Short Communication

2-((3-(chloromethyl) benzoyl) oxy) benzoic acid suppresses NF-κB expression in the kidneys and lungs of LPS-Induced BALB/C mice

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Abstract

Sepsis, a life-threatening systemic inflammatory condition, is a leading cause of mortality worldwide. Its pathophysiology involves the activation of nuclear factor kappa beta (NF-κB), which promotes the release of proinflammatory cytokines. Acetylsalicylic acid (ASA), a widely used nonsteroidal anti-inflammatory drug, inhibits NF-κB but poses risks of peptic ulcer disease and nephrotoxicity. This study evaluates the efficacy of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH_2Cl), a novel salicylate derivative, in reducing NF-κB expression in the kidneys and lungs of lipopolysaccharide (LPS)-induced septic BALB/C mice. Mice were divided into four groups: untreated, LPS only, LPS + ASA (60 mg/kg BW), and LPS + 3-CH_2Cl (60 mg/kg BW). NF-κB expression was assessed via immunohistochemistry. LPS significantly increased NF-κB expression in both renal and pulmonary tissues compared to controls ($P < 0.0001$). While ASA treatment reduced NF-κB levels ($P < 0.0001$), 3-CH_2Cl demonstrated superior suppression in the renal cortex, renal medulla, and alveolar regions ($P < 0.05$). In addition, 3-CH_2Cl alleviated hypothermia in septic mice, comparable to ASA. Given its enhanced anti-inflammatory efficacy and reduced gastrointestinal risk, 3-CH_2Cl presents a promising alternative to ASA for sepsis-related inflammation management. Further studies are warranted to explore its clinical applications.

Key words: 3-CH_2Cl, acetylsalicylic acid, immunohistochemistry, inflammation, lipopolysaccharide, NF-κB

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INTRODUCTION

Sepsis, ranked as the second leading cause of global mortality, is a systemic inflammatory response, leading to organ dysfunction due to an impaired immune reaction.[1] A central mediator of sepsis is nuclear factor kappa B (NF-κB), a key transcription factor in the inflammatory cascade. NF-κB includes various subunits, with the p50/RelA dimer being most commonly activated during inflammation.[2] NF-κB activation occurs through canonical, noncanonical,

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and atypical pathways. Lipopolysaccharide (LPS), a potent inflammatory trigger, activates NF-κB through toll-like receptor 4 (TLR-4) via the canonical pathway, inducing proinflammatory mediators such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and COX-2.

Acetylsalicylic acid (ASA) is widely used for its anti-inflammatory effects at a standard human equivalent dose of 300 mg/kg BW.[3] ASA works by non-selectively inhibits both COX-1 and COX-2, with a 10–100 times stronger affinity for COX-1. This nonselectivity contributes to adverse effects such as peptic ulcer disease and potential renal toxicity, particularly in individuals with preexisting kidney disorders.

Recently, a novel salicylate derivative, 2-((3-(Chloromethyl) benzoyl)oxy)benzoic acid (3-CH_2Cl), has been developed to address ASA limitations.[4] 3-CH_2Cl is synthesized using Schotten–Baumann acylation of salicylic acid with 3-chloromethylbenzoyl chloride using pyridine as catalysator.[5] Using *in silico* model, 3-CH_2Cl demonstrates preferential COX-2 binding (G-score: −9.48 kcal/mol vs. COX-1: −5.88 kcal/mol) and reduced gastric toxicity. Histopathological analysis confirmed that rats treated with 3-CH_2Cl (50 mg/kg BW) exhibited no gastric epithelial damage, unlike ASA-treated controls.[6] In addition to improved gastric safety, 3-CH_2Cl exhibits superior analgesic activity, attributed to its higher lipophilicity (LogP = 3.73) relative to ASA (LogP = 1.21).[7] At a dose of 60 mg/kg BW, 3-CH_2Cl significantly reduced IL-1β and TNF-α levels and showed greater efficacy in preventing intra-alveolar edema compared to ASA.

Given its pharmacological advantages, 3-CH_2Cl is a promising ASA alternative. This study aims to evaluate its effect on NF-κB expression in the kidneys and lungs of LPS-induced septic mice. Animals received LPS (2 mg/kg BW) to induce sepsis, followed by oral administration of ASA or 3-CH_2Cl (60 mg/kg BW) twice daily for 2 days.[5,8,9] NF-κB expression was assessed using immunohistochemistry method, providing both semiquantitative data and histological visualization.

MATERIALS AND METHODS

Synthesis and tabletation of 3-CH_2Cl

The synthesis and tabletation process of 3-CH_2Cl followed procedures previously optimized by Caroline *et al*. and Hadinugroho *et al*.[10,11] Tablets were prepared with a total weight of 800 mg, containing 300 mg of 3-CH_2Cl per tablet. Ferric chloride test was conducted, supported by thin-layer chromatography using silica gel F254 as the stationary phase and a mixture of ethanol:acetone (9:1, v/v) as the mobile phase to ensure that 3-CH_2Cl was not degraded during the treatment process.

Experimental animals and study design

Three male BALB/C mice (1–2 months old, 20–25 g) were obtained from the Pharma Veterinary Center, Indonesia, and the study was conducted in accordance with the Ethical Eligibility Statement (No. 001/EC-FKH/Eks/2022) from the Faculty of Veterinary Sciences, Gadjah Mada University. Mice were housed under controlled conditions (20°C–24°C, 65% humidity, 12 h light/dark cycle) with *ad libitum* access to food and water and acclimatized for 14 days before intervention. They were divided into four groups (n = 3/group): untreated, LPS only, LPS + ASA, and LPS + 3-CH_2Cl. LPS (*Escherichia coli* O111:B4, Sigma-Aldrich) was administered at 2 mg/kg BW to induce inflammation, while the untreated group received phosphate-buffered saline (PBS; Genaxxon Bioscience, Germany). Treated groups received oral ASA (Bayer, Germany) or 3-CH_2Cl at 60 mg/kg BW, equivalent to a human dose of 300 mg/kg BW to represent realistic human dose. The untreated and LPS-only groups received 3% *Acacia Gum Powder* (Brataco) as a placebo. During research, the researcher were blinded to reduce bias. Drugs were given twice daily for 2 days: the first dose 1 h after LPS injection and the second 6 h later. Body temperature was recorded at 0, 30, 60, 180, 240, 360, and 720 min post-LPS. At the end of the experiment, mice were euthanized using 90 mg/kg BW ketamine and 10 mg/kg BW xylazine, followed by kidney and lung collection.

Immunohistochemistry analysis

Lung and kidney tissues from BALB/C mice were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with Mayer's hematoxylin. The sections were deparaffinized with xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked using hydrogen peroxide, followed by PBS washes and antigen retrieval in citrate buffer (pH: 6). Slides were incubated with NF-κB p65 polyclonal antibody (1:200, Bioss, USA) at room temperature for 1 h and overnight at 4°C. After PBS washes, DAB staining was applied for 10 min and counterstained with Mayer's hematoxylin. Stained tissues were visualized under an Olympus CX-31 microscope with DP21 digital camera at 400× magnification. Lung sections were evaluated for alveolar and bronchial integrity, while kidney sections were assessed for glomerular and tubular structures.

Semiquantitative NF-κB expression analysis

NF-κB expression was analyzed semiquantitatively using ImageJ v1.54d (Bethesda, Maryland, USA) with the Color Deconvolution2 plug-in, applying the H-DAB vector. The resulting DAB-specific image was binarized and inverted to black-and-white format. The mean gray value from the processed image was used to quantify NF-κB expression levels.

Statistical analysis

All data were presented as mean values accompanied by standard deviation. The values were first assessed for normality using the Shapiro–Wilk test, followed by statistical analysis using an independent samples t-test with the limit of $P < 0.05$.

RESULTS

3-CH_2Cl and acetylsalicylic acid alleviates hypothermia in BALB/C mice

The graph of body temperature measurements in mice is shown in Figure 1, with temperature values at 180, 240, and 360 min as presented in Table 1. Statistical analysis of temperature at those time points is shown in Table 2. The LPS-treated group exhibited hypothermia at 60 min. Interestingly, both ASA- and 3-CH_2Cl-treated groups showed increased temperatures compared to the untreated group (32.16 ± 0.13°C) at 180 min (ASA: 34.66°C ± 0.57°C, $P < 0.0001$; 3-CH_2Cl: 35.74°C ± 0.13°C, $P < 0.0001$). A similar pattern was observed on the 2nd day, where the treated groups showed improved temperatures at 180 min (ASA: 34.10°C ± 0.10°C, $P < 0.0001$; 3-CH_2Cl: 34.83°C ± 0.43°C, $P < 0.0001$) compared to the untreated group (32.04°C ± 0.27°C).

3-CH_2Cl decreases expression of NF-kB in the kidney and lungs of lipopolysaccharide-induced mice

Representative images of immunohistochemical staining are shown in Figure 2. NF-κB expression values are listed in Table 3, and statistical analysis results are provided in Figure 3 and Table 4. The LPS-only group exhibited higher NF-κB expression than the negative control across all tissue regions. Both ASA- and 3-CH_2Cl-treated groups showed reduced expression in all organs. Notably, the 3-CH_2Cl group displayed significantly lower expression than the LPS-only group in the glomerulus ($P = 0.0014$), medulla ($P < 0.0001$), and alveolus ($P = 0.0426$), with no significant difference in the bronchus ($P = 0.1814$).

DISCUSSION

3-CH_2Cl alleviates hypothermia in BALB/C mice

LPSs have long been recognized as one of the main causes of sepsis. A dose of 2 mg/kg of LPS can induce systemic inflammation, known as sepsis, which is characterized by hypothermia.[8,12] To date, the mechanism of hypothermia remains unclear. However, it is suspected that proinflammatory mediators such as TNF-α and COX-1 play a significant role in inducing hypothermia.[2] Nuclear factor kappa beta (NF-κB) is the most frequently activated inflammatory transcription factor. NF-κB activation triggers

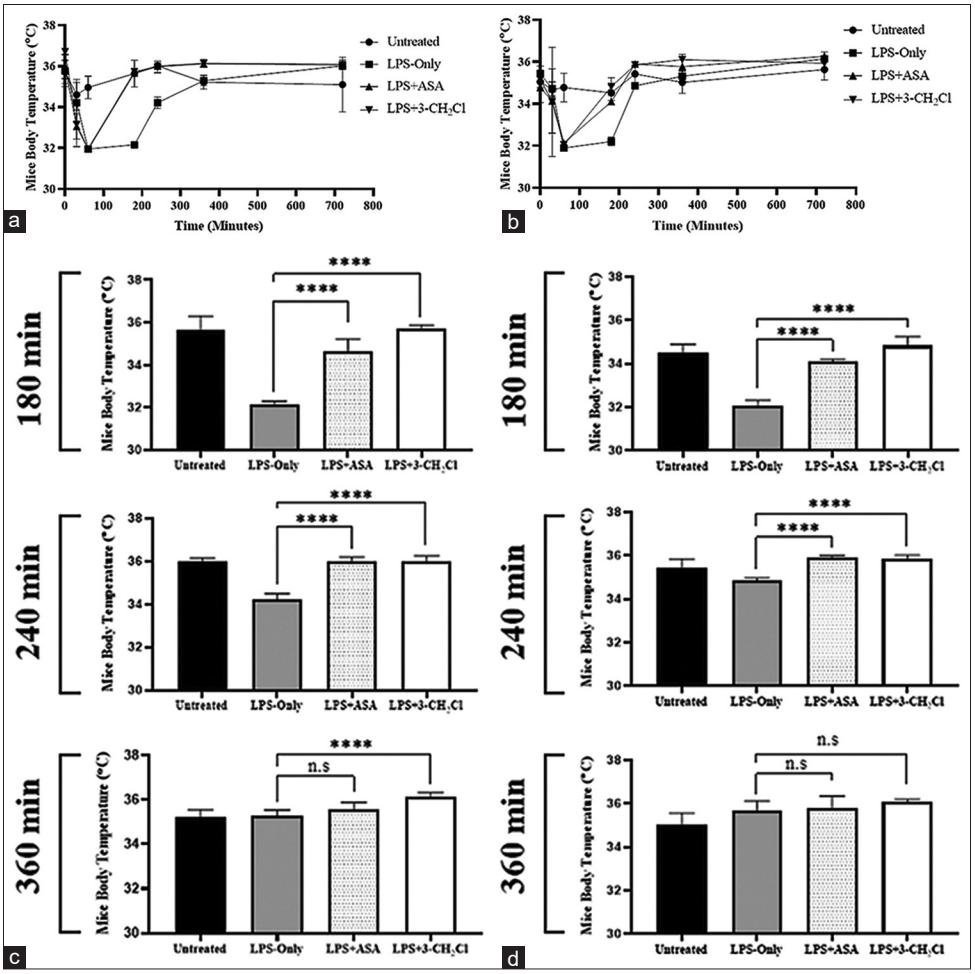

Figure 1: Lipopolysaccharide 2 mg/kg induces hypothermia in BALB/C mice. The mean body temperature of mice on (a) 1st day (b) 2nd day; body temperature comparison at 180, 240, and 360 min on (c) 1st day (d) 2nd day

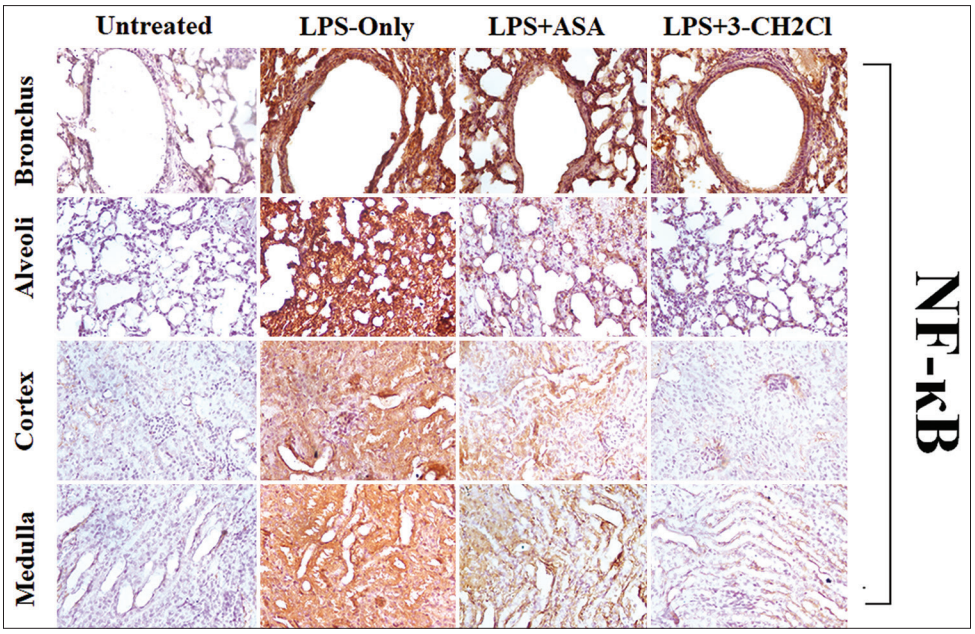

Figure 2: Representative images of NF-κB immunohistochemistry staining. Representative staining results of the lung bronchi and alveoli are presented in the upper row, while the lower row presents the renal cortex and medulla

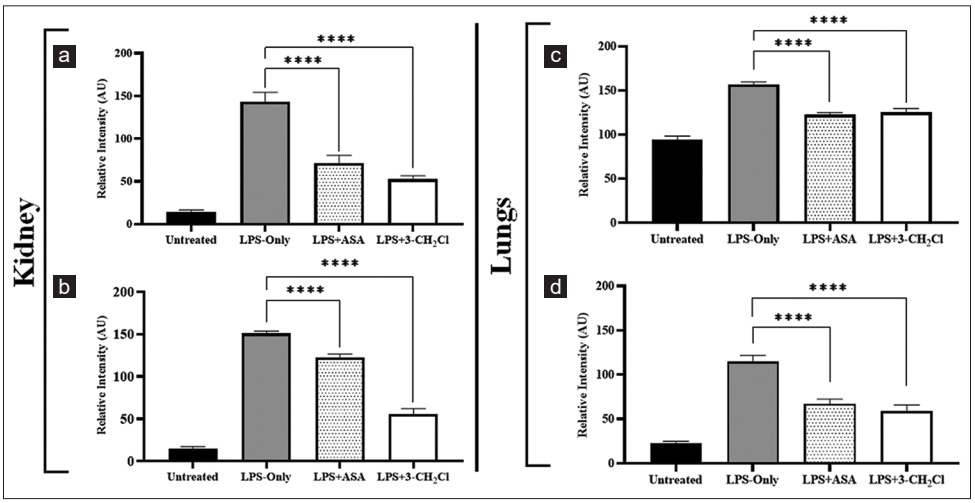

Figure 3: 3-CH_2Cl decreases NF-κB expression in kidney and lungs of lipopolysaccharide-induced BALB/C mice. Mean relative intensity of NF-kB in (a) renal cortex, (b) renal medulla, (c) lung bronchi, and (d) lung alveoli

Table 1: Mice body temperature

Group	180°C	240°C	360°C
Day 1			
Untreated	35.64±0.65	36.02±0.13	35.22±0.34
LPS-only	32.16±0.13	34.22±0.28	35.30±0.24
LPS + ASA	34.66±0.57	36.02±0.16	34.58±0.46
LPS + 3-CH_2CL	35.74±0.13	35.96±0.29	36.14±0.19
Day 2			
Untreated	34.53±0.36	35.43±0.41	35.03±0.53
LPS-only	32.04±0.27	34.86±0.11	35.66±0.46
LPS + ASA	34.10±0.10	35.90±0.10	35.77±0.59
LPS + 3-CH_2CL	34.83±0.43	35.87±0.15	36.10±0.10

Mean±SD. SD: Standard deviation, LPS: Lipopolysaccharide, ASA: Acetylsalicylic acid

the release of proinflammatory cytokines such as TNF-α, IL-6, and IL-10, as well as inflammatory mediator proteins such as COX-1 and COX-2.[13] This aligns with research conducted by Jüttler *et al.*, which states that NF-κB activation in brain neurons plays a role in the occurrence of hypothermia.

The mechanism of 3-CH_2Cl, a salicylate derivative, is believed to be similar to that of aspirin in alleviating hypothermia.[14] Salicylate class drugs (aspirin) are known to penetrate the blood–brain barrier.[15] Aspirin works by irreversibly inhibiting COX-1, COX-2, and IKBα, which are key proteins involved in NF-κB activation. The inhibition of IKBα and COX enzymes by aspirin is suspected to be the

primary mechanism, by which it alleviates hypothermia. The inhibition of NF-κB activation prevents the release of proinflammatory cytokines. Similarly, COX inhibition blocks the arachidonic acid pathway, preventing the formation of inflammatory mediators such as prostaglandins, prostacyclin, and thromboxane A2. The absence of these inflammatory mediators and proinflammatory cytokines helps alleviate hypothermia.

Table 2: Statistical analysis (*P*) of mice body temperature in each time point

Group	180	240	360
Day 1			
Untreated versus LPS-only	<0.0001	<0.0001	0.6819
Untreated versus LPS + ASA	0.0353	>0.9999	0.0361
Untreated versus LPS + 3-CH_2CL	0.7464	0.6826	0.0008
LPS-only versus LPS + ASA	<0.0001	<0.0001	0.0143
LPS-only versus LPS + 3-CH_2CL	<0.0001	<0.0001	0.0003
LPS + ASA versus LPS + 3-CH_2CL	0.0033	0.6964	0.0001
Day 2			
Untreated versus LPS-only	<0.0001	0.0205	0.0962
Untreated versus LPS + ASA	0.1088	0.1137	0.1400
Untreated versus LPS + 3-CH_2CL	0.3238	0.1429	0.0197
LPS-only versus LPS + ASA	<0.0001	<0.0001	0.7827
LPS-only versus LPS + 3-CH_2CL	<0.0001	<0.0001	0.1651
LPS + ASA versus LPS + 3-CH_2CL	0.0372	0.7676	0.3864

LPS: Lipopolysaccharide, ASA: Acetylsalicylic acid

3-CH_2Cl decreases NF-κB expression in kidneys and lungs of BALB/C mice

Overall, the pattern of NF-κB expression fluctuations in the kidneys and lungs follows a similar trend across different groups.[16] The LPS group exhibited a significantly higher NF-κB expression compared to the untreated group, both in the renal cortex and medulla. This aligns with the mechanism by which LPS binds to TLR-4 on tubular epithelial cells, triggering an inflammatory response through the canonical NF-κB signalling pathway [Figure 4]. Two key protein complexes involved in this pathway include the NEMO complex (NF-κB essential modulator), IKKα, and IKKβ (inhibitory kappa B kinase β), along with the IκBα complex (inhibitory subunit of NF-κBα), p50, p65, and PKAc (protein kinase A catalytic subunit).[17,18] Activation of IKKβ leads to the phosphorylation of IκBα, followed by its ubiquitination. Once ubiquitinated, IκBα

Table 3: Renal nuclear factor kappa beta expression in BALB/C mice

Group	Renal glomerulus (AU)	Renal medulla (AU)	Bronchus (AU)	Alveoli (AU)
Untreated	14.19±2.39	14.20±3.16	22.51±2.55	94.08±4.50
LPS-only	143.30±11.01	151.00±2.73	114.70±7.40	156.70±2.98
LPS + ASA	71.03±9.56	122.60±3.76	67.25±5.12	122.70±2.50
LPS + 3-CH_2CL	52.34±4.27	55.92±6.09	58.68±7.45	125.40±4.10

Mean±SD. SD: Standard deviation, AU: Arbitrary unit, LPS: Lipopolysaccharide, ASA: Acetylsalicylic acid, BALB/C: Bagg albino laboratory-bred / c substrain

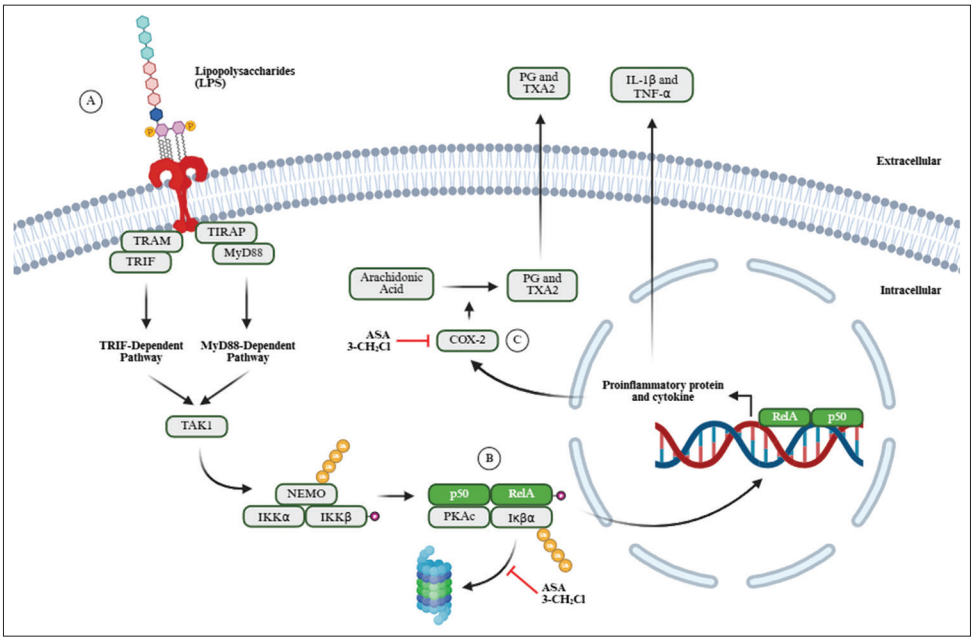

Figure 4: Mechanism of action of 3-CH_2Cl in the suppression of NF-κB expression. (A) The canonical inflammatory pathway at the cellular level is activated by the binding of a ligand to toll-like receptor-4. (B) 3-CH_2Cl decreases NF-kB expression, presumably by inhibiting IkBα degradation. (C) The antipyretic effect of 3-CH_2Cl is hypothesized to be due to its binding to the COX-2 receptor

Table 4: Statistical analysis (*P*) of nuclear factor kappa beta expression among groups

Group	Glomerulus	Medulla	Bronchus	Alveoli
Untreated versus LPS-only	<0.0001	<0.0001	<0.0001	<0.0001
Untreated versus LPS + ASA	<0.0001	<0.0001	<0.0001	<0.0001
Untreated versus LPS + 3-CH_2CL	<0.0001	<0.0001	<0.0001	<0.0001
LPS-only versus LPS + ASA	<0.0001	<0.0001	<0.0001	<0.0001
LPS-only versus LPS + 3-CH_2CL	<0.0001	<0.0001	<0.0001	<0.0001
LPS + ASA versus LPS + 3-CH_2CL	0.0014	<0.0001	0.1814	0.0426

LPS: Lipopolysaccharide, ASA: Acetylsalicylic acid

undergoes degradation via the proteasome, enabling NF-κB activation and its translocation into the nucleus. A key marker of canonical NF-κB activation is the phosphorylation of p65 at Ser276 and Ser536, a process facilitated by PKAc.

Groups treated with ASA and 3-CH_2Cl demonstrated significantly lower NF-κB expression levels compared to the LPS group, suggesting that these compounds effectively inhibit NF-κB activation. The mechanism underlying this inhibition is believed to be similar to that of ASA.[15] ASA (300 mg/kg BW) acts as an irreversible competitive inhibitor of ATP (adenosine triphosphate) binding to IKKβ, thereby reducing NF-κB activation. Furthermore, the LPS + 3-CH_2Cl group exhibited lower NF-κB expression than the LPS + ASA group, although the difference was not statistically significant.[6] This may be attributed to the higher LogP value of 3-CH_2Cl compared to ASA, indicating that 3-CH_2Cl is more lipophilic, allowing for greater cell membrane permeability. The increased intracellular concentration of 3-CH_2Cl enhances its ability to inhibit IκBα, leading to a greater reduction in NF-κB expression.

CONCLUSION

Our study demonstrates that both 3-CH_2Cl and ASA effectively counteract sepsis-induced hypothermia with no significant difference between them. However, 3-CH_2Cl showed superior ability to suppress NF-κB expression, likely due to its more favorable physicochemical properties. These findings support the potential development of 3-CH_2Cl as an alternative compound to ASA.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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