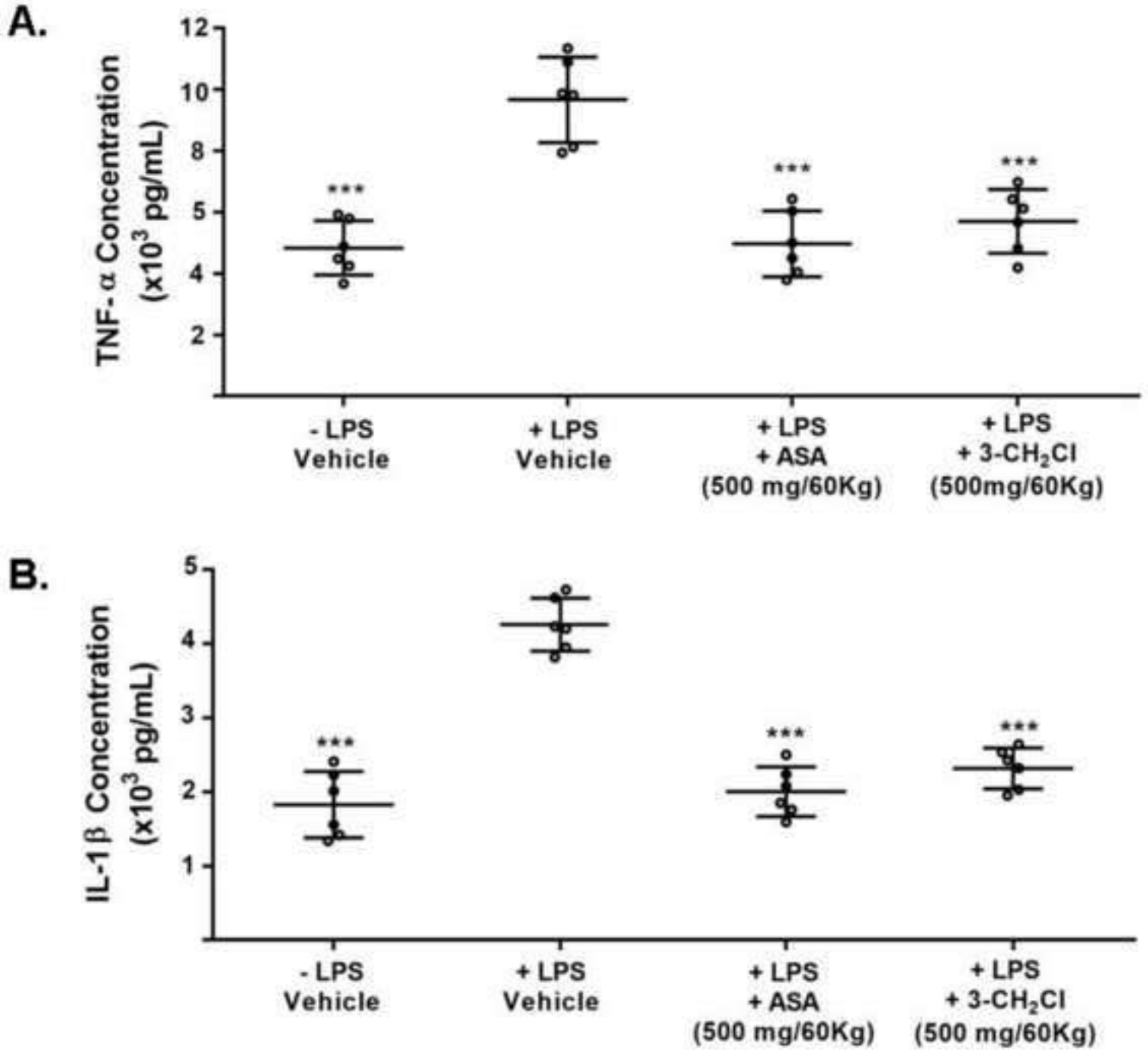

Figure 2

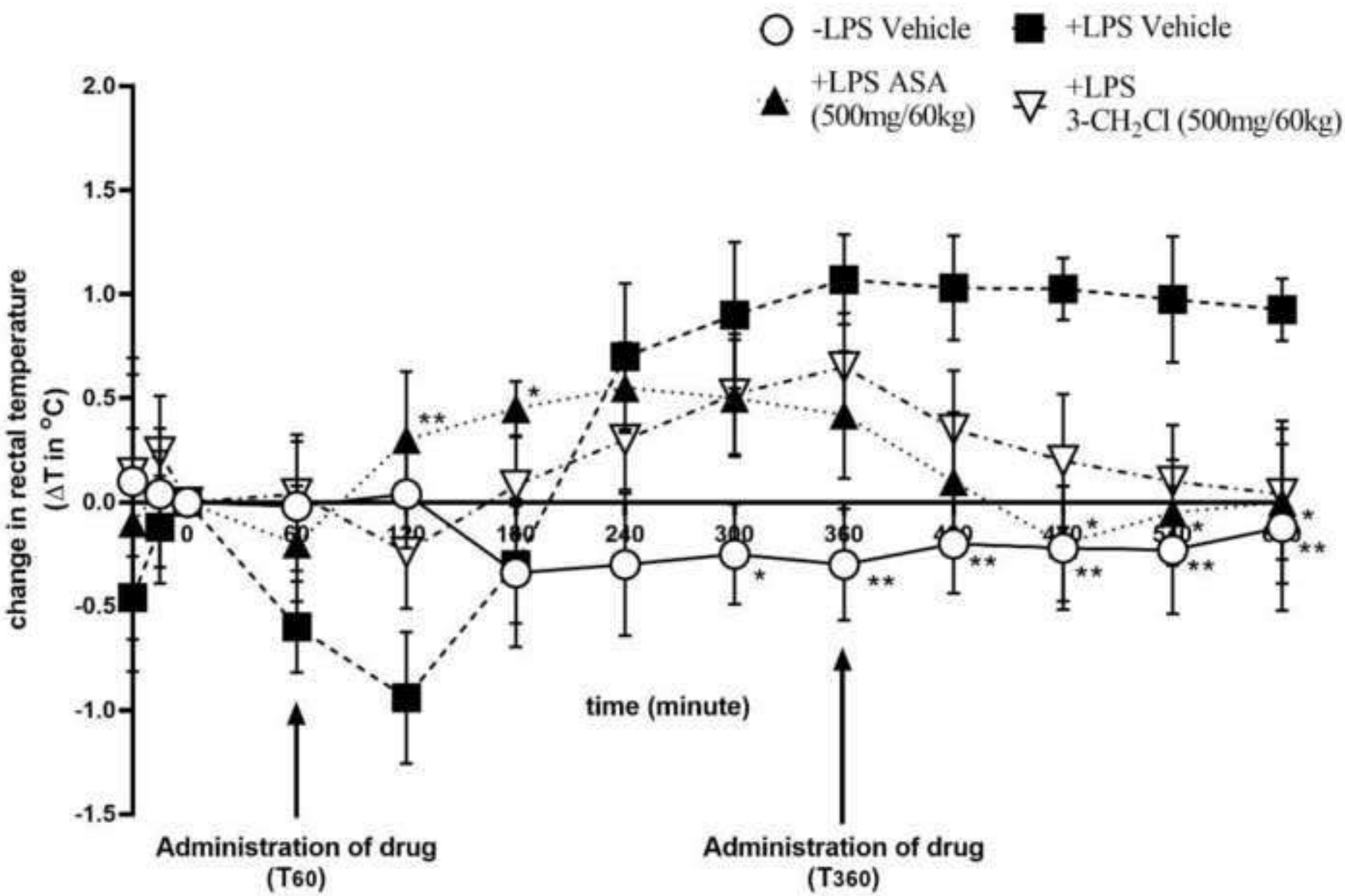

Figure 3

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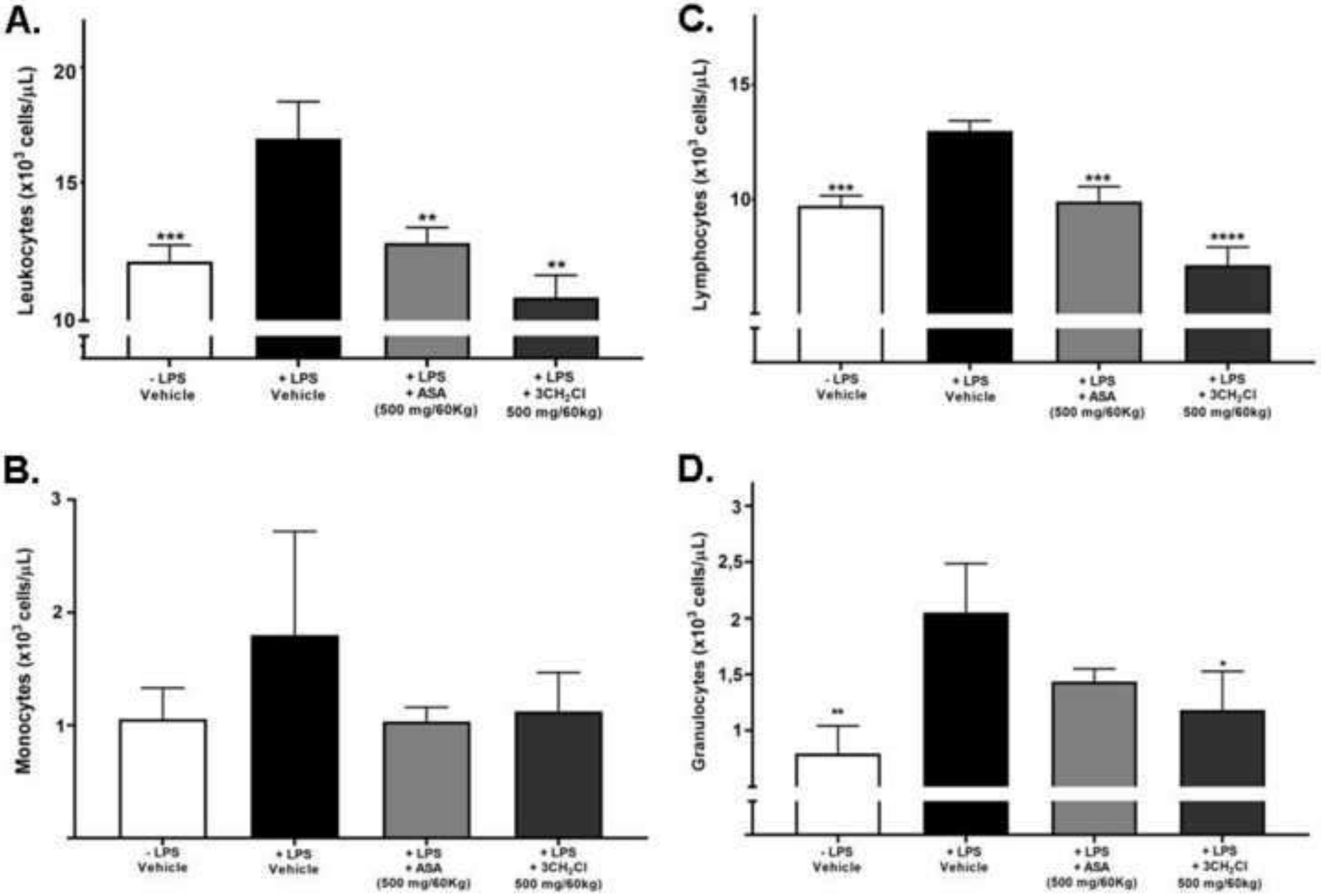

Figure 4a-d

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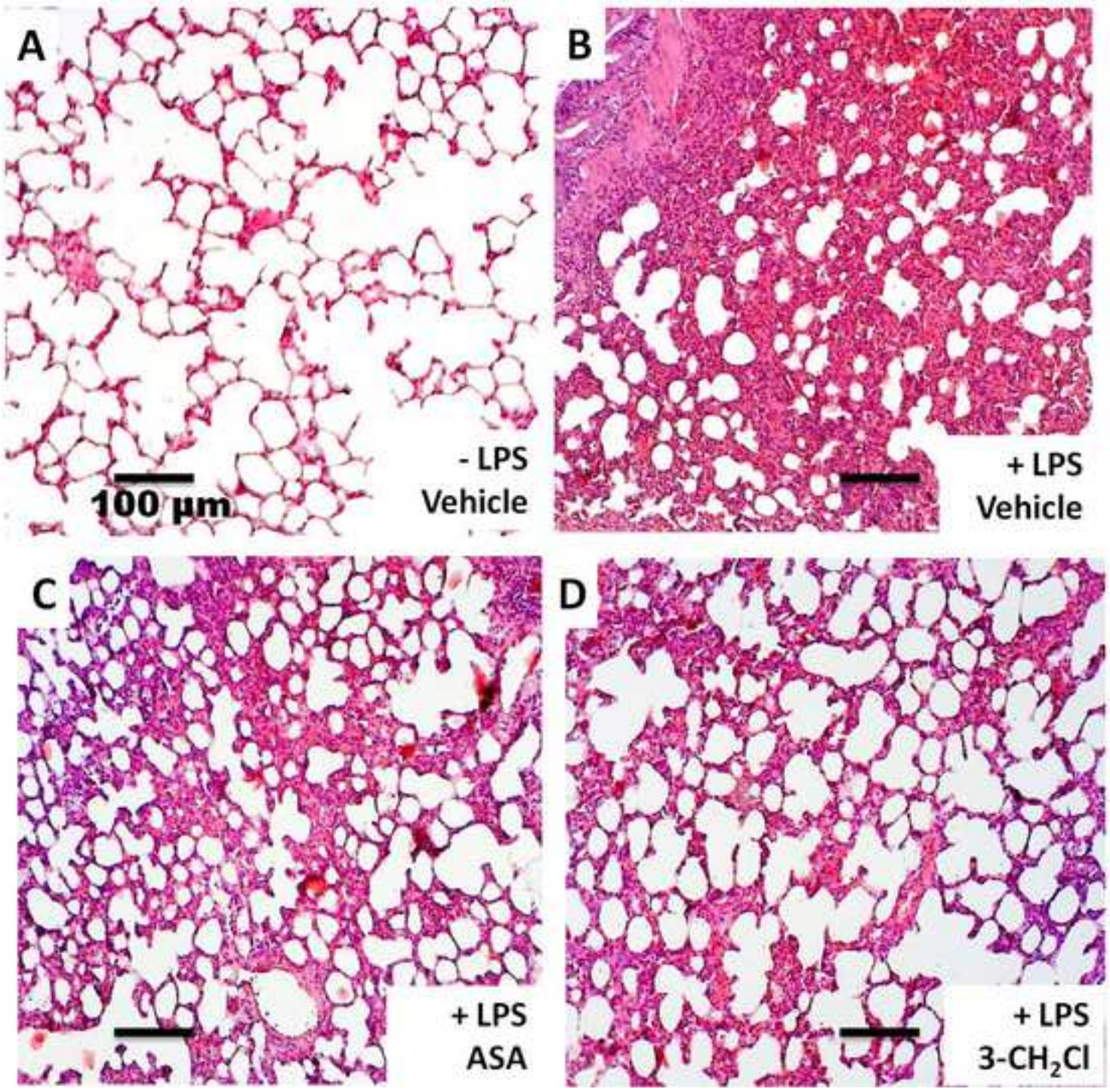

Figure 4e

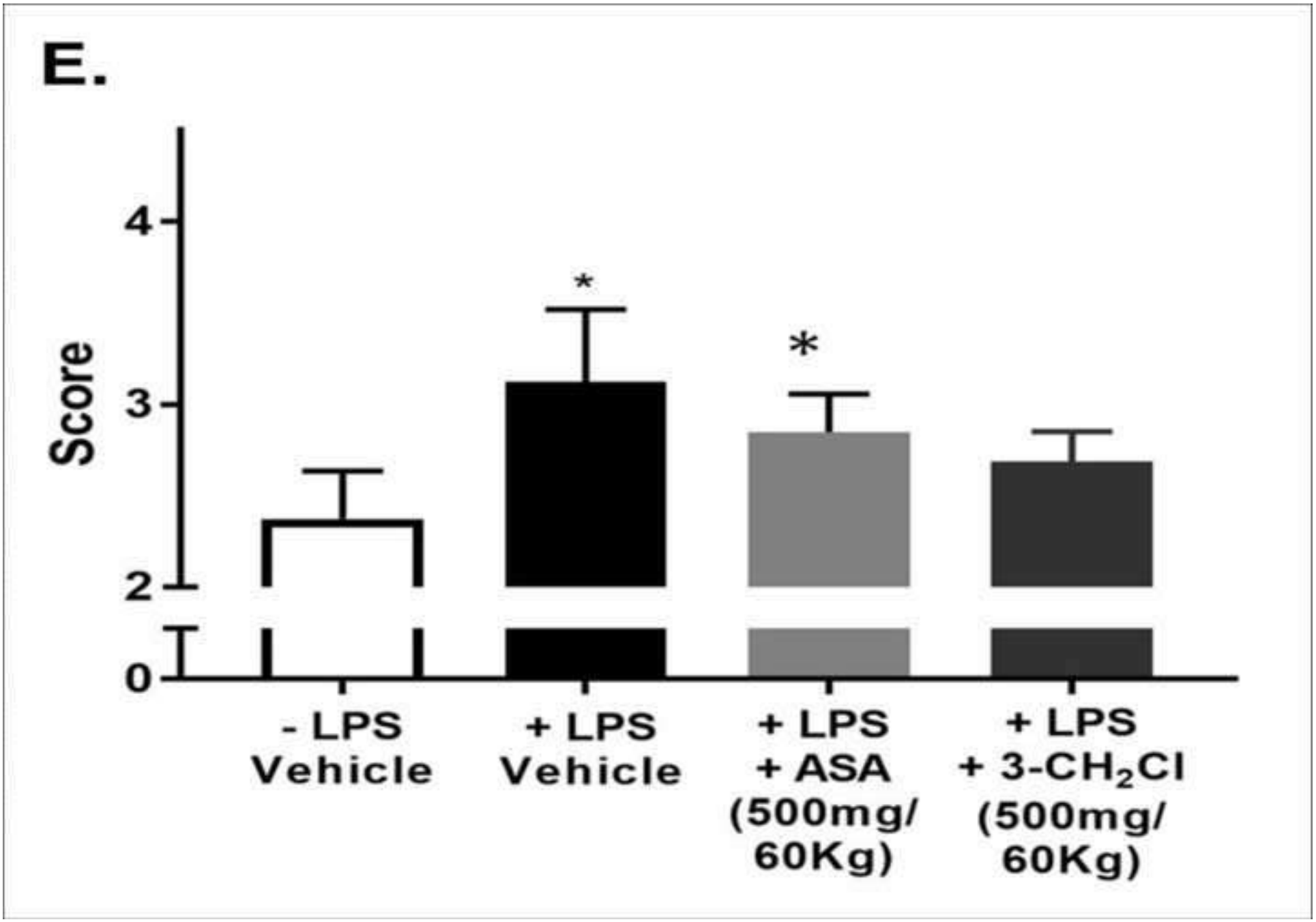

Figure 5a

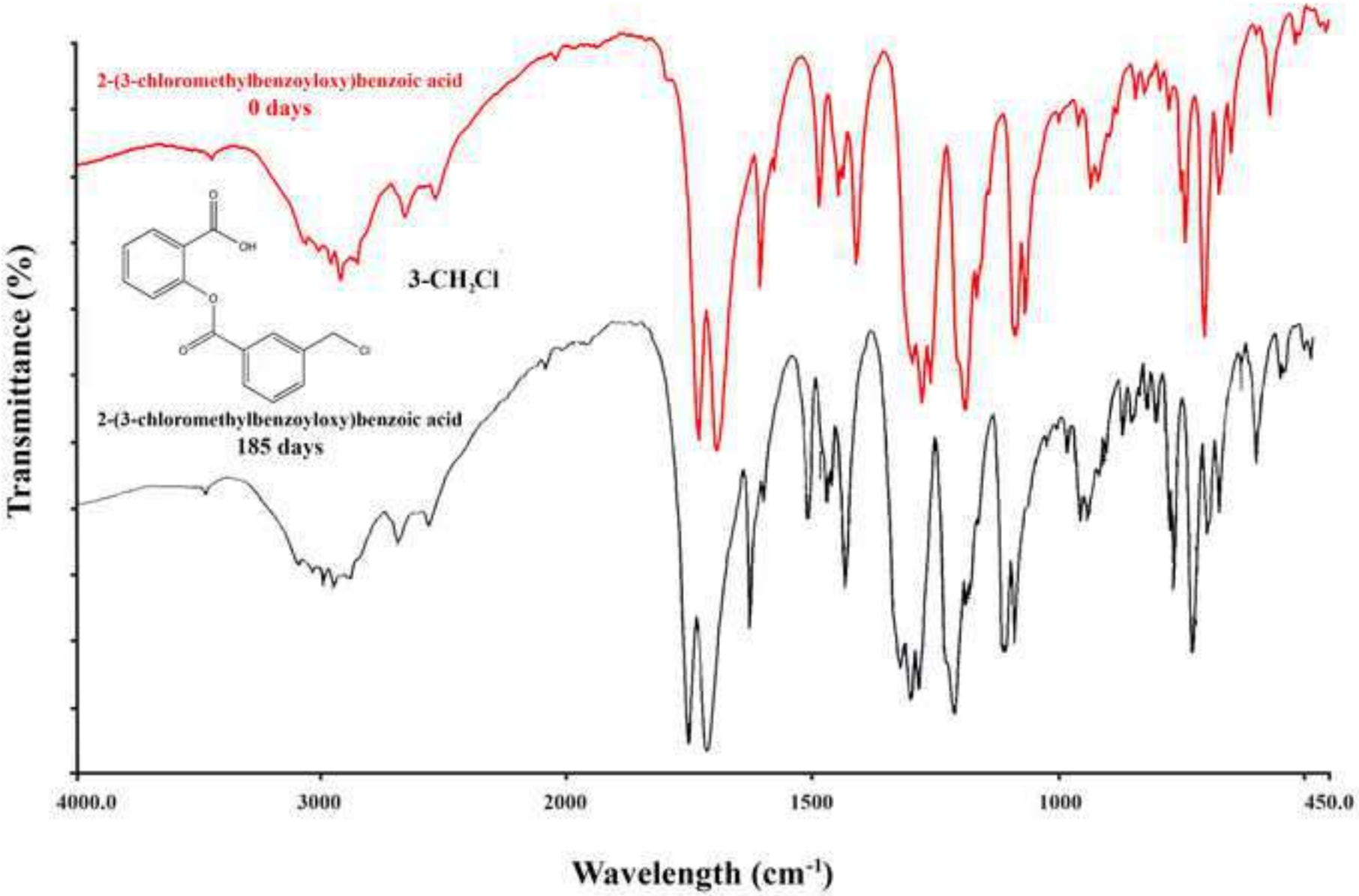

Figure 5b

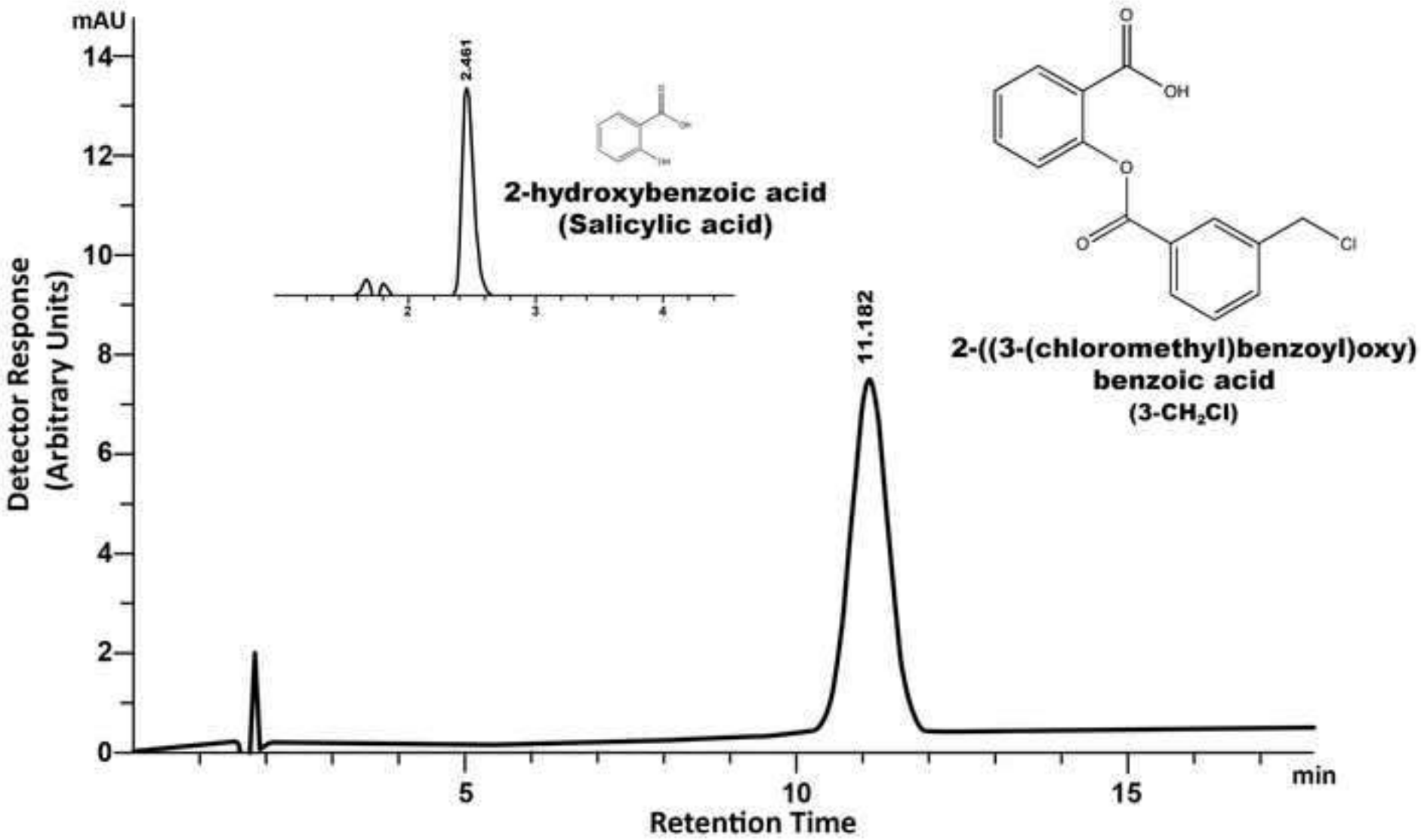

Figure 6

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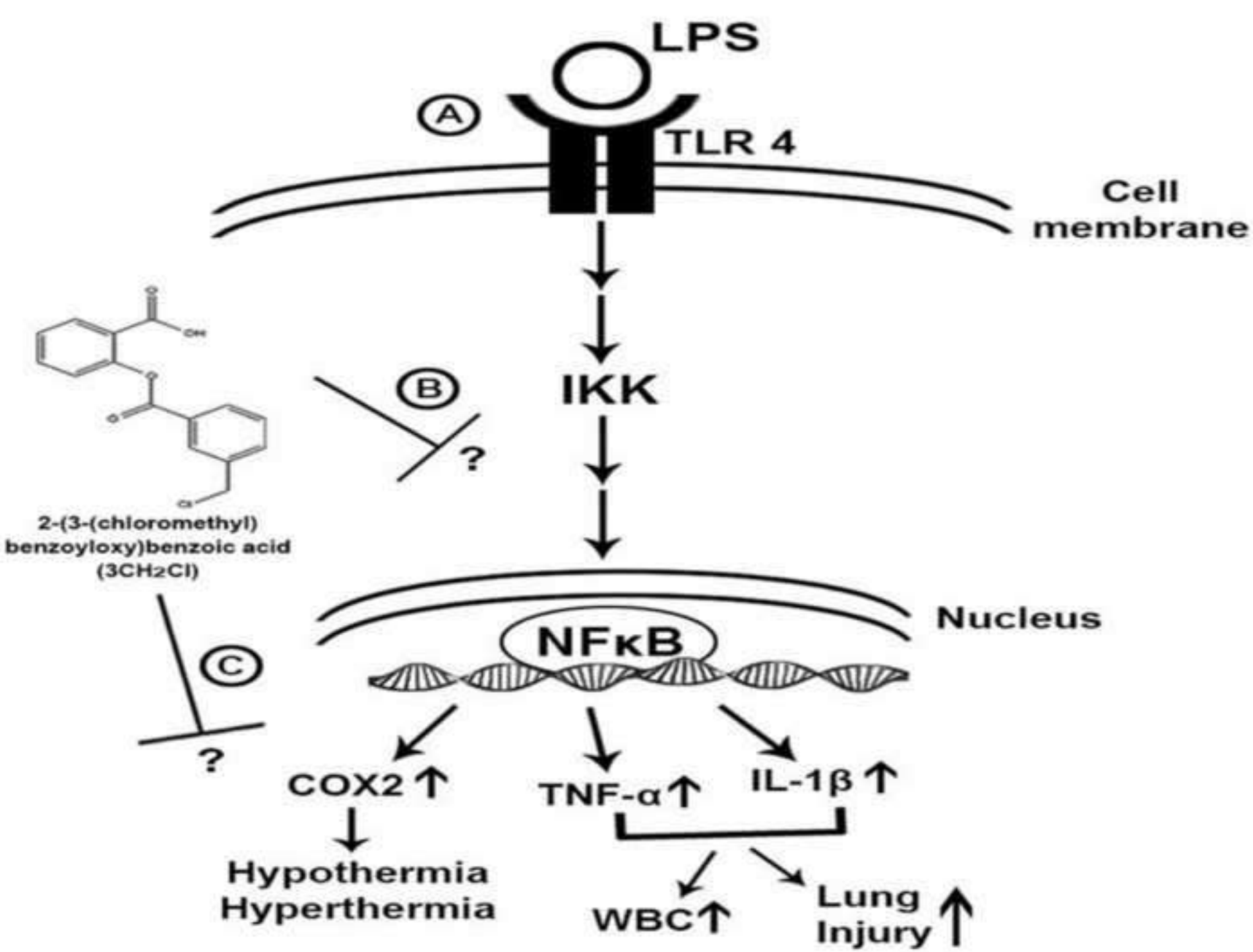

Supplementary Figure 1

Supplementary Figure 2