

# Evaluation of analgesic and antiplatelet activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid

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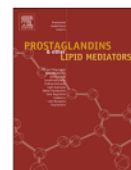
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## Evaluation of analgesic and antiplatelet activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid



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## ABSTRACT

Acetylsalicylic acid is used as a non-steroidal anti-inflammatory drugs (NSAID) and antiplatelet agents by inhibiting cyclooxygenases. However, therapy using acetylsalicylic acid could induce gastric bleeding and cause other gastrointestinal toxicity. The aim of this study was to demonstrate the synthesis of a new compound bearing salicylic acid residue namely 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid, to analyze its potential as a ligand for human cyclooxygenase-2 (COX-2) receptor, to evaluate its toxicity level and its effectiveness for analgesic and antiplatelet agent compared with acetylsalicylic acid.

Synthesis of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid was conducted by microwave irradiation. The purity of this compound was evaluated with TLC, IR, NMR, and EDS spectroscopy. The chemical characterization and docking studies against human COX-2 (PDB:5F1A) was performed *in-silico*. The acute oral toxicity assay was performed under OECD guidelines. The analgesic activity study was performed by plantar and writhing test on animal model. For anti-platelet activity study, we performed tail-bleeding assay and flow cytometry based platelet aggregation assay. We could successfully synthesize a pure white crystalline 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid. *In-Silico* G-Score result of those compounds gives us preliminary hint of the potential affinity of this compound as a ligand for COX-2 receptor (PDB: 5F1A). Acute toxicity and microscopic gastrointestinal assessments indicated non-observable harmful toxicity parameters. The plantar response time of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid treated groups showed a significant increment ( $P < 0.01$ ), and the nociceptive response in writhing test demonstrated a significant dose-dependent decrement. This indicated that its analgesic activity was better than acetylsalicylic acid. The platelet aggregation of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid was lower than its controls, indicating an aggregation inhibition pattern. The animals treated with 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid gave a longer bleeding time. Overall, this study demonstrated a successful synthesis of pure 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid. We postulated that this compound was better than acetylsalicylic acid, exhibiting excellent analgesic and antiplatelet activity with no toxicity impact.

## 1. Introduction

Over decades, a compound bearing salicylic acid residue namely acetylsalicylic acid is used as a non-steroidal anti-inflammatory drugs (NSAID) and antiplatelet agent due to its unique capability in inhibiting cyclooxygenases (COX). Recent studies showed that acetylsalicylic acid treatment can improve bone regeneration, particularly in inflammatory conditions [1]. One symptomatic character of inflammation such as

pain could be reduced by the analgesic action of acetylsalicylic acid [2].

Despite its cardioprotective effects and ability to inhibit inflammation, many studies have reported the dose-dependent harmful impact of acetylsalicylic acid on the gastrointestinal tract, ranging from mild upper gastrointestinal problem to severe peptic ulcer disease [3]. To maintain the benefit of acetylsalicylic acid and minimize its harmful effects,

the present work reported the synthesis of a new compound bearing

**Abbreviations:** COX, Cyclooxygenase; PRP, Platelet rich plasma; PPP, Platelet poor plasma; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); PGA, Pulvis Gummi Arabicum; FACS, Fluorescence Assisted Cell Sorting; 3CH<sub>2</sub>Cl, 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid; ASA, Acetylsalicylic Acid; CMR, Calculated Molar Refractivity; NSAID, Non steroidal anti inflammatory drugs

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salicylic acid residue, so-called 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid, followed by the evaluation of its pu<sup>1</sup>, analgesic and anti-platelet aggregation activities. The discovery of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid was based on preliminary in-silico drug modification study design. The structure of salicylic acid was modified, particularly the benzoyl-group attacking the phenolic OH-group within salicylic acid. The structure modification (supplemental Fig. 1) was conducted in 3 different zones with 4 different substituent (Cl, CH<sub>3</sub>, CH<sub>2</sub>Cl, and H). The choice of substituent was based on its positive lipophilic ( $\pi$ ) constants grades, which could increase the ability of the new compounds to enter the cell membranes. During the modification experiments, 64 new compounds were generated. In-silico studies of those compounds give preliminary hint of the potential activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid as a ligand for COX-2 receptor protein (PDB: 5F1A) in homeostasis such as inhibiting the platelet aggregation.

Platelet is the most essential blood cells responsible for homeostasis. The treatment of 150  $\mu$ mol/kg BW acetylsalicylic acid could prevent the platelet aggregation *in-vitro*, inhibit blood coagulation, and prevent thrombus formation inside the blood capillary. Thus it is very suitable for the treatment of patients with cardiovascular disease and stroke [4]. Platelet function stud<sup>2</sup> can be performed indirectly by bleeding time assay and directly by platelet aggregation assay. Recently, a novel flow cytometry-based platelet aggregation assay was developed to allow the detection of platelet aggregates with small blood volumes and low platelet numbers, enabling the assessment of its direct function in human and animal models [5]. This technique employs whole blood or plasma contains labeled platelets with different fluorochromes. After mixing and upon appropriate stimulation, *in-vitro* aggregation pattern can be interpreted from the formation of double-colored aggregate.

## 2. Materials and methods

### 2.1. General Procedure for the Synthesis of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid

An amount of 0.25 g salicylic acid (1.8 mmol) was mixed in a consecutive manner with 1 ml of 3-chloromethylbenzoylchloride (7.2 mmol), 0.14 ml of pyridine, 1.1 ml of acetone, and then was homogenized in an erlenmeyer tube. The reaction was generated by 5 min exposure of microwave irradiation with a Millstone Organic Synthesis Unit (MicroSYNTH) with a touch control terminal followed by further preparation [14]. The reaction mixture was placed in the microwave oven (600 W) for 1 min. Then, the mixture was removed from the microwave oven, stirred, and again placed in the microwave (600 W) for 1 min. After this period, microwave irradiation was continued and the reaction mixture was monitored every 1 min using thin-layer chromatography (TLC) with a stationary phase of silica gel F254 and a mobile phase hexane-ethanol (1:2, v/v). The ferric chloride (FeCl<sub>3</sub>) test was also used to identify the presence of salicylic acid in the reaction mixture every minute. The initial mixture formed slurry, and after microwave irradiation it became a homogeneous solution. The presence of the product was demonstrated by the formation of solid. The reaction condition has been optimized, by controlling the reaction time and the yield of the product.

### 2.2. Characterization of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid

Melting points were determined using a Melting Point Electrothermal Apparatus (Büchi B540, Sigma Aldrich, Switzerland) and were uncorrected. The elemental analysis (C, O, Cl) was performed using The Element Energy Dispersive Spectroscopy (EDS) EDAX-APOLLO-X (Ametex, Berwyn, PA, USA). IR spectra were recorded from 4000 to 400  $\text{cm}^{-1}$  with a Perkin Elmer System 60825 (Devon, UK). The spectra of <sup>1</sup>H and <sup>13</sup>CNMR were recorded on a Jeol JNM-ECS 400 spectrometer (Jeol Ltd., Akishima, Japan), operated at room

temperature (400 and 100 MHz for <sup>1</sup>H and <sup>13</sup>C spectroscopy respectively). Chemical shift ( $\delta$ ) is expressed in part per million.

### 2.3. In-silico analysis of physicochemical activities of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid

The physicochemical properties of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid compared to acetylsalicylic acid were analyzed by ChemDraw Ultra 8.0 and Chem3D Ultra 8.0 (Perkin Elmer, Waltham, USA) and MarvinSketch 15.2.16 (ChemAxon Ltd, Budapest, Hungary).

The docking of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (compared to acetylsalicylic acid) on the COX-2 receptor protein (PDB: 5F1A) was performed using Glide (Grid-Based Ligand Docking with Energetic) 4.0, Extra Precision (Glide 4.0 XP) docking (Schrödinger software, New York, USA). Ligand was minimized using OPLS-AA force field, meanwhile the cyclooxygenase protein was prepared by protein preparation wizard (Molecular Mechanics), provided with Glide. The system was minimized with Root Mean Square Deviation (RMSD) 0.30 Å.

### 2.4. Animal model

For pharmacological and physiological experiments, 6 to 8 week old mice weighing 20 g and Wistar rats weighing 200 g aged 6–8 weeks old were obtained from Pusvetma (Surabaya, Indonesia). Animals were housed in a temperature-controlled (20–24 °C) room, relative humidity 65%, with a 12-h light/dark cycle and allowed to consume food and water *ad libitum*. This study was approved by The University of Gadjah Mada Committee on the Use and Care of Animals. On the day of the experiment, a certain number of animal was divided into several appropriate groups and treated as described in each section below.

### 2.5. Acute oral toxicity test

An acute oral toxicity study was performed according to the Organization for Economic Cooperation and Development (OECD) Guidelines 423 (2001) with a slight modification in the number of experimental mice. Thirty animals were divided into three groups. Before the administration of compounds, mice were fasted for 24 h. In the treatment group, mice were treated with 2000 mg/kg bw of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid suspended in 1 ml of 3% PGA orally. The other 2 control groups were as follows: (1) mice were treated with 2000 mg/kg bw of acetylsalicylic acid suspended in 1 ml of 3% PGA as positive control group, and (2) mice were administered with 1 ml of 3% PGA suspension as negative control group. Following treatment, animals were continuously observed throughout the first 30 min and periodically observed during the early 24 h with particular attention during the first 4 h. Afterwards, the gross behavioral changes (loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality, and other signs of toxicity manifestation) of animals were observed daily for 14 days [6].

### 2.6. Gastric toxicity and histopathological analysis

Thirty male and female rats were divided into three groups equally and treated with the compounds as described above orally, with a single dose of 50 mg/kg BW of each compound. The dosage used was based on comparable dosage of 50 mg/kg rat body weight to ~500 mg/70 kg human body weight. After continuous treatment for seven days, the animals were anaesthetized with 0.1 ml of ketamine 100 mg/kg bw intraperitoneally for abdominal dissection. To obtain paraffin embedded whole gastric tissue, the fresh tissue was sunk directly into 0.5% formalin for 10 min. After fixation with paraffin block, microdissection with 6  $\mu$ m thickness and several dehydration steps, the probes were colored with routine Hematoxylin-Eosin technique. The gastric toxicity indices were measured through light-microscopical observation of

gastric mucosal layer disruption followed by visual scoring according to Zhang et al. [7].

### 2.7. Analgesic activity using plantar test and writhing test

The analgesic activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid was evaluated by using two methods, namely plantar test and acetic acid-induced writhing test [8]. In plantar test, cutaneous hyperalgesia to thermal stimulation in unrestrained rats was measured and especially used to evaluate the central action of the 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid. Animals were divided into three experimental groups. Before treatment, rats were fasted for 2 h. In the first group, six male rats were treated orally with vehicle namely 3% *Pubis Gummi Arabicum* (PGA) as negative control. In the second and third groups, animals were divided into five sub-groups received acetylsalicylic acid or 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid. Each sub-group consisting of six animals were treated orally with 5 different doses (12.5; 25; 50; 100; and 200 mg/kg body weight) to generate ED50 value. The dosage range was based on comparable dosage of 50 mg/kg rat body weight to 500 mg/70 kg human body weight with additional two stepwise half-value dosage ranges (lower and higher dosage). Thirty minutes after drug administration, rats were transferred to Plantar Aesthesiometer serial 7370 (Ugo Basile, Camerio, Italy), and adapted into the equipment for 10 min. After the heat induction of 55–56 °C, the animal response was observed and documented every 10 min for 1 h.

The analgesic activity of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid was evaluated in male mice with writhing test, as described by Turner [9] and Faujdar et al. [8] to evaluate the peripheral action of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in mice. The mice were divided into three experimental groups. Before treatment, mice were fasted for 2 h. In the first group, six male mice were treated with vehicle (3% PGA) intraperitoneally as negative control. In the second and third groups, animals were divided into five sub-groups receiving acetylsalicylic acid or 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid. Each sub-group consisting of six animals were treated with 5 different doses (12.5; 25; 50; 100; and 200 mg/kg bw). Thirty minutes after drug administration, 0.1 ml of 0.6% acetic acid was injected for the induction of writhings. The writhing effect was stimulated by stretching of at least one hind limb. The animal response was observed for 10 min, and the reduction in the number of writhings in each group was recorded and compared.

### 2.8. Animal treatment for bleeding time and flow cytometry assay

Sixty male mice were divided into 3 groups. Before the administration of compounds, mice were fasted for 2 h. Each group was then divided into 2 sub-groups in an equal number, used for bleeding time test and flow cytometry assay. In the first group, mice were treated orally with 0.58 mmol/kg bw of acetylsalicylic acid suspended in 1 ml of 3%PGA as positive control. In another groups, mice were treated with equimolar 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid suspended in 1 ml of 3% PGA or 1 ml of vehicle (3% PGA only). The experiments commenced 30 min after drug administration.

### 2.9. Bleeding time assay

To assess the blood anticoagulation effect of each compound *in vivo*, mice were anesthetized intraperitoneally using 0.1 ml of ketamine 100 mg/kg bw, and positioned horizontally on a platform allowing the tail to drop about 2 cm from the top of the platform. The experimental procedure in detail was performed as described with minor modifications. To evaluate bleeding from the incision, Whatman filter paper (Whatman International Ltd, Maidstone, UK) was applied to the edge of the forming clot every 30 s. The blood was allowed to fall separately from the cut during the 30-second interval. Data were expressed as

mean bleeding time (min) of each group after incision.

### 2.10. Preparation of mouse platelet-rich plasma (PRP) for flow cytometry assay

After bleeding time assay, whole blood was collected by cardiac puncture and collected in heparinized mouse blood collection tubes. The whole blood was diluted with 2 fold volume of HEPES medium. Diluted blood was centrifuged for 15 min at 50 g, and the collected platelet-rich plasma (PRP) was diluted further in HEPES medium to a final concentration of  $50 \times 10^6$  plt/ml. Mouse PRP was incubated at room temperature with 1:100 dilutions of fluorochrome-labeled platelet markers of CD31-APC and CD31-PECy7 monoclonal antibodies (Abcam, Cambridge, UK) for 15 min. After incubation, samples were centrifuged for 5 min at 2250 g and re-suspended in HEPES medium supplemented to a concentration of  $50 \times 10^6$  plt/ml.

### 2.11. Aggregation assay and flow cytometric analysis

For specific platelet aggregation function test, the two populations of labeled-washed mouse platelets were mixed in 1:1 ratio and then pre-incubated for 15 min at 37 °C while shaking (600 rpm). Pre-incubated platelets were activated using 10 mg/ml of type-I collagen (Sigma-Aldrich, St.Louis, MO, USA) at 37 °C while shaking at 1000 rpm. Fixed samples were measured and analyzed by BD FACS Calibur flow cytometry (BD Biosciences, San Jose, CA, USA), equipped with FlowJo version 9.2 software (Tree Star, inc., Ashland, OR, USA). For analysis, a quadrant was set in the dot plot of respective channels on non-stimulated platelets. The appearance of double-colored events in the upper right quadrant (Q2) was quantified as total aggregation events and calculated as described.

### 2.12. Statistics

Statistical analysis was performed with ANOVA. The data subsets were graphically presented with GraphPad Prism Software v.7 (La Jolla, CA, USA).

## 3. Results

### 3.1. Characterization of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid

The synthesis yielded white crystalline solid 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid with melting point of 129–131 °C. The absolute yield obtained from the above reaction was  $73.27 \pm 4.66\%$ .

Analytical calculation for 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (C<sub>15</sub>H<sub>11</sub>ClO<sub>4</sub>): C, 61.98; O, 22.02, Cl, 12.20%. Found: C, 62.10; O, 22.16, Cl, 12.87%

IR (KBr pellet, cm<sup>-1</sup>) data: 1732 (C = O ester), 1298, 1279, 1262 (C–O ester), 1694 (C = O carboxylic), 1262 (C–O carboxylic), 704 (C–Cl), 1606 (C = C aromatic).

<sup>1</sup>H NMR (Acetone-d<sub>6</sub>, J, δ): 8.22 (t, 1.9 Hz, 1H), 8.16–8.03 (m, 2H), 7.78 (d, 7.8 Hz, 1H), 7.70 (td, 7.8, 2.0 Hz, 1H), 7.51 (dt, 61.7, 7.6 Hz, 3H), 7.35 (d, 8.3 Hz, 1H), 4.83 (s, 1H).

<sup>13</sup>C NMR (Acetone-d<sub>6</sub>, δ, ppm): 20.553 (s, C = O), 164.98 (s, -C = O), 151.11 (s, -C-O), 138.87 (s, -C-CH<sub>2</sub>), 134.00 (s, -C = C-), 133.87 (s, -C = C-), 131.90 (s, -C = C-), 130.53 (s, -C = C-), 130.26 (s, -C = C-), 129.98 (s, -C = C-), 129.23 (s, -C = C-), 126.26 (s, -C = C-), 124.13 (s, -C = C-), 124.10 (s, -C = C-), 45.29 (s, -C-Cl).

### 3.2. Physicochemical Properties and in-Silico Analysis of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid

The physicochemical properties of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid were compared to acetylsalicylic acid by using Chemdraw Ultra 8.0, Chem3D Ultra 8.0 (Perkin Elmer, Waltham, USA)

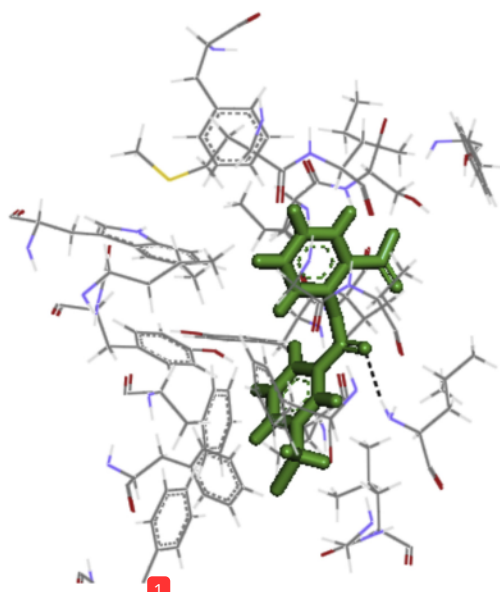


Fig. 1. *In-silico* docking of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid into cyclooxygenase-2 (COX-2) protein receptors (PDB: 5F1A). The green pattern represents one 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid. Grey pattern represents the cyclooxygenase-2 protein. The red lines represent oxygen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

and MarvinSketch 15.2.16 (ChemAxon Ltd, Budapest, Hungary). The general parameters of physicochemical properties, such as lipophilic parameter (CLogP), electronic parameter (polarization), and steric parameter (CMR), were based on previously reported symbols [10]. It was reported that 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid has CLogP value = 3.495; polarizability = 28.42; and CMR = 7.4602. Those values were better than acetylsalicylic acid values (CLogP value = 0.804; polarizability = 17.06; and CMR = 4.4576).

The docking of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid on the COX-2 receptor protein (PDB: 5F1A; Fig. 1) showed that 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (G Score - 9.48 kcal/mole) had better affinity than acetylsalicylic acid (GScore - 5.88 kcal/mole).

### 3.3. Acute oral toxicity and histopathological analysis

The acute toxicity test revealed that oral administration of a single dose (2000 mg/kg bw) of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in mice did not show any signs of toxicity or mortality in treated animals during 14 day observation period (data was not shown). In the

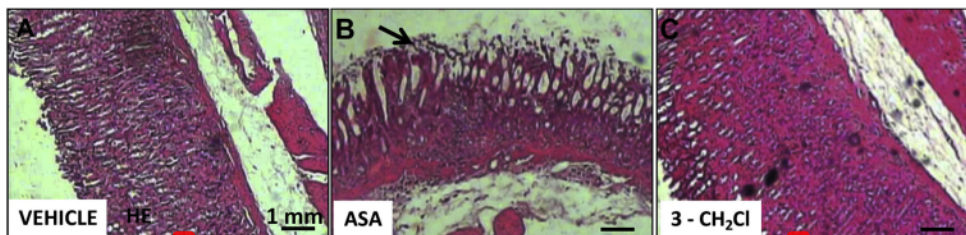


Fig. 2. Acute oral toxicity evaluation of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in rat gastric mucosal layer. The 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid did not disrupt rat gastric mucosal layer. Representative histochemical staining of rat gastric mucosal layer with H&E. Rats were treated continuously for 7 days with (A) Suspending agent of 3% PGA as vehicle, (B) 50 mg/kg bw of acetylsalicylic acid (ASA) and (C) 50 mg/kg bw of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl). Black arrow: mucosal layer with major disruption, as indicated in ASA treated rat. Scale bar = 1 mm.

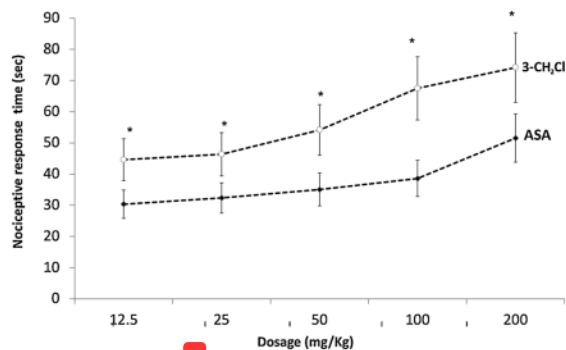


Fig. 3. Analgesic effects of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid and acetylsalicylic acid in rats with Plantar Anesthesiometer (Ugo Basile, Comerio, Italy). The line graphs show rat response time 30 min after oral administration of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl, white circle) and acetylsalicylic acid (ASA, black circle) in different dosages (12.5, 25, 50, 100, or 200 mg/kg bw). Data are presented as mean ± SEM (n = 6).

histopathological investigation of Wistar rats (Fig. 2), the micro-anatomy of gastric tissue did not demonstrate any significant treatment-related adverse effect in the animals receiving 50 mg/kg bw of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (Fig. 2C), comparison to vehicle control (Fig. 2A). As expected, the assessment result of animals receiving 50 mg/kg bw dose of acetylsalicylic acid showed significant disruption of mucosal epithelial layer, indicating the formation of gastric ulcer (Fig. 2B).

### 3.4. Analgesic activity

Oral administration of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid on heat induced rats in Plantar Anesthesiometer showed the dose-dependent increase of nociceptive response time (Fig. 3). The response time of each 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid groups in various dosages showed a significant (P < 0.01) increase compared with acetylsalicylic acid group. Following the induction of 0.6% acetic acid in writhing test (Fig. 4), 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid group showed a significant dose-dependent decrease of nociceptive response count compared with acetylsalicylic acid treated group (P < 0.01).

### 3.5. Flow cytometry based platelet aggregation assay

Fig. 5a shows representative dot-plot data of murine platelet aggregation pattern after *in-vitro* incubation of platelet with various compounds using CD31-APC and CD31-PECy7 as antibodies. Upon stimulation with collagen, the visual appearance of double-colored events (upper right box) in 2-((3-(chloromethyl)benzoyl)oxy)benzoic

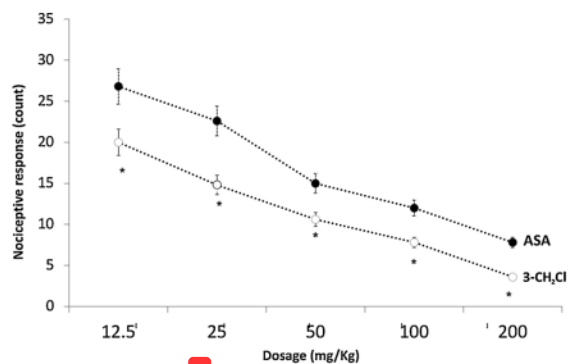


Fig. 4. Analgesic effects of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl) and acetylsalicylic acid (ASA) in mice with writhing test. Before experimental set-up, mice were administered orally with 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl; white circle) or acetylsalicylic acid (ASA, black circle) for 30 min in different dosages (12.5, 25, 50, 100, or 200 mg/kg bw). The line graphs show mice nociceptive response count after stretching of at least one hind limb to stimulate the writhing. Data are presented as mean  $\pm$  SEM (n = 6).

acid (3-CH<sub>2</sub>Cl) and acetylsalicylic acid (ASA) treated platelets was decreased compared with vehicle. The percentage of double-colored events from 30 mice (10 mice per group) were analyzed as shown in Fig. 5b. In accordance with its representative data, the total percentage of platelet aggregation events of acetylsalicylic acid (ASA)  $0.92 \pm 0.03\%$  and 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl)  $0.87 \pm 0.03\%$  was significantly lower than its negative control (vehicle;  $2.7 \pm 0.06\%$ ), indicating a significant aggregation inhibition pattern on those two compounds ( $P < 0.05$ ).

### 3.6. Tail bleeding time assay

Representative tail bleeding time analysis of 30 mice (10 mice per group) as shown in Fig. 6a showed a longer bleeding time pattern in mice after the oral administration of acetylsalicylic acid ( $t = 840$  s) and 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl;  $t = 870$  s), compared with its negative control (vehicle;  $t = 870$  s). The statistical analysis of multiple data as illustrated in Fig. 6b showed the same results. Acetylsalicylic acid (ASA;  $t = 750 \pm 130.8$  s) and 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl;  $t = 860 \pm 45.8$  s) treated animals demonstrated significantly longer bleeding time than its negative control (vehicle;  $480 \pm 103.9$  s). The bleeding pattern of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in the treated animals seemed to be more in amount compared with acetylsalicylic acid treated group.

## 4. Discussion

### 4.1. Characterization of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid

We have successfully synthesized a pure salicylic acid derivative compound, namely 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid. The synthesis of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid was carried out based on modified Schotten-Baumann acylation reaction [11,12], with the use of biphasic aqueous basic conditions.

2-((3-(chloromethyl)benzoyl)oxy)benzoic acid was formed spontaneously when 3-chloromethylbenzoyl chloride reacted with salicylic acid. However, the esters were sensitive to acid and diminished the yield of reaction. A tertiary amine like pyridine was added to the reaction mixture as a solvent to neutralize the acid (Scheme 1). As a catalyst for the reaction, we used pyridine directly to salicylic acid. Pyridine could form a stable pyridinium chloride ion by binding to free hydrogen ion from salicylic acid and free chloride ion from 3-chloromethylbenzoyl

chloride to prevent the spontaneous hydrolysis of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (supplemental Fig. 2). Pyridine could convert benzoyl chloride moieties into a good leaving group [11,12].

In this paper, we have modified our previously reported reflux method [13] with microwave irradiation method that has been described previously with acetylsalicylic acid and many other compounds ([14] [15]). The use of acetone as a solvent of the reaction and the resulting residue was significantly reduced, and therefore resulting in a better environmental impact.

The product yield of this method ( $73.27 \pm 4.66\%$ ) was higher than the reflux method ( $67.75 \pm 1.77\%$ ). The time required to accomplish a complete reaction was very rapid and significantly reduced in comparison to the previous method. Meanwhile, the reflux-irradiation took up to 6 h reaction time, whereas microwave irradiation took only 5 min reaction time.

### 4.2. Physicochemical Properties and in-Silico Analysis of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid

Based on the theoretical lipophilic parameter results, our newly synthesized compound 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (CLogP = 3.495) showed a greater value, indicating a more non-polar properties than acetylsalicylic acid (CLogP = 0.804). The higher lipophilic parameter of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid could increase its capability to enter the cytoplasm through the cell membranes [16].

Furthermore, the electronic and steric parameters of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (polarizability = 28.42; CMR = 7.4602) also demonstrated a higher value than acetylsalicylic acid (polarizability = 28.42; CMR = 7.4602). These results open a new hypothesis that our newly synthesized compound 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid could interact better with its cellular receptor with higher affinity and activity [10].

The *in-silico* docking experiment of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid to its hypothetical receptor cyclooxygenase-2 (COX-2) was conducted using Glide 4.0 XP licensed by Schrödinger (New York, USA). Glide has numerous advantages compared with numerous commercial software. This program has been designed to perform as close to an exhaustive search of the conformational, orientational and positional space of the docked ligand and was found to be more accurate than the GOLD, FlexX and DOCK [17]. To generate preliminary data regarding the interaction of our compound with COX-2, we considered GlideScore (GScore) as the scoring parameter to predict its affinity. GlideScore measures the approximate ligand binding free energy, including its electrostatic and van der Waals force fields [18]. Therefore, a smaller GScore value shows better affinity between ligand and its receptor. Theoretically, our results predicted that 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (G Score - 9.48 kcal/mole) has better affinity to its target receptor COX-2 than acetylsalicylic acid (GScore - 5.88 kcal/mole).

### 4.3. Acute oral toxicity and histopathological analysis

In this study, acute oral toxicity test indicated no harmful toxicity parameters following the administration of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid. Furthermore, the lethal dose (LD<sub>50</sub>) of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid ranged below 2000 mg/kg bw, which is identical to acetylsalicylic acid.

Numerous NSAIDs targeted on cyclooxygenases inhibition to generate analgesic effect and alleviate the symptoms of inflammation. It is well known that the therapeutic administration of non-selective COX inhibitor such as acetylsalicylic acid could induce gastric ulcer. Our histopathological results as shown in Fig. 2 particularly on 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid treatment suggested that we could exclude the harmful effect of acetylsalicylic acid by adding a new substitute 3-chloromethylbenzoyl, to its main component of salicylate.

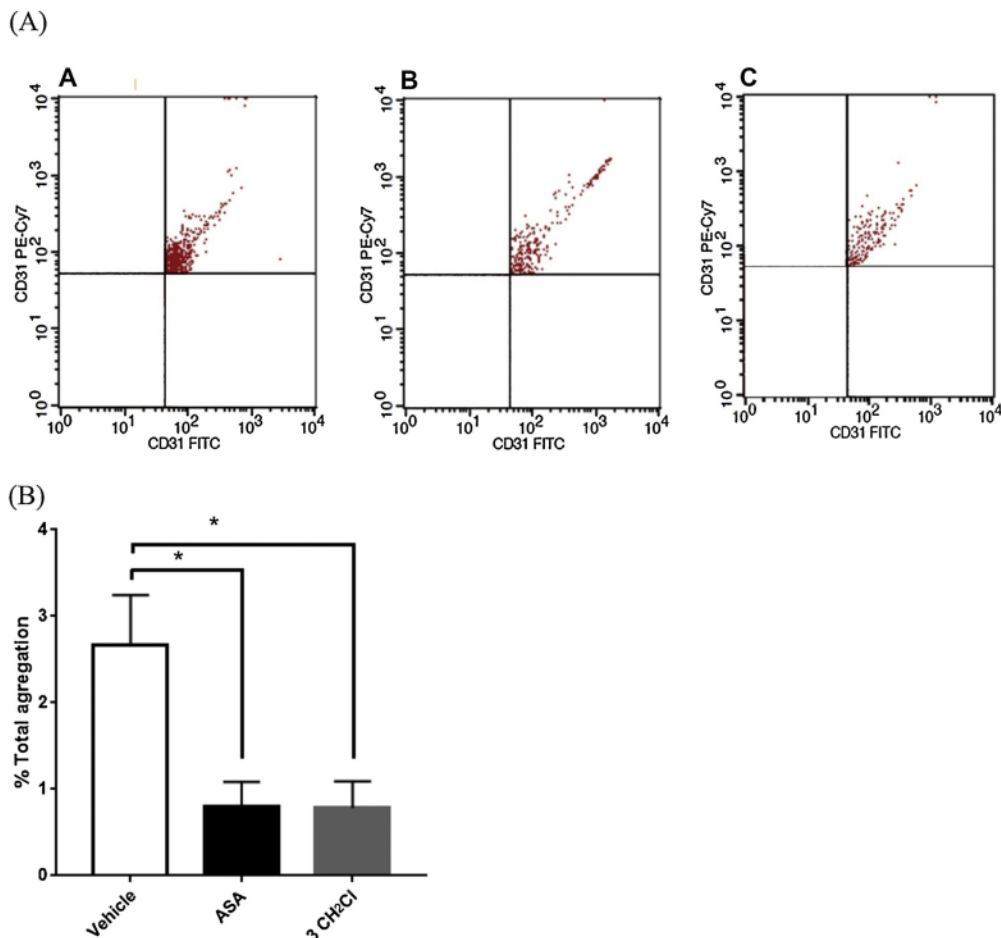


Fig. 5. Mouse platelet aggregation test with whole blood. Platelets in mouse plasma were labeled with CD31-APC or CD31-PE and mixed in 1:1 ratio before pre-incubation and stimulation. Aggregation was measured by flow cytometry, 15 min after pre-incubation with vehicle: suspending agent 3% PGA; ASA: with 0.58 mmol/kg bw of acetylsalicylic acid and 3-CH<sub>2</sub>Cl: equimolar 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl). Each pre-incubation probe was stimulated simultaneously with collagen. A. Dot Plot representative of double-colored event 15 min after pre-incubation and stimulation with collagen. B. Quantitative analysis of double-colored events (box upper right only). Data are presented as the mean of % Total aggregation (double-coloured events in box upper right divided with total events) ± SEM (n = 10). An asterisk indicates a significant difference compared with vehicle controls (P < 0.05).

On the account of the fact that the inhibition of COX-2 could successfully exhibit analgesic and antiinflammatory effect, the inhibition of COX-1 by acetylsalicylic acid leads to disruption of the integrity of healthy gastric mucosal layer [19]. Our results showed a trend that the action of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid had a lower interaction with gastric COX-1, presumably resulted in the partial inhibition of its function and therefore it could maintain the integrity of gastric mucosal membrane (supplemental Fig. 3).

#### 4.4. Analgesic activity

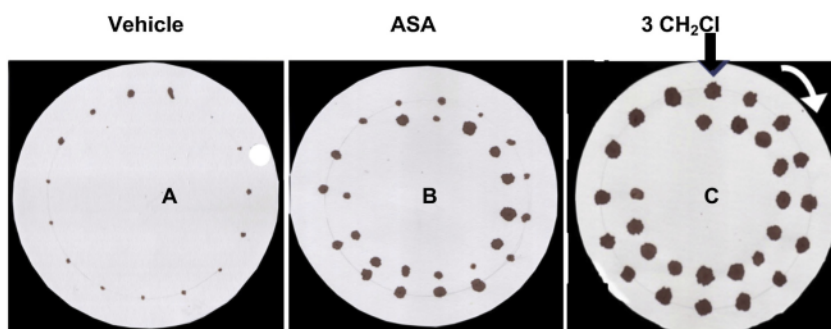
The dose-dependent increase of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid nociceptive response on heat induced rats in Plantar Anesthesiometer gave us a primary evidence of the analgesic effect of our newly synthesized drug to the peripheral nervous system response. Under peripheral analgesic effect, the animal models were significantly more resistant to peripheral heat sensors better than its control group, acetylsalicylic acid. Interestingly, the writhing test of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid with 0.6% acetic acid showed a dose-dependent decrease and it significantly gave a lower nociceptive

response count compared with acetylsalicylic acid treated groups. It has been considered that the writhing test could stimulate the signals transmitted to central nervous system in response to pain generated by irritation agents such as 0.6% acetic acid [20], the inhibition of pain by common NSAIDs could inhibit the production of prostaglandins in central nervous system by inhibiting its synthesized enzyme of COX-2 which contributes to the decreased sensitivity to nociceptors time [21]. Therefore, summarizing the results from Plantar Anesthesiometer and *in-silico* docking experiment, we postulated that our newly synthesized drug, 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid, could act as a better analgesic agent than acetylsalicylic acid through the peripheral and central nervous systems by inhibiting the production of prostaglandins by common mechanism of action such as COX-2-inhibition.

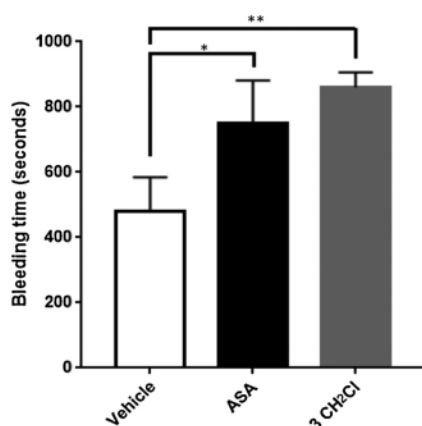
#### 4.5. Flow cytometry based platelet aggregation assay

To discover further advantage of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid in cardiovascular disease, we postulated that in accordance with acetylsalicylic acid, 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid could also play a role as an anti platelet-aggregation drug

(A)



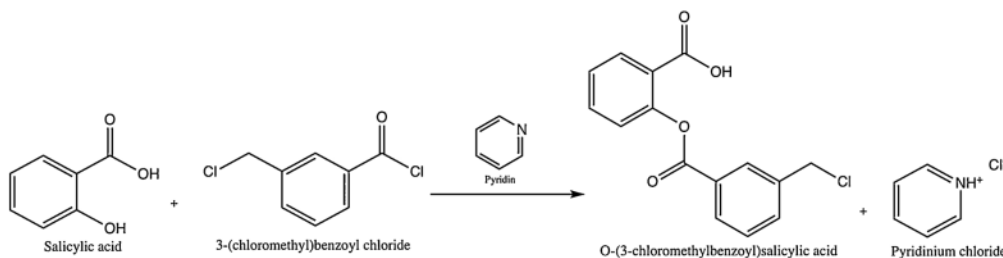
(B)



**Fig. 6.** Tail bleeding time assay of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid and acetylsalicylic Acid by a filter paper method. A. Representative graphics are shown for blood spot of incised mice tails (A) with suspending agent 3% PGA acted as vehicle, (B) with 0.58 mmol/kg bw of acetylsalicylic acid (ASA), or (C) equimolar O-(3-chloromethylbenzoyl)salicylic acid (3-CH<sub>2</sub>Cl), 30 min after drug administration. Black arrow indicates preliminary blood drop spot. White arrow indicates the clockwise blood drop position. Each spot represents 30 s interval. Continuing blood flow from the cut during the 30-second interval was allowed to fall separately. B. Quantitative analysis of tail bleeding time by filter paper method. Blood spot from 6a was counted as described and represented as bleeding time (in second) in this diagram. Graphical result of vehicle: suspending agent 3% PGA, ASA: with 0.58 mmol/kg bw of acetylsalicylic acid and 3-CH<sub>2</sub>Cl: equimolar 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid (3-CH<sub>2</sub>Cl). Data are presented as mean ± SEM (n = 6). An asterisk indicates a significant difference compared with vehicle controls (P < 0.05). Two asterisks indicate a significant difference compared with vehicle controls (P < 0.05).

for cardioprotective effect by minimizing the thrombus formation. Indeed the platelet aggregation phenomenon tested in flow cytometry-based assay was significantly decreased in 0.58 mmol/kg bw of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid treated mice (equivalent to human dosage 500 mg/60 kg bw), in comparison to the equimolar acetylsalicylic acid treated animal group. It is already well known, that the antithrombotic action of aspirin (acetylsalicylic acid) is due to the inhibition of platelet function by acetylation of the platelet COX-1 [2]. However, the histopathological results as shown in Fig. 2 led us to another hypothesis that the mechanism of action of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid was presumably not directly or

partially associated with COX-1 inhibition due to the intact integrity of gastric mucosal layer [22]. Therefore the antithrombotic action of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid could be caused by another common anti-aggregation mechanism associated with salicylic acid derivative drugs. Tehrani et al. [23] demonstrated the influence of acetylsalicylic acid on the disruption of blood plasma coagulation factors network such as fibrin formation. Other studies showed that another salicylate derived anti-platelet agent namely triflusal has minor gastrointestinal toxicity due to weaker COX-1 inhibition than acetylsalicylic acid [24], which may be another possible reason for the dual advantage of 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid as an



**Scheme 1.** Salicylic acid (IUPAC name: 2-hydroxybenzoic acid) reacted with 3-(chloromethyl)benzoyl chloride, with pyridine as the catalyst and the reactive group, by using MicroSYNTH microwave (600 W, 5 min), yielded 2-((3-(chloromethyl)benzoyl)oxy)benzoic acid and pyridin-1-ium chloride. The pyridin-1-ium chloride was solved in water, nor was the products.



analgesic-antiplatelet drug with significantly lower gastrointestinal toxicity profile.

#### 4.6. Tail bleeding time assay

To verify the anti-aggregation results, we conducted the classical murine tail bleeding time assay with 0.58 mmol/kg bw of 2-((3-(chloromethyl)benzoyloxy)benzoic acid). The results generated from this assay did not only indicate the platelet functionality, but also gave us a broader spectrum to be considered regarding the coagulation factors and vascular integrity issue [25]. As expected with salicylic acid-related drugs, the bleeding time was prolonged in 2-((3-(chloromethyl)benzoyloxy)benzoic acid) treated animal group. Additionally, the bleeding pattern of 2-((3-(chloromethyl)benzoyloxy)benzoic acid) treated animals seemed to be more in amount compared with the acetylsalicylic acid treated group. Therefore until further laboratory and clinical investigation, we postulated that 2-((3-(chloromethyl)benzoyloxy)benzoic acid is a better candidate of anti-platelet aggregation drugs, which could contribute better than acetylsalicylic acid in treating the patients with cardiovascular problems. Additionally, our results as shown in Fig. 6 gave us some clue regarding the finding of another mechanism apart from platelet COX-1 aggregation cascade which could induce higher bleeding, simultaneously disrupt the platelet function and has greater impact on blood homeostasis. The disruption of plasma coagulation factors integrity was one of possible mechanism caused by oral administration 2-((3-(chloromethyl)benzoyloxy)benzoic acid to induce the antiplatelet aggregation.

#### 5. Conclusion

The present study demonstrated that we could successfully synthesize a new compound related to salicylic acid namely 2-((3-(chloromethyl)benzoyloxy)benzoic acid with a relative simple and easy method, having higher purity grade, and higher reaction yield. Through the observation of our animal models, we postulated that this new compound had an excellent analgesic effect with no toxicity impact, better than acetylsalicylic acid. Since this compound could induce antiplatelet aggregation, it seemed that 2-((3-(chloromethyl)benzoyloxy)benzoic acid could play another role as an anti-platelet aggregation drug presumably by binding to its hypothetical target: COX-2 receptor protein.

#### Author contribution

Caroline and Yudy Tjahjono designed the experiments, carried out experiments, analyzed the data, and wrote the manuscript. Kuncoro Foe, Senny Yesery Esar, Ami Soewandi, Ratna Megawati Widharna, Wahyu Dewi Tamayanti and Elisabeth Kasih assisted the experiments. Hevi Wihadmadyatami analyzed the experimental data.

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