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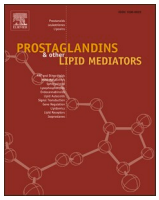

2-(3-(Chloromethyl)benzoyloxy)benzoic Acid reduces prostaglandin E-2 concentration, NOX2 and NFKB expression, ROS production, and COX-2 expression in lipopolysaccharide-induced mice

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ABSTRACT

Introduction: Inflammation is a fundamental response to various insults, including microbial invasion and tissue injury. While aspirin (ASA) has been widely used for its anti-inflammatory properties, its adverse effects and limitations highlight the need for novel therapeutic alternatives. Recently, a novel salicylic acid derivative, 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH_2Cl), has emerged as a potential substitute for ASA, offering a simpler, environmentally friendly synthesis and a promising safety profile.

Aim of the study: This research aims to evaluate the anti-inflammatory mechanism of 3-CH_2Cl in a lipopolysaccharide (LPS)-induced mouse model, focusing on its effects on prostaglandin E-2 (PGE-2) concentration, NOX2 and NFkB expression, ROS production, and COX-2 expression.

Material and methods: Utilizing BALB/C mice subjected to LPS-induced inflammation, we investigated the therapeutic potential of 3-CH_2Cl. The study included synthesis and tablet preparation, experimental design, peripheral blood plasma PGE-2 measurement, splenocyte isolation and COX-2 expression analysis, nitric oxide and ROS measurement, and immunohistochemical analysis of NOX2 and NFkB expression.

Results: 3-CH_2Cl significantly reduced PGE-2 levels ($p = 0.005$), NO concentration in liver homogenates ($p = 0.005$) and plasma ($p = 0.0011$), and expression of NOX2 and NFkB in liver ($p < 0.0001$) and splenocytes ($p = 0.0036$), demonstrating superior anti-inflammatory activity compared to ASA. Additionally, it showed potential in decreasing COX-2 expression in splenocytes.

Conclusion: 3-CH_2Cl exhibits potent anti-inflammatory properties, outperforming ASA in several key inflammatory markers in an LPS-induced inflammation model. The reduction of COX-2 expression, alongside the reduction of pro-inflammatory cytokines and oxidative stress markers, suggest it as a promising therapeutic agent for various inflammatory conditions.

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1. Introduction

Inflammation constitutes a crucial adaptive response to various internal and external insults, including microbial invasion and tissue injury. Aspirin, or acetylsalicylic acid (ASA) derived from salicylic acid, has been a cornerstone in medicine for decades due to its potent anti-inflammatory and analgesic properties. However, several drawbacks associated with its use underscore the urgency of finding a novel salicylic acid derivative to replace it. These drawbacks include risks of gastrointestinal irritation, bleeding disorders, and potential complications like Reye's syndrome, particularly in pediatric populations [23]. Additionally, aspirin shows limitations due to resistance in preventing cardiovascular events [9].

The development of new derivatives of salicylic acid holds promises in addressing these shortcomings, potentially retaining the benefits of aspirin while minimizing its adverse effects. Recently, a novel salicylic acid derivative called 2-((3 (chloromethyl)benzoyl)oxy)benzoic acid (3-CH_2Cl, MR = 277.31 g/mol) was introduced as a potential alternative compound to substitute ASA. The synthesis of 3-CH_2Cl is relatively simple and environmentally friendly. Using biphasic aqueous basic conditions (with acetone as a solvent), 3-CH_2Cl forms spontaneously when the precursor compound 3-chloromethylbenzoylchloride reacts with SA under 5 min of exposure to 600-Watt microwave irradiation or heat-induced reflux method [6,25].

To deliver an accurate dosage orally, Hadinugroho et al. [11] suggested the tablet-form of 3-CH_2Cl using sodium lauryl sulfate (SLS) as surfactants, and croscarmellose sodium (CS) as a disintegrating agent [11]. Pharmacokinetics studies indicate the high lipophilic properties (log P = 3.73) and longer elimination time of 3-CH_2Cl, suggesting extensive distribution of this compound in deep tissues during absorption [6,26,28]. Unlike ASA, which produces salicylic acid degradation products rapidly, no degradation product such as salicylic acid was observed with 3-CH_2Cl until 3 years at 25°C with a relative humidity of 75 ± 5 % [27]. Furthermore, the lethal dose (LD50) of 3-CH_2Cl is below 2000 mg/60 kg body weight (BW), similar to ASA. Histopathological observations of mice treated with 3-CH_2Cl (60 mg/kg BW) showed a significant reduction of gastric mucosal erosion compared to ASA at equal concentrations [26]. Additionally, treatment with 3-CH_2Cl did not harm liver, heart, lung, or kidney functions in rats. Although this was a toxicity observation, it warrants investigating 3-CH_2Cl as a potential ASA substitute.

NSAIDs inhibit pain by suppressing prostaglandin production, primarily via COX inhibition [16]. The nonselective inhibition of ASA and other compounds towards COX-1 enzyme is the main reason for its adverse effect due to disruption of cellular homeostasis [4,5,28]. Therefore, many studies shifted towards the discovery of drugs with better selectivity towards COX-2 inhibition. Induction of COX-2 expression is triggered by several pathways, such as activated toll-like receptors (TLR) and NFkβ pathways [17,31]. The in-silico docking experiment of diverse salicylic acid bearing compound with human COX-2 receptor protein (PDB: 5F1A) using Grid-Based Ligand Docking with Energetic (Glide) scoring system shows that 3-CH_2Cl (Glide Score 3-CH_2Cl = – 9.48 kcal/mole) had better affinity towards COX-2 than ASA (Glide Score - 5.88 kcal/mole) [6]. Moreover, studies in rats show that 3-CH_2Cl exhibits dose-dependent increases in nociceptive response time and decreases in response count, indicating potential analgesic superiority over ASA. The optimal analgesic, antipyretic and anti-inflammatory activity of 3-CH_2Cl peaked at 120 mg/kg in rat dosage [25] or 60 mg/kg in mice dosage [26], equivalent with ~ 350 mg/60 kg body weight in human. However, the direct impact of 3-CH_2Cl on blood prostaglandin concentration remains under investigation. Additionally, 3-CH_2Cl shows promise as an anti-thrombotic agent, as evidenced by reduced platelet aggregation events and prolonged tail-bleeding time following oral administration. Its interaction with platelet COX suggests a mechanism involving inhibition of $TBXA_2$ production, but further research is needed to confirm its specific effects on platelet receptors, particularly the $TBXA_2$ receptor [24].

Further investigations into the anti-inflammatory properties of 3-CH_2Cl have revealed promising results. Administering a single dose 120 mg/kg of 3-CH_2Cl to Wistar rats subjected to intravenous LPS significantly decreased pro-inflammatory cytokine levels, stabilized body temperature, and prevented LPS-induced septic shock [26]. Additionally, 3-CH_2Cl treatment reduced cardiac white blood cell count, pulmonary oedema, and hepatocyte injury, while histological examination showed a significant decrease in lung fibroblasts. These findings suggest that 3-CH_2Cl may be effective in treating a range of acute lung injury-related conditions, including sepsis, pneumonia, and COVID-19. Although the molecular mechanisms underlying 3-CH_2Cl's anti-inflammatory effects require further investigation, preliminary studies suggest its potential involvement in the canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway through reduction the plasma concentration of pro-inflammatory cytokines TNF-α, and IL-1β, which are associated with LPS-induced inflammation via the Toll Like receptor-4 (TLR-4) [27]. Salicylic acid bearing molecule such as ASA has been shown to inhibit the LPS-activated NFkB pathway [21], and indirectly reduce the NADPH oxidase (NOX2) expression and subsequent ROS production. This effect can help to mitigate oxidative stress during inflammation and might be linearly related to 3-CH_2Cl's anti-inflammatory mechanism.

Interestingly, the administration of a single dose of 60 mg/kg 3-CH_2Cl could decrease the CD4+ T-cell population and increase CD4+ Treg population mediated by the increase of FoxP3 expression in LPS-induced inflammation mice [27]. CD4+ Tregs have been known to respond to natural (e.g., pathogenic bacteria or self-antigen during autoimmunity or graft rejection) or inducible inflammatory conditions (e.g., LPS) by increasing their suppressive function [29]. This finding leads us to another potential of 3-CH_2Cl, which may act as a potential therapeutic drug to reduce inflammatory conditions, especially Treg-mediated autoimmunity, and graft rejection.

Overall, 3-CH_2Cl represents a fascinating compound with diverse chemical and pharmacological properties, holding promise for various applications in both research and industry. This potential arises from the limitations and adverse effects associated with aspirin (ASA), particularly its gastrointestinal irritation, bleeding disorders, and potential complications like Reye's syndrome. However, to support this statement, the molecular mechanism of 3-CH_2Cl-mediated anti-inflammatory activity needs elucidation. To further explain the mechanism of action of previously reported observations, the aim of this study is to investigate whether 3-CH_2Cl's anti-inflammatory properties could inhibit plasma PGE-2 concentration, COX-2 expression, inflammatory-related oxidative stress inducer NOX2, free radical's nitrite oxide (NO) and superoxide ($O_2^{\bullet}$), and canonical inflammatory transcription factor NFκB. To address this question, we stimulate the Balb/C mice with high dose LPS (2 mg/kg BW), and a single oral analgesic and anti-inflammatory dose (60 mg/kg) of 3-CH_2Cl.

2. Material and methods

2.1. Synthesis and tablet preparation of 3-CH_2Cl

The synthesis of 2-(3-(chloromethyl)benzoyloxy) benzoic was performed according to the previously published methods [6,26,27]. In brief, 1.8 mmol salicylic acid (Brataco, Surabaya, Indonesia), 7.2 mmol 3-chloromethylbenzoylchloride (Sigma-Aldrich, St. Louis, MO, USA), 1.7 × 10^{-6} mmol pyridine (Merck, Darmstadt, Germany), and 1.48 ×10^{-6} mmol acetone (Merck) were mixed homogenously. After heat induction for 1 min × 2 times (with stirring in-between) at 600 W using a Millstone Organic Synthesis Unit microwave (MicroSYNTH, Göttingen, Germany), the mixture was characterized by thin layer chromatography methods using silica gel F254 (Merck) as stationary phase, and hexane-ethanol (1:2 v/v) as mobile phase. Furthermore, the Salicylic acid impurity test was performed using ferric chloride ($FeCl_3$) test. To

prepare the 3-CH_2Cl tablet, direct compression methods was performed [11]. In brief, 9.38 % Neusilin (Gangwal Chemicals, Mumbai, India) was used to homogenize 300 mg of 3-CH_2Cl. After the mixture was moved to a cubic mixer, 0.92 % sodium lauryl sulfate (Sigma-Aldrich), 2.33 % croscarmellose sodium (FMC Biopolymer, Philadelphia, USA), 5 % microcrystalline cellulose (Gujarat Microwax, Gujarat, India), and spray dried lactose (Foremost Farms, Pasig, Philippines) were added, and rotated for two minutes at 100 rpm (Erweka). A single punch machine (Jenn Chian Machinery, FengYuan, Taiwan) was used to compress the final tablet. The final tablet, containing 300 mg of ASA or 3-CH2Cl, was assessed with regard to hardness, friability, disintegration time, and drug dissolution parameters.

2.2. Experimental animals and design

Twelve BALB/C male mice (1–2 months old; 20–25 g) were obtained from the Pharma-Veterinary Center of Surabaya, Indonesia. The mice were housed in a temperature-controlled room (20–24 °C) with 65 % relative humidity and a 12-h light/dark cycle, and were acclimatized for 21 days before the intervention. The animal procedures and handling were performed in accordance with institutional animal care and use committee (IACUC) guidelines and ethically approved protocols. The consumption of food and water was given ad libitum for 24 days, including 21 days of acclimatization, and the temperature was recorded twice a day. The mice were grouped into 4 groups (n = 3 for each group) named untreated, LPS-only, LPS + ASA, and LPS + 3-CH_2Cl. To induce inflammation in the LPS-only, LPS + ASA, and LPS + 3-CH_2Cl groups, lipopolysaccharide (LPS) from Escherichia coli O111:B4 (Sigma-Aldrich) was administered intraperitoneally with a dose of 2 mg/kgBW. Meanwhile, the untreated group was injected with Phosphate-Buffered Saline (Genaxxon Bioscience, Ulm, Germany) only. ASA (Bayer, Leverkusen, Germany) or 3-CH_2Cl was given orally to the mice with a single dose of 60 mg/kgBW, respectively, in accordance to the previously published works [26,27,6] The untreated and LPS-only group were given 3 % Pulvis Gummi Arabicum (Brataco) as the placebo for the drugs. 3-CH_2Cl and ASA were given twice a day, one hour after LPS administration and six hours after the first treatment. The treatment was repeated for three consecutive days. At the end of the experiment, the animals were euthanized using 90 mg/kgBW ketamine and 10 mg/kgBW xylazine to isolate the blood plasma, spleen, liver, kidney, and lung.

2.3. Peripheral blood plasma PGE-2 measurement

Peripheral blood plasma was collected by inserting a heparin-coated microhematocrit blood capillary tube (Marienfeld, Königshofen, Germany) into the sinus orbitals. After centrifugation (1000 × *g*, 5 min.) 50 µl of peripheral blood plasma was diluted with ELISA-dilution buffer (1:1) to evaluate the PGE-2 concentrations (catalog no. E-EL-0034c, Elabscience). Following manufacturer's instructions, each sample was evaluated in duplicate. Thermo Scientific Multiskan FC systems (402 nm detection wavelength) were used to detect levels of PGE-2. All procedures were performed according to the manufacturer's instructions.

2.4. Splenocyte isolation

Splenocyte isolation was performed according to the previously optimized protocol [27]. The spleen was incubated for 30 min with a buffer containing 100 U/ml collagenase type IV (Worthington, Lakewood, NJ, USA), 0.2 % DNAse (Worthington), and RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA). The resulting cell suspensions were filtered through a 70 µm cell strainer (Biologix, Shandong, China). To lyse the red blood cells, ammonium-chloride-potassium buffer was added to the cell suspensions. After 2 min of incubation on ice, the splenocytes were centrifuged for 5 min at 1500 rpm, and the cell pellet was washed with 0.5 % bovine serum albumin (BSA) in PBS. The pellet was then resuspended in 0.5 % BSA in PBS. To adjust the splenocyte concentration until it reached 5×10^6 cells/ml, the splenocytes were stained with 0.5 % trypan blue (Contacare Ophthalmics and Diagnostics, Vadodara, India) and counted under a light microscope (100x magnification).

2.5. Splenic endogen COX-2 measurement with flow cytometry

For extracellular staining and specific sorting of T-lymphocytes, splenocytes (5×10^6 cells/ml) were incubated with 10 µg of PE Anti-Mouse CD3 Antibody (Elabscience) at room temperature for 20 min in the dark. The splenocytes were washed with 0.5 % BSA in PBS, centrifuged for 5 minutes at 1800 rpm, and the supernatant was discarded. For intracellular staining, splenocytes were resuspended with eBioscience Permeabilization Buffer (Thermo Fisher Scientific) and incubated for 10 minutes in the dark. The splenocytes were washed twice and resuspended in 0.5 % BSA in PBS. Ten µg of APC Anti-Mouse COX-2 Antibody clone sc-19999 (Santa Cruz Biotechnology, Texas, USA) was added to the splenocytes. After further washing and the addition of permeabilization buffer, the splenocytes were fixed with BD Cytofix fixation buffer (Becton Dickinson, Franklin Lakes, NJ, USA), and analyzed with flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson) and Flowing Software (Turku Bioscience, Turku, Finland). The data from 10,000 events were acquired. Firstly, the area with the densest cell population was determined. The population of CD3+ T-cells was then selected by selecting the CD3 option in FL-1 channel. Then the T-cells specific COX-2 expression was measured using histogram analysis of CD3+ populations by selecting the COX-2 option in FL-2 channel and the mean fluorescence intensity was acquired.

2.6. Nitric oxide measurement using Griess assay

100 µl of test samples (undiluted plasma or 100 mg/ml liver homogenate) were centrifuged (1000 × *g* for 15 min), then transferred to a 96-well plate. To precipitate protein [30], 100 µl of 8 g/liter Vanadium chloride (VCl3) solution dissolved in 1 molar hydrochloric acid (HCl) was used. Next, the Griess reagent consisted of freshly prepared 50 µl N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) 0.1 % dissolved in distilled water (v/v), and 50 µl Sulfanilamide (SULF) 2 % (wt/v) dissolved in 5 % HCl was used. Color changes were read using a spectrophotometric tool (540 nm), after incubation for 10 min at room temperature, proportional to optical density (OD). Quantification and validation of NO in samples were carried out by creating a linear OD equation formed from various concentrations of $NaNO_2$ (0, 50 µM, 100 µM, 150 µM, 200 µM in PBS) and entering the sample OD value in the linear equation. After the molar conversion, the data was presented as NO in µg/ml plasma or homogenate.

2.7. Immunohistochemistry analysis

Organ specimens used included spleen, lung, kidney, and liver from BALB/C mice. They were fixed in 10 % neutral-buffered formalin, embedded in paraffin, cut into 5-µm sections, and stained with Mayer's hematoxylin. For the immunohistochemical analysis, the staining steps of the paraffin sections following dewaxing and hydration were as follows: Immunohistochemistry slides were deparaffinized in a standard fashion, washed in xylene 3 times for 5 minutes each, followed by absolute alcohol, alcohol 96 %, alcohol 90 %, alcohol 80 %, and alcohol 70 %. They were then treated with Hydrogen Peroxide Block for 5 min, washed in PBS 2 times for 5 min each. Afterward, the slides were treated in citrate buffer (composition: 10 mM sodium citrate, 0.05 % Tween-20, and HCl 1 N for adjusting pH to 6.0) for antigen retrieval at pH 6 for 5 min, followed by another wash in PBS. Slides were then incubated for 1 hour at room temperature with the primary antibody NF-kB p65 Polyclonal Antibody from Bioss, (Massachusetts, USA), and mouse monoclonal antibody rabbit anti- NOX2/gp91phox Polyclonal Antibody

clone BS-3889R from Bioss (Massachusetts, USA), at 4 °C overnight and 1:200 antibody dilution factor. The slides were washed in PBS 2 times for 5 min each at room temperature and then treated with impact DAB for slides with the primary antibody, incubating sections for 10 min at room temperature, followed by another wash with PBS 2 times for 5 min each. Counterstaining was done using Mayer's modified hematoxylin solution. The results were observed using a microscope (Olympus CX-31; Olympus, Tokyo, Japan) with a digital camera (Olympus DP21) at 400 × optical magnification. Observation of the renal cortex, including glomerulus and medulla with tubulus section, was conducted for the kidney. For the liver organ, observation was done on the perivascular and pericentral areas. Meanwhile, for the spleen, the red and white pulp in the central area of the sample was observed, along with alveoli and bronchi for the lungs sample.

2.8. Semi-quantitative NFkB and NOX2 expression analysis

For semi-quantitative NFkB and NOX2 expression data, ImageJ v1.54d software (Bethesda, Maryland, USA) was utilized. The Color Deconvolution2 plug-in with H.DAB was used as the color vector and the output eight-bit transmittance was used to separate image into 3 color panels [12]. Color 1 represent haematoxylin staining (blue or purple), color 2 represent DAB staining (brown), and color 3 represent another unspecific staining. The DAB staining image was chosen, binarized, resulting in a black and white image, and then inverted using the invert tools. Finally, the processed image was measured and with the mean gray value was chosen as representative value of NFkB or NOX2-expression. The box size used to measure the liver was 800 × 600 pixels, except for the selected red pulp and white pulp area, where a 140 × 140 pixels box was used.

2.9. In-vitro reactive oxygen species (ROS) measurement

ROS production in splenocytes was assessed using the ferricytochrome-c assay as described [2]. In brief, Microtiter wells were coated with 50 μL of BSA, (10 μg/ml) overnight at 4 °C and washed twice with 150 μL Hank's Balance Salt Solution (HBSS; Gibco BRL) containing Mg2+ and Ca2+ (PAA Laboratories). After washing, wells were blocked with 50 μL of 3 % BSA for 1 h at 37°C and washed. Aliquots of 50 μL splenocytes (5×10^6 cells in HBSS) were incubated for 5 min at room temperature with 20 μL of superoxide dismutase (SOD; 3000 U/ml, Sigma Aldrich, Saint Louis, MO) or 20 μL HBSS. Subsequently, each sample was treated with 5 μL LPS (5 μg/ml in HBSS) with or without 5 μL salycylic acid derivate (50 or 500 μM). After transfer into microtiter wells, a mixture of 10 μL of 1 mM ferricytochrome (Sigma Aldrich) and 65 μL HBSS was added, and superoxide production was measured at 550 nm in duplicates using a microtiter reader (Thermo Scientific Multiskan FC systems) for 2 h after incubation at 37 °C and 5 % CO_2. The superoxide production (nmol $O_2^{\bullet-}$/5 × 10^6 splenocytes) was calculated as mean OD "with SOD" minus "without SOD" × (100/8.39), as recommended by the manufacturer (Sigma Aldrich). Data was subtracted with values from untreated samples and blank OD for normalization.

2.10. Statistical analysis

GraphPad Prism version 8.0.1 was used for data analysis. Unpaired t-tests were employed to compare relative intensity differences between specific groups. A p-value < 0.05 is considered significant. All data were presented as mean ± standard deviation (SD).

3. Results

3.1. 3-CH_2Cl decreases the PGE-2 concentration in the peripheral blood plasma of LPS-induced mice

To analyze the PGE-2 level as a common inflammatory marker, the peripheral blood plasma was assayed with ELISA, and the calculated PGE-2 concentration (pg/ml) is displayed in Fig. 1**A**. The PGE-2 level in the untreated cohort (72.57 ± 7.05 pg/ml) is significantly lower compared to the LPS-only cohort (121.17 ± 33.04 pg/ml; p = 0.0282), indicating that the elevated PGE-2 level was mediated by intravenous LPS administration. Meanwhile, the PGE-2 level in the LPS + ASA cohort (40.02 ± 14.42) showed a significant decrease compared with the untreated mice (p = 0.0067) and LPS-only (p = 0.0041). Moreover, the oral administration of 3-CH_2Cl in LPS + 3-CH_2Cl mice (34.18 ± 21.52) showed a significant decrease compared with the untreated mice (p = 0.0147). Both results indicate that the addition of a salicylic acid-bearing compound, even in the inflammatory condition, could dramatically reduce the PGE-2 level in blood plasma below the normal condition. The significant decrement of PGE-2 was observed dramatically when the result of LPS + ASA (p = 0.0041) and LPS + 3-CH_2Cl (p = 0.0045) was compared to the LPS-only mice. Even though the 3-CH_2Cl cohort shows the lowest mean PGE-2 level, statistically, there is no significant difference in the plasma PGE-2 level between LPS + 3-CH_2Cl and LPS + ASA (p = 0.6678).

3.2. 3-CH_2Cl decreases the endogen COX-2 expression in the splenocytes of LPS-induced mice

To investigate whether oral treatment of 3-CH_2Cl during the inflammatory induction could regulate the splenic endogenous COX-2 expression, flow cytometry analysis was performed, and the mean fluorescence intensity of histogram analysis (in arbitrary unit /AU) as a parameter of COX-2 expression is displayed in Fig. 1**C**. The graphical analysis of replicates is presented in Fig. 1**B**. The endogenous COX-2 expression in the LPS-only cohort (13.94 ± 1.88 AU) is significantly increased (p = < 0.0001) compared to the Untreated cohort (3.89 ± 0.35 AU). Meanwhile, the COX-2 expression in the LPS + ASA cohort (5.58 ± 1.09 AU), compared with the untreated cohort, showed a significant increase (p = 0.0253), while the LPS + 3-CH_2Cl cohort (4.05 ± 0.50 AU), showed no significant changes (p = 0.6349). Interestingly, compared to the LPS-only cohort, the addition of a single dose (60 mg/kgBW) of salicylic acid-bearing compounds ASA (LPS + ASA; p = 0.0003) and 3-CH_2Cl (LPS + 3-CH_2Cl; p = < 0.0001) showed significantly lower splenic COX-2 expression. Furthermore, oral treatment of 3-CH_2Cl in LPS-mice could significantly reduce the COX-2 expression compared with the ASA-treatment, indicating that the treatment of the 3-CH_2Cl cohort exhibited a slightly better ability to reduce COX-2 expression.

3.3. 3-CH_2Cl decreases the NO-concentration in the liver homogenate and plasma of LPS-induced mice

The NO level in the liver homogenate and blood plasma was measured with the Griess-reagent, calculated, and are presented in Fig. 2. In the liver homogenate (see Fig. 2**A**), the nitrite concentration of the LPS-only cohort is significantly increased compared to the Untreated cohort (2590 ± 0.482 μg NO/ml lysate vs 1279 ± 0.165 μg NO/ml lysate; p = 0.0021). While the LPS + 3-CH_2Cl cohort (1432 ± 0.232 μg NO/ml lysate) showed no significant difference (p = 0.3240), interestingly, the LPS + ASA cohort (3340 ± 0.847 μg NO/ml lysate) showed a significantly higher NO level (p = 0.0031). The NO production of the LPS + ASA cohort showed no significant difference compared to the LPS-only cohort (p = 0.1746), meanwhile, the LPS + 3-CH_2Cl cohort showed a significant decrease (p = 0.0049) in NO production. The oral treatment of a single dose (60 mg/kgBW) of 3-CH_2Cl (LPS + 3-CH_2Cl cohort) exhibited a significantly better ability to reduce the concentration of NO compared to the ASA-treated cohort (p = 0.0048). In the mice peripheral blood plasma (see Fig. 2**B**), compared to the Untreated cohort (0.405 ± 1.440 μg NO/ml plasma), the LPS-only cohort (1.734 ± 4.56 μg NO/ml plasma) showed a significant increase in nitrite concentration (p = 0.0014), while the LPS + ASA cohort (0.223±0.18 μg

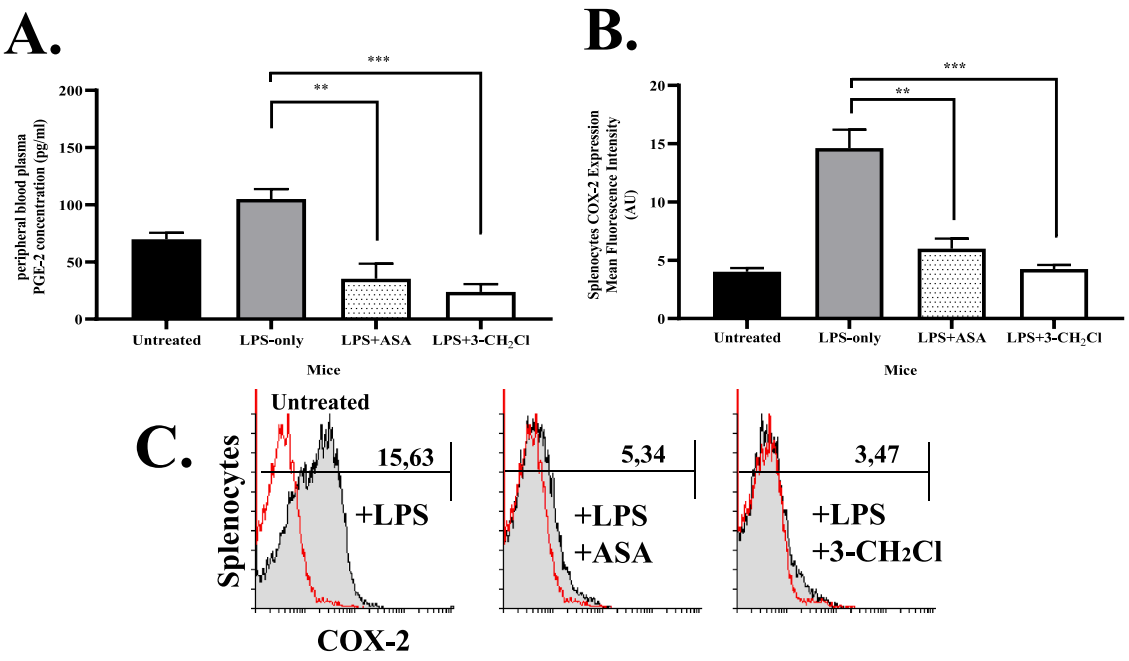

Fig. 1. PGE-2 concentration in the peripheral blood plasma of LPS-induced mice and COX-2 expression in the splenocytes of LPS-induced mice. Mice (n = 3) were treated with or without intraperitoneally administered LPS (2 mg/kgBW), and orally treated with or without ASA (60 mg/kgBW) or 3-CH_2Cl (60 mg/kgBW). The plasma and the spleen of the mice were isolated, processed and measured. (A) Representative PGE-2 concentration in peripheral blood plasma (pg/ml) from each group. (B) Representative Splenic Endogen COX-2 Expression Mean Fluorescence Intensity (AU) of each group. Statistical analysis was conducted using unpaired t-test: * ($0.05 \leq p > 0.01$); ** ($0.01 \leq p > 0.001$); *** ($0.001 \leq p > 0.0001$); **** ($p \leq 0.0001$). (C) Representative histogram with Splenic Endogen COX-2 Expression Mean Fluorescence Intensity (AU) value obtained from each group.

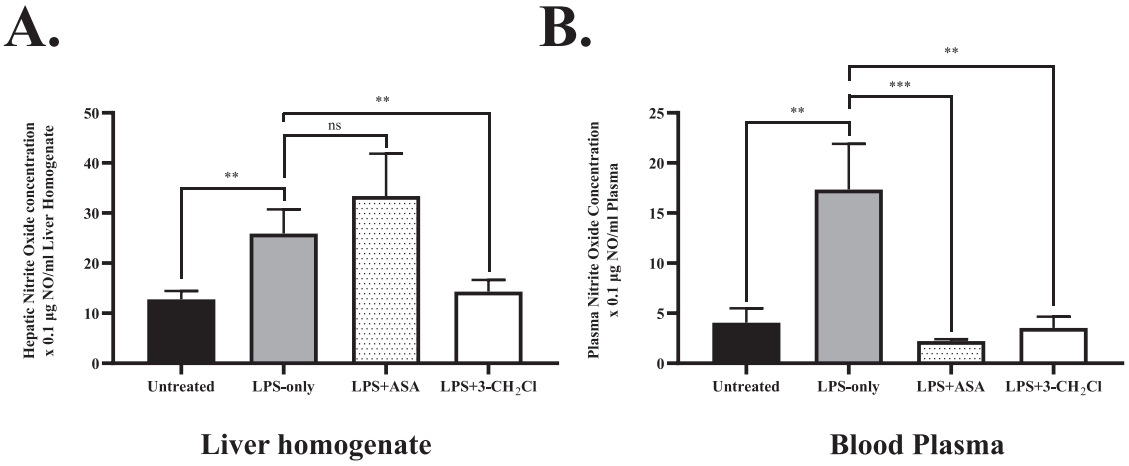

Fig. 2. NO-concentration in the liver homogenate and plasma of LPS-induced mice. Mice (n = 3) treated either with or without intraperitoneally administered LPS (2 mg/kgBW), and then orally treated with or without ASA or 3-CH_2Cl (60 mg/kgBW each). The NO-concentration were detected using colorimetric methods and a spectrophotometer. (A) Nitrite Oxide Concentration in Liver homogenate (× 0.1 µg/ml Liver Homogenate) (B) NO Concentration in plasma (× 0.1 µg/ml Plasma). Statistical analysis was conducted using an unpaired t-test: * ($0.05 \leq p > 0.01$); ** ($0.01 \leq p > 0.001$); *** ($0.001 \leq p > 0.0001$); **** ($p \leq 0.0001$).

NO/ml plasma) shows a significant decrease in NO production ($p = 0.0458$), the LPS + 3-CH_2Cl cohort (0.353 ± 1.12 µg NO/ml plasma) showed no significant NO concentration differences ($p = 0.5927$). While the LPS + ASA cohort and the LPS + 3-CH_2Cl cohort demonstrated a significant decrease compared to the LPS-only cohort ($p = 0.0006$ and $p = 0.0011$, respectively), there is no significant difference in the ability of ASA (LPS + ASA cohort) and 3-CH_2Cl (LPS + 3-CH_2Cl cohort) to reduce the LPS-triggered NO production ($p = 0.0606$).

3.4. 3-CH_2Cl decreases the expression of NOX2 and NFkB in the liver perivascular area and the expression of NFkB in the splenocytes of LPS-induced mice

To elucidate the role of 3-CH_2Cl in inhibiting the reactive oxygen species generator enzyme NOX2 and inflammatory transcription factor NFkB expression, an immunohistochemical assay focused on the liver perivascular areas and spleen (NFkB only) of the mice cohort was performed (see Fig. 3, black arrow). The representative histological analysis of the liver perivascular area can be seen in Fig. 3**A** (upper and middle row), while the splenic red-pulp and white pulp area could be seen on the lower panel (red box for red-pulp area, black box for white-pulp

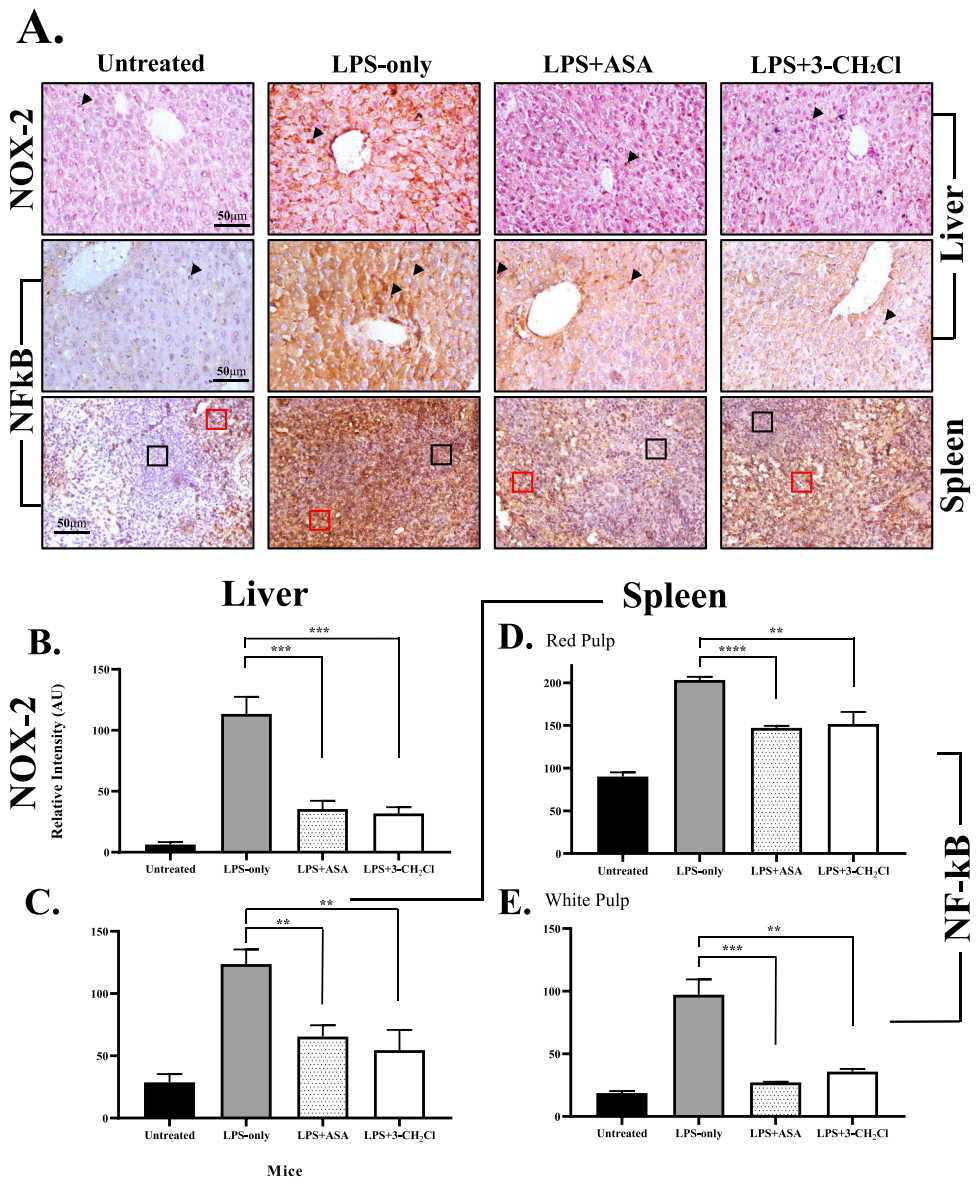

Fig. 3. NOX-2 and NF-kB expression in the liver perivascular area of LPS-induced mice and NFkB expression in the splenocytes of LPS-induced mice. Mice (n = 3) were treated with or without LPS (2 mg/kgBW) intraperitoneally and treated orally with or without ASA (60 mg/kgBW) or 3-CH_2Cl (60 mg/kgBW). Liver and spleen of the mice were processed, stained, and analyzed with immunohistochemistry as described in Methods. The expression value of NOX2 and NFkB expression is presented as relative intensity (AU). (A) Representative staining results of the liver perivascular area were in the upper and middle row while the splenic red-pulp and white-pulp area were in the lower row. Black arrow represents hepatocytes of liver perivascular area, red box represents splenic red-pulp area, and black box represents splenic white-pulp area. (B) Scoring of relative intensity of NOX-2 in liver perivascular area, (C) Scoring of relative intensity of NF-kB in liver perivascular area, (D) Scoring of relative intensity of NF-kB in splenic red-pulp area, and (E) Scoring of relative intensity of NF-kB in splenic white-pulp area. Statistical analysis was conducted using an unpaired t-test: * ($0.05 \leq p > 0.01$); ** ($0.01 \leq p > 0.001$); *** ($0.001 \leq p > 0.0001$); **** ($p \leq 0.0001$).

area). The semi-quantitative relative expression analysis (presented as relative intensity/AU) of each cohort replicates is presented in Fig. 3**B–E**. In comparison to the untreated cohort, the LPS-only group showed a significant increase (6.316 ± 1.940 AU vs 113.4 ± 13.99 AU; p = < 0.0001) of NOX2 expression in the liver perivascular area (see Fig. 3**B**). Both the LPS + ASA and LPS + 3-CH_2Cl groups showed higher NOX2 expression compared to the untreated cohort (LPS + ASA = 35.48 ± 6.76 AU; p = < 0.0001, LPS + 3-CH_2Cl = 31.84 ± 5.15 AU; p = < 0.0001). Meanwhile, the expression of NOX2 showed a significant decrease in the LPS + ASA (p = < 0.0001) and LPS + 3-CH_2Cl (p = < 0.0001) cohorts compared to LPS-only. However, the NOX2 expression of the 3-CH_2Cl treated mice cohort shows no significant difference than that of the LPS + ASA group (p = 0.2571). The NFkB-expression pattern of the liver pericentral area of all cohorts has an identical pattern (see Fig. 3**C**). The relative intensity of NFkB in the LPS-only is significantly increased compared with the Untreated cohort (LPS-Only = 123.7 ± 11.68 AU vs Untreated = 28.74 ± 6.62 AU; p = 0,0003). LPS + ASA show significantly higher values of relative NFkB-relative expression compared to the untreated cohort (LPS + ASA = 65.45 ± 9.12 AU; p = 0.0049), while LPS + 3-CH_2Cl shows slight increment (LPS + 3-CH_2Cl = 54.59 ± 17.19 AU; p = 0.0626). Meanwhile, the expression of NFkB significantly decreases in the LPS + ASA cohort (p = 0.0024) and LPS + 3-CH_2Cl (p = 0.0039) cohort compared to LPS-Only. Although both cohorts statistically show no significant difference (p= 0.3688), LPS + 3-CH_2Cl exhibited a slightly better ability to reduce the expression of NFkB in the pericentral area of Hepatocytes. The Analysis of splenic Red-pulp area, which has more activated lymphocytes, shows a higher NFkB expression pattern than the white pulp area. While in the red pulp

area (Fig. 3**D**), the LPS-only (203.3 ± 3.56 AU; $p < 0.0001$), LPS + ASA (147.1 ± 2.38 AU; $p < 0.0001$), and LPS + 3-CH_2Cl cohorts (151.8 ± 14.08 AU; $p = 0.002$) show significantly higher expression of NFkB than the untreated cohort (90.03 ± 5.06 AU), and in the white pulp area (Fig. 3**E**), the LPS-only (97.18 ± 12.31 AU; $p = 0.0004$), LPS + ASA (27.17 ± 0.47; $p = 0.0009$), and LPS + 3-CH_2Cl cohorts (35.83 ± 2.13 AU; $p = 0.0004$) show significantly higher NFkB expression than the untreated cohort (18.74 ± 1.58 AU). The treatment of salicylic acid-bearing compounds could significantly decrease the NFkB expression in both the red pulp and white pulp area, compared with the LPS-only cohort (LPS + ASA red pulp $p < 0.0001$; white pulp $p = 0.0006$; LPS + 3-CH_2Cl red pulp $p = 0.0036$; white pulp $p= 0.001$). Interestingly, treatment of 3-CH_2Cl reduces the splenic NFkB expression less than LPS + ASA (red pulp 3.2 % less reduction or higher NFkB expression; $p = 0.6016$; white pulp 31.87 % less reduction or higher NFkB expression; $p = 0.0024$).

3.5. In-vitro treatment of 3-CH_2Cl decreases ROS in the LPS-activated splenocytes

To analyze the reactive oxygen species (ROS) manifested by the measurement of superoxide generation, spleen from mice were isolated from the LPS-induced mice, activated with 200 μg/ml LPS in-vitro, and then incubated with ASA 50 μM, ASA 500uM, 3-CH_2Cl 50uM or 3-CH_2Cl 500uM. After further incubation with or without SOD, the superoxide generated was calculated through the difference of "with" or "without" SOD-assay, and the optical quantification of ferricytochrome-C mediated color change (see Fig. 4). The superoxide generated by LPS-activated splenocytes (192.5 ± 6.87 nmol $\Delta O_2^{\bullet}$) showed a significant increase ($p = 0.0004$), compared with the untreated splenocytes (148.3 ± 10.46 nmol $\Delta O_2^{\bullet}$). The in-vitro treatment of both salicylic acid-bearing compounds (ASA and 3-CH_2Cl) could significantly reduce the LPS-mediated superoxide generation ($p < 0.001$). Furthermore, the addition of ASA and 3-CH_2Cl in LPS-activated splenocytes could significantly reduce ($p < 0.0001$ for all treatments) the superoxide generation in a dose-dependent manner (ASA 500 μM = 103.7 ± 11.46 nmol $\Delta O_2^{\bullet}$; ASA 50 μM = 28.03±8.35 nmol $\Delta O_2^{\bullet}$; 3-CH_2Cl 500 μM = 121.6 ± 5.02 nmol $\Delta O_2^{\bullet}$; 3-CH_2Cl 50 μM = 62.90 ± 10.33 nmol $\Delta O_2^{\bullet}$). The treatment of an equimolar concentration of 3-CH_2Cl significantly reduces the splenic superoxide generation less than ASA (124.40 % less superoxide generation in 50 μM concentration, $p = 0.029$; 17.26 % less superoxide generation in 500 μM concentration, $p = 0.002$).

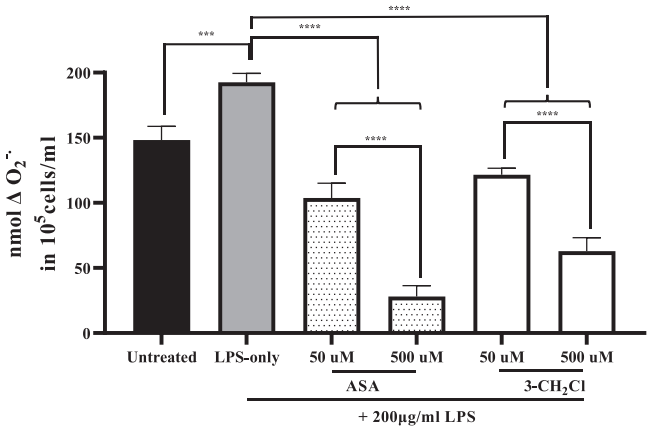

Fig. 4. ROS in the splenocytes of LPS-induced mice was inhibited by 3-CH_2Cl. Spleen of the mice (n = 5) were isolated, activated with or without LPS (200 μg/ml), then incubated with or without ASA (50 or 500 μM) or 3-CH_2Cl (50 or 500 μM). Reactive Oxygen Species (ROS) productions were assessed by ferricytochrome-c assay and the superoxide generations were measured. The data represent the difference of each assay between with and without SOD (Δ nmol $O_2^{\bullet}$) Statistical analysis was conducted using unpaired t-test: * ($0.05 \leq p > 0.01$); ** ($0.01 \leq p > 0.001$); *** ($0.001 \leq p > 0.0001$); **** ($p \leq 0.0001$).

4. Discussion

The results of this study highlight the potential of 3-CH_2Cl as a novel salicylic acid derivative with anti-inflammatory properties. We propose a mechanism for 3-CH_2Cl, based on experimental data, that includes reducing blood plasma prostaglandin E-2 (PGE-2) concentration, liver NOX2 expression, liver and splenic NFkB expression, ROS production, and increasing splenic COX-2 expression in lipopolysaccharide (LPS)-induced mice. Analysis of peripheral blood plasma PGE-2 concentration (Fig. 1**A**) revealed a significant decrease in PGE-2 levels in both the LPS + ASA and LPS + 3-CH_2Cl groups compared to the LPS-only group. This demonstrates the effectiveness of both compounds in mitigating systemic inflammation, with 3-CH_2Cl showing comparable efficacy to ASA. This suggests 3-CH_2Cl's potential as an alternative to ASA in managing inflammation-related conditions. The decrease in PGE-2 levels suggests a mechanism involving the inhibition of COX enzymes [15].

Flow cytometry analysis (Fig. 1**B and** 1**C**) showed that 3-CH_2Cl treatment significantly decreased endogenous COX-2 expression in splenocytes compared to the LPS-only group. This aligns with the reduction in PGE-2 levels and further highlights the anti-inflammatory properties of 3-CH_2Cl. The analysis of spleen from COX-2 luciferase knock-in reporter mice ($Cox2^{fLuc/+}$) visualized with bioluminescent imaging [17] supports our flow cytometry findings by showing that LPS significantly increased COX-2 expression in the spleen. The ability of 3-CH_2Cl to suppress COX-2 expression may contribute to its efficacy in attenuating inflammation. The enhanced selectivity of 3-CH_2Cl towards COX-2 over ASA, as indicated by in-silico docking experiments [6] provides promising insights into its potential to reduce side effects associated with non-selective COX inhibition.

LPS-induced expression of NOX2 and NFκB is a critical aspect of the inflammatory response [21]. LPS stimulates phagocytes, leading to the activation of the NOX2 complex and the production of inflammatory radicals such as NO and $O_2^{\bullet}$ [7,13,22]. Furthermore, LPS triggers NFκB activation through the TLR4 signaling pathway [8]. Measurements of NO concentration in liver homogenate and plasma (Fig. 2) and splenic supernatant (Fig. 4) showed that 3-CH_2Cl significantly reduced NO and $O_2^{\bullet}$ levels compared to both the LPS-only and LPS + ASA groups. This indicates 3-CH_2Cl's potential as a therapeutic agent for inflammatory disorders. Immunohistochemical analysis showed that 3-CH_2Cl treatment reduced NOX2 and NFkB expression in the liver (Fig. 3), supporting its anti-inflammatory effects and potential to mitigate inflammation-induced tissue damage. The significant reduction in pro-inflammatory markers by oral treatment with 3-CH_2Cl could explain the inhibition of TNF-α and IL-1β in LPS-induced inflammatory mice [26]. The regulation of splenic inducible COX-2 was promoted through the increase of splenic NFkB during LPS-induced inflammation [18].

The spleen, particularly in the red pulp, is involved in the innate immune response, including the activation of macrophages and cytokine production [1,20]. NF-kB activation in the white pulp may be involved in the adaptive immune response [10]. The attenuation of splenic NFkB expression by 3-CH_2Cl upon inflammation suggests its ability to modulate immune responses and reduce inflammation at both the adaptive and innate levels, aligned with the COX-2 expression inhibitory effect [27]. While the spleen does produce ROS, it is not typically characterized as a "good superoxide producer" compared to other cell types or organs [3]. In the red pulp, activated splenocytes and phagocytes induce NOX2 expression, contributing to antimicrobial activities [19]. Based on those facts, the ROS generation assay presented in this manuscript was conducted in vitro and in the presence of an ROS activator to ensure the detectable amount of $O_2^{\bullet}$.

Interestingly, compared with ASA, 3-CH_2Cl shows a slightly reduced inhibition activity in NFkB expression in the spleen and NO production in blood plasma. This difference in effects can be attributed to their distinct chemical structures and mechanisms of action. 3-CH_2Cl might

target different molecules or pathways, leading to slightly reduced efficacy in these specific outcomes. The higher inhibition of NO production by 3-CH2Cl in the liver suggests a potentially different or additional therapeutic mechanism, which could be beneficial in reducing NO production in inflammatory or autoimmune diseases. The increased NO-levels mediated by anti-inflammatory dosages of ASA might be attributed to higher liver toxicity [14], compared with 3-CH_2Cl. Further studies are needed to clarify this matter, particularly regarding the nitric oxide synthase (NOS) pathways.

The results of this study collectively suggest that 3-CH_2Cl possesses potent anti-inflammatory properties that are comparable to, or even surpass, those of ASA. Its ability to reduce PGE-2 levels, suppress COX-2 expression, decrease NO production, and inhibit NFkB activation highlights its potential as a promising therapeutic agent for various inflammatory conditions. These findings have significant clinical implications, especially in the management of inflammatory diseases where current treatments, such as ASA, may be associated with adverse effects. The superior efficacy and favorable safety profile of 3-CH_2Cl make it a promising candidate for further preclinical and clinical investigations.

However, it is important to acknowledge certain limitations of this study. The experimental design primarily focused on acute systemic inflammation induced by LPS administration in mice, which may not fully represent the chronic inflammatory conditions observed in clinical settings. Additionally, further studies are needed to elucidate the underlying molecular mechanisms of 3-CH_2Cl-mediated anti-inflammatory effects and to assess its long-term safety and efficacy in diverse inflammatory disease models.

5. Summary

In conclusion, this study provides compelling evidence for the anti-inflammatory potential of 3-CH_2Cl (Fig. 5), a novel salicylic acid derivative. Its ability to modulate LPS-induced TLR4-inflammatory (1) pathways through inhibited NFkB activation (2,3), suppress NOX2 expression (4), suppressed COX-2 expression (5), reduced PGE-2 levels (6), and decreased NO production (7) underscores its promise as a therapeutic agent for a wide range of inflammatory disorders. These findings highlight the potential of 3-CH_2Cl to address limitations of current NSAIDs, including ASA, by offering a more selective mechanism of action, reduced side effects, and broader therapeutic applications. Future research should explore its clinical utility and address remaining questions about its mechanism of action and safety profile.

CRediT authorship contribution statement

Claritta Angelina Wiyanto Putri: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Michelle Angelina Henrikus:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Wuryanto Hadinugroho:** Writing – review & editing, Visualization, Project administration, Methodology, Formal analysis. **Caroline Caroline:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **yufita ratnasari wilianto:** Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Formal analysis. **Yudy Tjahjono:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision,

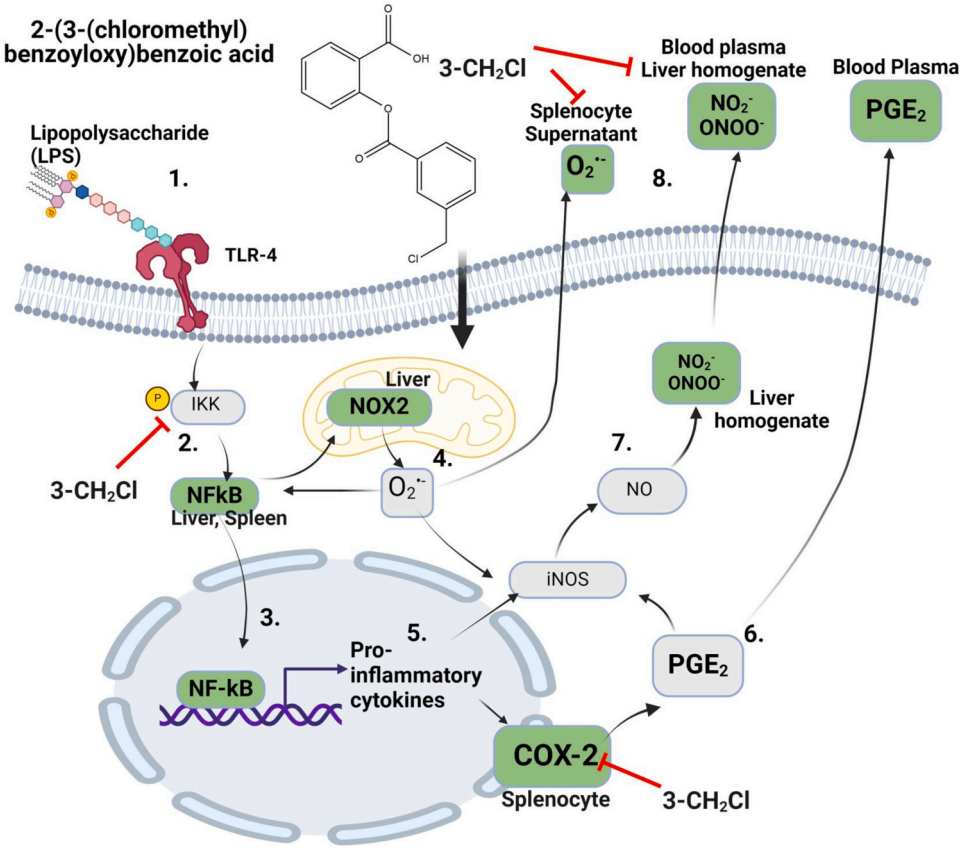

Fig. 5. Mechanism of action of 2-(3-(chloromethyl)benzoyloxy)benzoic acid (3-CH_2Cl) as a novel salicylic acid derivative with anti-inflammatory potential. Upon entering the cells, this compound modulates Lipopolysaccharide (LPS)-induced Toll-like receptor 4 (TLR4) inflammatory pathways (**1**) by inhibiting Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB) activation (**2,3**), suppressing NADPH oxidase (NOX2) expression (**4**), suppressing Cyclooxygenase-2 (COX-2) expression (**5**), reducing Prostaglandin E2 (PGE-2) levels (**6**), and decreasing Nitric Oxide (NO, ONOO-) and Superoxide ($O_2^{\bullet}$) production (**7,8**). These actions underscore 3-CH_2Cl potential as a therapeutic agent for a wide range of inflammatory disorders.

Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dwi Aris Agung Nugrahaningsih:** Writing – review & editing, Visualization, Validation, Formal analysis. **Hendy Wijaya:** Writing – review & editing, Validation, Investigation, Formal analysis. **Jusak Nugraha:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Kuncoro Foe:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Formal analysis. **Hevi Wihadmadyatami:** Writing – review & editing, Supervision, Project administration, Investigation, Formal analysis, Data curation. **Srikanth Karnathi:** Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Dwi Liliek Kusindarta:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation. **Bernadette Dian Novita Dewi:** Writing – review & editing, Validation, Investigation, Data curation, Conceptualization. **Philipus Karel:** Writing – original draft, Visualization, Validation, Resources, Methodology, Data curation. **Senny Yesery Esar:** Writing – review & editing, Resources, Investigation, Formal analysis, Conceptualization. **Fransiskus Regis Partana:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation.

Conflict of interest

All authors declare no conflict of interest.

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Authors contribution

Yudy Tjahjono, Caroline and Hevi Wihadmadyatami designed the experiments, carried out experiments, Philipus Karel, Fransiskus Regis Partana, Michelle Angelina Henrikus, Claritta Angelina Wiyanto, and Yufita Ratnasari Wilianto carried out the experiments and analyzed the data. Kuncoro Foe, Hendy Wijaya, Bernadette Dian Novita, Srikanth Karnati, Süleyman Ergün, Wuryanto Hadinugroho, Jusak Nugraha, Dwi Aris Agung Nugrahaningsih, Dwi Liliek Kusindarta analyzed the data, and prepared the manuscript.

Data availability

No data was used for the research described in the article.

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Institutional review board statement

This study was ethically approved by preclinical ethic committee Faculty of veterinary sciences, Gadjah Mada University, Yogyakarta-Indonesia No. 001/ECFKH/Eks/2022.

References

[1] M. Aliyu, F. Zohora, A. Akbar Saboor-Yaraghi, Spleen in innate and adaptive immunity regulation, AIMS Allergy Immunol. 5 (1) (2021) 1–17, https://doi.org/10.3934/Allergy.2021001.

[2] B. Bayat, Y. Tjahjono, H. Berghöfer, S. Werth, H. Deckmyn, S.F. De Meyer, U. J. Sachs, S. Santoso, Choline transporter-like protein-2: new von willebrand factor-binding partner involved in antibody-mediated neutrophil activation and transfusion-related acute lung injury, Arterioscler. Thromb. Vasc. Biol. 35 (7) (2015), https://doi.org/10.1161/ATVBAHA.115.305259.

[3] A.V. Belikov, B. Schraven, L. Simeoni, T cells and reactive oxygen species, J. Biomed. Sci. 22 (1) (2015) 85, https://doi.org/10.1186/s12929-015-0194-3.

[4] S. Bindu, S. Mazumder, U. Bandyopadhyay, Non-steroidal anti-inflammatory drugs (NSAIDs) and organ damage: a current perspective, Biochem. Pharm. 180 (2020) 114147, https://doi.org/10.1016/j.bcp.2020.114147.

[5] Brune, P. Patrignani, New insights into the use of currently available non-steroidal anti-inflammatory drugs, J. Pain Res. 8 (2015) 105, https://doi.org/10.2147/JPR.S75160.

[6] Caroline, K. Foe, S. Yesery Esar, A. Soewandi, H. Wihadmadyatami, R.M. M. Widharna, W.D.D. Tamayanti, E. Kasih, Y. Tjahjono, Evaluation of analgesic and antiplatelet activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid, Prostaglandins Other Lipid Mediat. 145 (2019) 106364, https://doi.org/10.1016/j.prostaglandins.2019.106364.

[7] J. Checa, J.M. Aran, Reactive oxygen species: drivers of physiological and pathological processes, J. Inflamm. Res. 13 (2020) 1057–1073, https://doi.org/10.2147/JIR.S275595.

[8] A. Ciesielska, M. Matyjek, K. Kwiatkowska, TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling, Cell. Mol. Life Sci. 78 (4) (2021) 1233–1261, https://doi.org/10.1007/s00018-020-03656-y.

[9] A.Y. Gasparyan, T. Watson, G.Y.H. Lip, The role of aspirin in cardiovascular prevention, J. Am. Coll. Cardiol. 51 (19) (2008) 1829–1843, https://doi.org/10.1016/j.jacc.2007.11.080.

[10] S. Gerondakis, U. Siebenlist, Roles of the NF- B pathway in lymphocyte development and function, Cold Spring Harb. Perspect. Biol. 2 (5) (2010), https://doi.org/10.1101/cshperspect.a000182.

[11] W. Hadinugroho, K. Foe, Y. Tjahjono, C. Caroline, S.Y. Esar, H. Wijaya, M. A. Jessica, Tablet formulation of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid by linear and quadratic models, ACS Omega (2024) (in press).

[12] E. Heyman, M. Meeremans, B. Devriendt, M. Olenic, K. Chiers, C. De Schauwer, Validation of a color deconvolution method to quantify MSC tri-lineage differentiation across species, Front. Vet. Sci. 9 (2022), https://doi.org/10.3389/fvets.2022.987045.

[13] J. Huang, V. Canadien, G.Y. Lam, B.E. Steinberg, M.C. Dinauer, M.A.O. Magalhaes, M. Glogauer, S. Grinstein, J.H. Brumell, Activation of antibacterial autophagy by NADPH oxidases, Proc. Natl. Acad. Sci. 106 (15) (2009) 6226–6231, https://doi.org/10.1073/pnas.0811045106.

[14] Y. Iwakiri, M.Y. Kim, Nitric oxide in liver diseases, Trends Pharm. Sci. 36 (8) (2015), https://doi.org/10.1016/j.tips.2015.05.001.

[15] X. Jiang, H. Renkema, B. Pennings, S. Pecheritsyna, J.C. Schoeman, T. Hankemeier, J. Smeitink, J. Beyrath, Mechanism of action and potential applications of selective inhibition of microsomal prostaglandin E synthase-1-mediated PGE2 biosynthesis by sonlicromanol's metabolite KH176m, Sci. Rep. 11 (1) (2021) 880, https://doi.org/10.1038/s41598-020-79466-w.

[16] Z. Ju, M. Li, J. Xu, D.C. Howell, Z. Li, F.-E. Chen, Recent development on COX-2 inhibitors as promising anti-inflammatory agents: the past 10 years, Acta Pharm. Sin. B 12 (6) (2022) 2790–2807, https://doi.org/10.1016/j.apsb.2022.01.002.

[17] N.S. Kirkby, M.V. Chan, A.K. Zaiss, E. Garcia-Vaz, J. Jiao, L.M. Berglund, E. F. Verdu, B. Ahmetaj-Shala, J.L. Wallace, H.R. Herschman, M.F. Gomez, J. A. Mitchell, Systematic study of constitutive cyclooxygenase-2 expression: role of NF-κB and NFAT transcriptional pathways, Proc. Natl. Acad. Sci. USA 113 (2) (2016), https://doi.org/10.1073/pnas.1517642113.

[18] A. Kulesza, L. Paczek, A. Burdzinska, The role of COX-2 and PGE2 in the regulation of immunomodulation and other functions of mesenchymal stromal cells, Biomedicines 11 (2) (2023) 445, https://doi.org/10.3390/biomedicines11020445.

[19] D. Kurotaki, T. Uede, T. Tamura, Functions and development of red pulp macrophages, Microbiol. Immunol. 59 (2) (2015) 55–62, https://doi.org/10.1111/1348-0421.12228.

[20] S.M. Lewis, A. Williams, S.C. Eisenbarth, Structure and function of the immune system in the spleen, Sci. Immunol. 4 (33) (2019), https://doi.org/10.1126/sciimmunol.aau6085.

[21] Y. Liu, S. Fang, X. Li, J. Feng, J. Du, L. Guo, Y. Su, J. Zhou, G. Ding, Y. Bai, S. Wang, H. Wang, Y. Liu, Aspirin inhibits LPS-induced macrophage activation via the NF-κB pathway, Sci. Rep. 7 (1) (2017), https://doi.org/10.1038/s41598-017-10720-4.

[22] J. Lv, X. He, H. Wang, Z. Wang, G.T. Kelly, X. Wang, Y. Chen, T. Wang, Z. Qian, TLR4-NOX2 axis regulates the phagocytosis and killing of Mycobacterium tuberculosis by macrophages, BMC Pulm. Med. 17 (1) (2017) 194, https://doi.org/10.1186/s12890-017-0517-0.

[23] B.G. Magrum, K.K. Pickworth, Aspirin rechallenge in an adult patient previously diagnosed with Reye syndrome, Am. J. Health-Syst. Pharm. 77 (2) (2020) 123–127, https://doi.org/10.1093/ajhp/zxz276.

[24] A. Morel, E. Miller, M. Bijak, J. Saluk, The increased level of COX-dependent arachidonic acid metabolism in blood platelets from secondary progressive multiple sclerosis patients, Mol. Cell. Biochem. 420 (1–2) (2016) 85–94, https://doi.org/10.1007/s11010-016-2770-6.

[25] W.D. Tamayanti, R.M. Widharna, C. Caroline, B. Soekarjo, Uji Aktivitas Analgesik Asam 2-(3-(Klorometil)Benzoiloksi)Benzoat dan Asam 2-(4-(Klorometil) Benzoiloksi)Benzoat Pada Tikus Wistar Jantan dengan Metode Plantar Test, J. Pharm. Sci. Community 13 (01) (2016), https://doi.org/10.24071/jpsc.2016.130103.

[26] Y. Tjahjono, S. Karnati, K. Foe, E. Anggara, Y.N.N. Gunawan, H. Wijaya, Steven, H. Suyono, S.Y.Y. Esar, W. Hadinugroho, H. Wihadmadyatami, S. Ergün, R.M.

M. Widharna, Caroline, S. Ergun, R.M.M. Widharna, Caroline, Anti-inflammatory activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in LPS-induced rat model 154 (2021) 106549, https://doi.org/10.1016/j.prostaglandins.2021.106549.

[27] Yudy Tjahjono, C. Caroline, J. Nugraha, K. Foe, S. Karnati, S. Ergün, N. Jafet, O. Chrisantia, I.M.A.B. Kusuma, H. Wijaya, W. Hadinugroho, D.A. A. Nugrahaningsih, D.L. Kusindarta, Y.R. Wilianto, S.Y. Esar, H. Wihadmadyatami, 2-(3-(chloromethyl)benzoyloxy)benzoic acid increases CD4+ regulatory T-cell population and FoxP3 expression in lipopolysaccharide-induced mice, Indones. Biomed. J. 15 (4) (2023), https://doi.org/10.18585/inabj.v15i4.2429.

[28] Yudy Tjahjono, Caroline, K. Foe, S. Karnati, S. Ergün, S.Y. Esar, H. Wijaya, W. Hadinugroho, D.L. Kusindarta, H. Wihadmadyatami, Synthesis, characterization, and application of 2-((3-(chloromethyl)-benzoyl)oxy)benzoic acid: a review, ACS Omega 8 (1) (2023) 42–47, https://doi.org/10.1021/acsomega.2c05566.

[29] J. van der Veeken, A.J. Gonzalez, H. Cho, A. Arvey, S. Hemmers, C.S. Leslie, A. Y. Rudensky, Memory of inflammation in regulatory T cells, Cell 166 (4) (2016) 977–990, https://doi.org/10.1016/j.cell.2016.07.006.

[30] N.I. Vargas-Maya, F. Padilla-Vaca, O.E. Romero-González, E.A.S. Rosales-Castillo, Á. Rangel-Serrano, S. Arias-Negrete, B. Franco, Refinement of the Griess method for measuring nitrite in biological samples, J. Microbiol. Methods 187 (2021), https://doi.org/10.1016/j.mimet.2021.106260.

[31] H. Yu, L. Lin, Z. Zhang, H. Zhang, H. Hu, Targeting NF-κB pathway for the therapy of diseases: mechanism and clinical study, Signal Transduct. Target. Ther. 5 (1) (2020) 209, https://doi.org/10.1038/s41392-020-00312-6.