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SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF ACYLHYDROQUINONE DERIVATIVES AS POTENT ANTIPLATELET AGENTS

Running title: Antiplatelet activity of acylhydroquinone

By

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ABSTRACT

Platelets are the smallest blood cells, and their activation (platelet cohesion or aggregation) at sites of vascular injury is essential for thrombus formation. Since the use of antiplatelet therapy is an unsolved problem, there are now focused and innovative efforts to develop novel antiplatelet compounds. In this context, we assessed the antiplatelet effect of an acylhydroquinone series, synthesized by Fries rearrangement under microwave irradiation, evaluating the effect of diverse acyl chain lengths, their chlorinated derivatives, and their dimethylated derivatives both in the aromatic ring and also the effect of the introduction of a bromine atom at the terminus of the acyl chain. Findings from a primary screening of cytotoxic activity on platelets by lactate dehydrogenase assay identified 19 non-toxic compounds from the 27 acylhydroquinones evaluated. A large number of them showed IC_{50} values less than 10 μM acting against specific pathways of platelet aggregation. The highest activity was obtained with compound **38**, it exhibited sub-micromolar IC_{50} of 0.98±0.40, 1.10±0.26, 3.98±0.46, 6.79±3.02 and 42.01±3.48 μM against convulxin-, collagen-, TRAP-6-, PMA- and arachidonic acid-induced platelet aggregation, respectively. It also inhibited P-selectin and granulophysin expression. We demonstrated that the antiplatelet mechanism of compound **38** was through a decrease in a central target in human platelet activation as in mitochondrial function, and this could modulate a lower response of platelets to activating agonists. The results of this study show that the chemical space around *ortho*-carbonyl hydroquinone moiety is a rich source of biologically active compounds, signaling that the acylhydroquinone scaffold has a promising role in antiplatelet drug research.

Keywords: platelets; antiplatelet; synthesis; acylhydroquinone; acylchlorohydroquinones.

1. INTRODUCTION

Atherothrombosis, characterized by atherosclerotic lesion disruption with superimposed thrombus formation, is the common underlying process for numerous progressive manifestations of acute coronary syndromes and cardiovascular death. Following rupture, fissure, or erosion of an atherosclerotic plaque; atherothrombotic events are essentially platelet-driven processes [1, 2]. Platelets are the smallest blood cells, numbering 150 to 350 × 10^9/L in healthy individuals [3]. In addition to being the first step for the maintenance of normal hemostasis, the ability of platelets to form stable adhesion contacts with other activated platelets (platelet cohesion or aggregation) at sites of vascular injury is essential for thrombus formation, myocardial infarction, and stroke [4-7]. Thrombus development can involve the initial formation and recruitment of reversible platelet aggregates independent of platelet activation [8].

Antiplatelet therapy represents the cornerstone of the clinical treatment of atherothrombotic events [9]. Currently, independent of classes of antiplatelet drugs (e.g. clopidogrel, aspirin, and ticlopidine), these reduce the vascular events by about 10-12% in secondary prevention [10]. However, it is necessary to further knowledge on antiplatelet scaffolds and their structural requirements to obtain new active compounds [11].

Several phenols and FDA-approved drugs exert antiplatelet effects by different mechanisms resulting from their antioxidant properties [12]. Among them is

hydroquinone which is a powerful antioxidant used in cosmetics [13] that inhibits thromboxane A2 (TXA2) production and suppresses arachidonic acid (AA)-induced platelet aggregation [14]. Besides, some hydroquinone derivatives such as arbutin, the natural β-glucoside, inhibit platelet aggregation induced by different agonists (adenosine diphosphate [ADP], AA, collagen, and thrombin) [15], although it is quickly metabolized in blood in rats [16], 2,5-di-(*tert*-butyl)-*p*-hydroquinone also inhibits platelet aggregation, in this case, it is stimulated by protease-activated receptor (PAR)-1 or PAR-4 agonist peptides (SFLLRN and AYPGKF) [17], and avarol, sesquiterpenoid hydroquinone isolated from the sponge *Dysidea avara*, inhibits platelet aggregation stimulated by AA and A23187 (calcium ionophore) (Figure 1) [18].

Some antiplatelet mechanisms involving inhibition of oxidative phosphorylation (OXPHOS) through the interaction of hydroquinone derivatives with the electron transport chain (ETC) have been described [19]. Several o*rtho*-carbonylhydroquinone scaffold-containing compounds affect mitochondrial bioenergetics, by inhibiting mitochondrial electron transport and/or by uncoupling of OXPHOS, with consequences on the proliferation of cancer cells [20-24] and the antioxidant activity of some of them has also been reported [25]. The strong hydrogen bond between the carbonyl group and one of the phenolic hydroxyl groups is an important feature regarding these activities [26-28]. Recently, the effects of the acylhydroquinone scaffold, the simplest *ortho*-carbonylhydroquinone structure, against leukemia cell lines, have been reported [29].

Taking into account that *ortho*-carbonyl hydroquinone moiety is a structural feature that, besides other activities, confers potent antiplatelet activity via differential

inhibition of platelet aggregation induced by collagen or thrombin receptor activator peptide 6 (TRAP-6) [30], also considering that, small structural changes modify these properties including the effect on OXPHOS [23], and given that, most of the assessed acylhydroquinones against leukemic cells do not exhibit significative cytotoxicity, it was decided to assess the antiplatelet activity of a wider series of acylhydroquinones.

2. MATERIALS AND METHODS

2.1. Chemical Methods

^{1}H and ^{13}C NMR spectra were obtained from a spectrometer (Bruker Avance 400 NMR, Rheinstetten, Germany) operating at either 400.13 MHz (^{1}H) or 100.61 MHz (^{13}C), or from one operating at 300.13 MHz (^{1}H) or 75.47 MHz (^{13}C). Chemical shifts are reported as ppm downfield from the tetramethylsilane dissolved in $CDCl_3$ (Merck, DA, Germany) for ^{1}H NMR and relative to the central $CDCl_3$ resonance (77.0 ppm) for ^{13}C NMR. All melting points are uncorrected and were determined using an Electrothermal 9100 apparatus. IR spectra (KBr discs) were recorded on an FT-IR spectrophotometer (Thermo Nicolet Corp, Madison, WI, USA); wavenumbers reported in cm^{-1}. High-resolution mass spectra (HRMS) were obtained from an orthogonal time-of-flight (TOF) mass spectrometer (Waters/Micromass Q-TOF micro, Manchester, UK). Silica gel 60 (230 - 400 mesh ASTM) (Sigma-Aldrich, DA, Germany) and thin-layer chromatography (TLC) sheets silica gel 60 F254 (Merck, DA, Germany) were used for flash-column chromatography and analytical TLC, respectively [30].

2.2. Synthesis of acylhydroquinones and acylchlorohydroquinones

The synthetic methodology for obtaining acylhydroquinones has been already

reported [29, 30] and was used to synthesize the compounds **13-27** (Table 1). Briefly, acylhydroquinones **13-27** were obtained using microwave irradiation at 90°C, at a fixed power of 150W as follows. To a 10 mL CEM microwave (CEM Discovery SP Labmate, NC, USA) process vial equipped with a magnetic bar, a mixture of one equivalent of hydroquinone was added (**1**) or dimethyl hydroquinone (**2**), 1.5 equivalent of the corresponding carboxylic acid (**3-11**), and 4 mL of boron trifluoride dihydrate (Sigma-Aldrich, DA, Germany). The mixture was irradiated under microwave for 20 min. After that, the mixture was extracted with ethyl acetate (Panreac Quimica, BCN, Spain), washed with water, and dried with anhydrous sodium sulfate (Sigma-Aldrich, DA, Germany), then it was filtered and concentrated under vacuum. Afterward, the corresponding acylhydroquinone was purified by flash chromatography with hexane/ethyl acetate (Panreac Quimica, BCN, Spain) 4:1 as eluent. In this manner, the acylhydroquinones **13-27** were obtained. As shown in Table 1, compounds **28-38** were obtained by chlorination, with hydrogen chloride (obtained by reaction of concentrated sulfuric acid with sodium chloride) [31] of the corresponding quinones obtained by oxidation of the hydroquinones **12-15**, **17-22,** and **24**, with Ag_2O (obtained by adding concentrated sodium hydroxide to a silver nitrate solution) [32] in dichloromethane (Merck, DA, Germany) [29], these compounds were also purified by flash chromatography with hexane/ethyl acetate 4:1 as eluent.

The syntheses of the new analogs **23-27**, **29**, and **38** are described as follows:

*Synthesis of 5-bromo-1-(2,5-dihydroxyphenyl)pentan-1-one (**23**)*

Hydroquinone (500 mg, 4.54 mmoles) (Sigma-Aldrich, DA, Germany), 5-bromopentanoic acid (1.23 g, 6.81 mmoles) (Sigma-Aldrich, DA, Germany) and 3

mL of boron trifluoride dihydrate (Sigma-Aldrich, DA, Germany) react yielding 409 mg of **23** (4.54 mmol, 33 % yield). ^{1}H-NMR δ(400.13 MHz, $CDCl_3$): 1.87-2.03 (m, 4H, 2xCH_2); 2.99 (t, 2H, *J* = 7.4Hz, CH_2-CO); 3.47 (t, 2H, *J* = 6.4Hz, BrCH_2); 4.82 (s, 1H, 5-OH); 6.90 (d, 1H, *J* = 8.9Hz, 3-H); 7.04 (dd, 1H, J_1 = 8.9Hz; J_2 = 2.8Hz, 4-H); 7.22 (d, 1H, *J* = 2.8 Hz, 6-H); 11.86 (s, 1H, 2-OH). ^{13}C-NMR δ(100.61 MHz, $CDCl_3$): 22.71; 32.01; 33.11; 37.24; 114.67; 119.48; 124.90; 147.37; 177.69; 205.24. m.p. 58.7-60.7 °C. IR(KBr) ν_{max}: 1491.00; 1650.16; 1708.04; 2871.38; 2943.73; 3380.71. HRMS (ESI): m/z calcd for $C_{11}H_{13}O_3Br$ M^+: 272.0048, found: 272.0048.

*Synthesis of 5-bromo-1-(2,5-dihydroxy-3,4-dimethylphenyl)pentan-1-one (**24**)*

2,3-Dimethylhydroquinone (500 mg, 3.61 mmol) (Sigma-Aldrich, DA, Germany), 5-bromopentanoic acid (980 mg, 5.42 mmol) (Sigma-Aldrich, DA, Germany) and 3 mL of boron trifluoride dihydrate (Sigma-Aldrich, DA, Germany) react yielding 233 mg of **24** (3.61 mmol, 21 % yield). ^{1}H-NMR δ(400.13 MHz, $CDCl_3$): 1.86-2.01 (m, 4H, 2XCH_2); 2.21 (s, 3H, Ar-CH_3); 2.24 (s, 3H, Ar-CH_3); 2.95 (t, 2H, *J* = 6.6Hz, CO-CH_2); 3.46 (t, 2H, *J* = 6.4Hz, Br-CH_2); 4.53 (s, 1H, 5-OH); 7.02 (s, 1H, 6-H); 12.36 (s, 1H, 2-OH). ^{13}C-NMR δ(100.61 MHz, $CDCl_3$): 11.58; 13.02; 23.02; 32.07; 33.18; 37.08; 111.16; 115.86; 127.13; 134.17; 145.43; 155.43; 204.98. m.p. 125.2-126.5 °C. IR(KBr) ν_{max}: 1572.03; 1669.65; 2923.47; 2958.20; 3377.81. HRMS (ESI): m/z calcd for $C_{13}H_{17}O_3Br$ M^+: 300.0361 found 300.0359.

*Synthesis of 6-bromo-1-(2,5-dihydroxyphenyl)hexan-1-one (**25**)*

Hydroquinone (453 mg, 4.12 mmol) (Sigma-Aldrich, DA, Germany), 6-bromohexanoic acid (1.2 g, 6.15 mmol) (Sigma-Aldrich, DA, Germany) and 3 mL of

boron trifluoride dihydrate (Sigma-Aldrich, DA, Germany) react yielding 526 mg of **25** (1.83 mmol, 22% yield). ^{1}H-NMR δ(300.13 MHz, $CDCl_3$): 1.54 (quint, 2H, *J* = 7.3 Hz; CH_2); 1.76 (quint, 2H, *J* = 7.3 Hz, CH_2); 1.92 (quint, 2H, *J* = 7.3 Hz, CH_2); 2.96 (t, *J* = 7.4 Hz, 2H, $COCH_2$); 3,43 (t, 2H, *J* = 6.6 Hz, Br-CH_2); 5.84 (s, 1H, 5'-OH), 6.88 (d, 1H, *J* = 8.9 Hz, 3-H); 7.05 (dd, 1H, J_1 = 8.9 Hz, J_2 = 2.9 Hz, 4-H); 7.23 (d, 1H, *J* = 2.9 Hz, 6-H); 11.92 (s, 1H, 2-OH). ^{13}C-NMR δ(75.45 MHz, $CDCl_3$): 23.32; 27.77; 32.49; 33.58; 38.11; 114.75; 118.94; 119.40; 124.87; 147.48; 156.57; 205.86. m.p. 156,1-160,4 °C; IR(KBr) ν_{max}: 1728.30; 2859.81; 2943.73; 3010.29; 3073.96; 3395.18. HRMS (ESI): m/z calcd for $C_{12}H_{15}BrO_3$ M^+: 286.0205 found 286.0205.

*Synthesis of 6-bromo-1-(2,5-dihydroxy-3,4-dimethylphenyl)hexan-1-one (**26**)*

2,3-Dimethylhydroquinone (500 mg, 3.61mmol) (Sigma-Aldrich, DA, Germany), 6-bromohexanoic acid (1.06 g, 5.43 mmol) (Sigma-Aldrich, DA, Germany) and 3 mL of boron trifluoride dihydrate (Sigma-Aldrich, DA, Germany) react yielding 481mg of **26** (3.61 mmol, 42 mmol, 42% yield). ^{1}H-NMR δ(400.13 MHz, $CDCl_3$): 1.48-1.59 (m, 2H, CH_2); 1.76 (quint, 2H, *J* = 7.5Hz, CH_2); 1.92 (quint, 2H, *J* = 6.8Hz, CH_2); 2.21 (s, 3H, Ar-CH_3); 2.24 (s, 3H, Ar-CH_3); 2.93 (t, 2H, *J* = 7.5Hz, CH_2CO); 3.44 (t, 2H, *J* = 6.6Hz, CH_2-Br); 4.59 (s, 1H, 5-OH); 7.02 (s, 1H, 6-H); 12.41 (s, 1H, 2-OH). ^{13}C-NMR δ(100.61 MHz, $CDCl_3$): 11.58; 13.02; 23.63; 27.83; 32.53; 33.53; 37.95; 111.23; 115.92; 127.08; 134.06; 145.40; 155.41; 205.46. m.p. 125.2-125.9 °C. IR (KBr) ν_{max}: 1609.65; 2856.91; 2932.15; 2961.09; 3366.24. HRMS (ESI): m/z calcd for $C_{14}H_{19}BrO_3$ M^+: 314.0518, found 314.0517.

*Synthesis of 10-bromo-1-(2,5-dihydroxy-3,4-dimethylphenyl)decan-1-one (**27**)*

2,3-Dimethylhydroquinone (500 mg, 3.61mmol) (Sigma-Aldrich, DA, Germany), 10-bromodecanoic acid (1.36 g, 5.43 mmol) (Sigma-Aldrich, DA, Germany) and 3 mL of boron trifluoride dihydrate (Sigma-Aldrich, DA, Germany) react yielding 228 mg of **27** (3.61 mmol, 17% yield). ^{1}H-NMR δ(400.13 MHz, $CDCl_3$): 1.27-1.39 (m, 8H, 4xCH_2); 1.43 (quint, 2H, *J* = 7.5Hz, CH_2); 1.73 (quint, 2H, *J* = 7.5Hz, CH_2); 1.86(quint, *J* = 7.0Hz, CH_2); 2.21(s, 3H, Ar-CH_3); 2.24 (s, 3H, Ar-CH_3); 2.90 (t, 2H, *J* = 7.5Hz, CO-CH_2); 3.41 (t, 2H, *J* = 7.0Hz, Br-CH_2); 4.53 (s, 1H, 5-OH); 7.03(s, 1H, 6-H); 12.45 (s, 1H, 2-OH). ^{13}C-NMR δ(100.61 MHz, $CDCl_3$): 11.58; 13.00; 24.70; 28.12; 28.68; 29.23; 29.26; 29.28; 32.79; 34.03; 38.27; 111.39; 115.98; 127.01; 133.89; 145.34; 155.42; 206.10. m.p. 97.5-98.1 °C. IR (KBr, cm^{-1}) ν_{max}: 1572.03; 1609.65; 2848.23; 2926.37; 3366.24. HRMS (ESI): m/z calcd for $C_{18}H_{27}O_3Br$ M+: 370.1144 found 370.1131.

*Synthesis of 1-(2-chloro-3,6-dihydroxy-4,5-dimethylphenyl)ethan-1-one (**29**)*

A mixture of acylhydroquinone **13** (100 mg, 0.55 mmol) and Ag_2O (380 mg) in dichloromethane (6 mL) (Merck, DA, Germany) was stirred for 30 min, after the mixture was filtered through Celite and dry hydrogen chloride was bubbled through the filtrate for 5 min. Column flash chromatography on silica gel, allowed the isolation of 101 mg of **29** (0.47 mmol, 85% yield); m.p. 117.3-118.4 °C; IR νmax: 1615.43; 2926.37; 2628.30; 2544.37; 3453.05. ^{1}H-NMR δ(400 MHz, $CDCl_3$): 2.19 (s, 3H, Ar-CH_3); 2.30 (s, 3H, Ar-CH_3); 2.81 (s, 3H, $COCH_3$); 5.54 (s, 1H, 3-OH); 12.62(s, 1H, 6-OH). ^{13}C-NMR δ(100.17 MHz, $CDCl_3$): 11.79; 13.72; 33.30; 114.38; 115.60; 126.44; 133.46; 143.03; 155.94; 203.95. HRMS (ESI): m/z calcd for $C_{10}H_{11}ClO_3$ M^+: 214.0397 found 214.0375.

*Synthesis of 5-bromo-1-(2-chloro-3,6-dihydroxy-4,5-dimethylphenyl)pentan-1-one (**38**)*

A mixture of acylhydroquinone **24** (100 mg, 0.33 mmol) and Ag_2O (380 mg) in dichloromethane (6 mL) (Merck, DA, Germany) was stirred for 30 min, after the mixture was filtered through Celite and dry hydrogen chloride was bubbled through the filtrate for 5 min. Column flash chromatography on silica gel allowed the isolation of 59 mg of 38 (0.18 mmol, 55 % yield). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.86-2.00(m, 4H, 2xCH_2), 2.19 (s, 3H, Ar-CH_3); 2.29(s, 3H, Ar-CH_3); 3.20(t, 2H, *J* = 6.5Hz, $COCH_2$); 3.45(t, 2H, *J* = 6.3Hz, $BrCH_2$); 5.54(s, 1H, 3-OH); 12.23(s, 1H, 6-OH). ^{13}C-NMR δ(100.61 MHz, $CDCl_3$): 11.87, 13.70, 23.51, 29.70, 32.16, 33.20, 43.26, 113.43, 115.67, 126.58, 133.15, 143.09, 155.31, 205.80. m.p.: 97.2-98.3 °C. IR (KBr) ν_{max}: 1592.28, 1618.33, 2923.47, 2966.88, 3418.33. HRMS (ESI) M+: m/z calcd for $C_{13}H_{16}BrClO_3$ M^+: 333.9971 found 333.9984.

Besides, the monomethyl ethers of some of these hydroquinones (**12**, **13**, **17**, **25**, and **26**) were obtained by the standard methodology of protecting the phenolic hydroxyl group [33].

2.3. Isolation of human platelets from whole blood

Venous whole blood was obtained with anticoagulant acid citrate-dextrose (ACD) (Sigma-Aldrich, MO, USA) from apparently healthy volunteers (during the last 7 days without consumption of non-steroidal anti-inflammatory drugs and absence of chronic diseases), used at a 1:4 ratio for ACD: venous whole blood [34]. Then the sample was centrifuged (Eppendorf 5804, CT, USA) for 10 min at 200 g to obtain platelet-rich plasma (PRP). One part of the PRP (two thirds) was centrifuged for 8

min at 800 g, the supernatant was removed and the platelet pellet was resuspended in modified Tyrode's buffer (Sigma-Aldrich, MO, USA) without calcium. Platelets were centrifuged once more (8 min at 800 g) and used within 3 h. A Hematology analyzer (Hematological counter Mindray BC-3000 Plus, Japan) was used to count the washed platelets [30]. The study was approved by the Scientific Ethics Committee from the University of Talca.

2.4. Lactate dehydrogenase (LDH)-based cytotoxicity assay

To assess the cytotoxicity of the compounds under study, LDH Cytotoxicity Assay Kit (Cayman Chemical, MI, USA) was used [30]. Briefly, washed platelets $300x10^9$ (platelets/L) were used under different conditions: negative control with dimethyl sulfoxide (DMSO) 0.2% (Duchefa Biochemie, Haarlem, Netherlands), positive control with triton 10% (Sigma-Aldrich, MO, USA), reactive control and synthesized compounds (200 µM). All conditions were incubated for 10 min at 37°C and then centrifuged at 800 g for 8 min at 4°C. After that, 100 µL of the supernatant was mixed with 100 µL of LDH reaction solution in a 96-well microplate. The microplate was incubated for 30 min at 37°C under constant orbital shaking and the reaction was measured at 490 nm in a microplate reader (Microplate Reader Thermo Scientific Multiskan Go, Finland). Based on positive control, the level of LDH released was considered as 100%.

2.5. Platelet aggregation and secretion of adenosine triphosphate (ATP)

The real-time analysis of platelet aggregation and secretion of ATP *in vitro*, monitored by light transmission and luminescence, was performed using a lumi-aggregometer (Chrono-Log, Haverton, PA, USA) [34]. Briefly, synthesized compounds (0.02 to 200 µM) were pre-incubated with washed platelets ($300×10^9$

platelets/L) or DMSO 0.2%, and $CaCl_2$ (2 mM) (Sigma-Aldrich, MO, USA) for 5 min at 37°C. For ATP secretion, platelets were pre-incubated for 30 s at 37°C with chrono-lume probe (Chrono-Log, Haverton, PA, USA). Then real-time monitoring of platelet aggregation and ATP secretion was started by the addition of agonist (TRAP-6 5 µM [Cayman Chemical, MI, USA], collagen 1 µg/mL [Helena Laboratories, TX, USA], convulxin 5 ng/mL [Santa Cruz Biotechnology, TX, USA], phorbol-myristate-acetate [PMA] 100 nM [Cayman Chemical, MI, USA] or arachidonic acid 30 ng/mL [Helena Laboratories, TX, USA]) for 5 min at 37°C. Results of platelet aggregation and ATP secretion were determined by the software AGGRO/LINK (Chrono-Log, Havertown, PA, USA) and expressed as a percentage of maximal amplitude. The synthesized compounds were solubilized in DMSO 0.2%.

2.6. Flow cytometry assays

Washed platelets (200×10^9 platelets/L) were pre-incubated for 10 min at 37°C with synthesized compounds (2 and 20 µM) or DMSO 0.2%, and $CaCl_2$ (2 mM), and then platelets were stimulated by TRAP-6 5 µM, convulxin 5 ng/mL or PMA 100 nM. Subsequently, 50 µL aliquots were taken per condition to be evaluated: (i) platelet activation (dense granules and α–granules) were evaluated by CD63PE and CD62PE antibodies (BD Biosciences, CA, USA), (ii) mitochondrial membrane potential ($\Delta\Psi m$) was determined by Accuri C6 flow cytometer (BD Biosciences, CA, USA), using the potentiometric probe tetramethylrhodamine, methyl ester, perchlorate (TMRM) 100 nM (Invitrogen, MA, USA), (iii) intraplatelet reactive oxygen species (ROS) level was determined using the dihydroethidium probe 10 µM (Thermo Fisher Scientific, MA, USA), (iv) intraplatelet calcium levels were

measured by flow cytometry with the Fluo-3 AM 0.44 µM (Invitrogen, MA, USA) and (v) externalization of phosphatidylserine on platelet membrane was detected by FITC Annexin V Apoptosis Detection Kit (BD Biosciences, CA, USA). Finally, the conditions were incubated with anti-CD61FITC (BD Biosciences, CA, USA) for 30 min at room temperature in the dark and analyzed by Accuri C6 flow cytometer [30, 34].

2.7. Statistical analysis

Data were obtained from experiments from at least 3 healthy volunteers and analyzed using Prism 5.0 software (GraphPad Inc., San Diego CA, USA). The half-maximal inhibitory concentration (IC_{50}) was calculated from the dose-response curves. Differences between samples were analyzed using ANOVA analysis and Tukey's posthoc test. P values <0.05 were considered significant.

3. RESULTS AND DISCUSSION

3.1. Chemistry of synthesized compounds

The chemical structures of acylhydroquinones and acylchlorohydroquinones studied in this work are depicted in Table 1. The reported methodology for obtaining acylhydroquinones [29, 30] was used to synthesize the previously reported compounds **13**-**27** and **28**-**37**. Meanwhile, compound **12** was obtained from a commercial source, and compound **13** and **28** were obtained as reported [35, 36]. Compounds **14**-**22** were recently synthesized by Fries rearrangement under microwave irradiation, their chloro-derivatives were also obtained **30**-**37**, and their antileukemic activity studied [29]. The syntheses of the new analogs **23**-**27**, **29**, and **38** were described in the Materials and Methods section.

3.2. Cytotoxicity of synthesized compounds on platelets

The cytotoxicities of acylhydroquinone derivatives were evaluated in human washed platelets. The LDH is present in numerous cell types, including platelets, which have high LDH activity [37]. In the LDH assay, cytotoxicity of synthesized compounds or substances is measured by the release of cytosolic LDH upon damage of the cell membrane, which is different from platelet activation by agonists [38]. As a positive control, incubation of membrane detergent triton X-100 with human washed platelets was expressed as 100%. As shown in Figure 2, LDH assay revealed that the synthesized compounds **36** ($p<0.001$) > **33** ($p<0.001$) > **34** ($p<0.001$) > **32** ($p<0.001$) > **37** ($p<0.001$) > **21** ($p<0.001$) > **35** ($p<0.001$) > **19** ($p<0.01$) at 200 μM incubated for 10 min with washed platelets significantly increased the percentage of LDH release, compared with the control respectively. In acylhydroquinones, the cytotoxicity of compounds **19** and **21** showed that long acyl chains, with R_1 and R_2 = H, confer cytotoxic activity. This was also observed, although not statistically, in compound **17** (chain with n = 6) which showed a higher cytotoxicity than the control. While in acylchlorohydroquinones derivatives, compounds **32**, **33**, **34**, **35**, **36,** and **37** were toxic with carbon chains of n = 6, 7, and 8, independently if they had Me or H as R_1 substituent. LDH leakage has been reported to correlate well with the degree of decrease in platelet count, suggesting that these toxic compounds may decrease platelet count directly through platelet cytotoxicity [39]. Meanwhile, LDH leakage assay by synthesized compounds **12**, **13**, **14**, **15**, **16**, **17**, **18**, **20**, **22**, **23**, **24**, **25**, **26**, **27**, **28**, **29**, **30**, **31**, and **38** did not induce human platelet cytolysis and was not significantly different than DMSO-treated platelets (8.19±1.67%). We continued testing for antiplatelet activity with these nineteen non-toxic compounds.

3.3. Antiplatelet activity of non-toxic compounds

To determine the antiplatelet activity of the 19 non-toxic compounds, the platelet aggregation induced by TRAP-6, collagen, or convulxin was evaluated by light transmission [40]. TRAP-6 acts as a PAR1 agonist in platelet activation [41]. Platelet GPVI is the major platelet collagen receptor in the activation of the platelet. Also, integrin $\alpha 2\beta 1$, and CD36 bind to collagen directly, whereas GP $Ib\alpha$ and integrin $\alpha IIb\beta 3$ interact with collagen-bound vWF [42]. Meanwhile, convulxin, a protein purified from the venom of *Crotalusdurissus terrificus*, is known to specifically bind to GPVI [43].

None of the synthesized compounds exhibited proagreggant effect on platelets on their own. Active compounds that inhibited platelet aggregation in a dose-dependent manner are summarized with IC_{50} values in Table 2. A large number of the compounds studied showed IC_{50} values less than 10 μM, acting against specific pathways of platelet aggregation induced by TRAP-6, collagen, or convulxin. A general view of Table 2 shows that the most active compounds were **38** (IC_{50} 3.98 ±0.46 μM), **17** (IC_{50} 0.04±0.03 μM), and **38** (IC_{50} 0.98±0.40 μM) for inhibition of platelet aggregation induced TRAP-6, collagen, and convulxin, respectively. The platelet aggregation, in a dose-dependent manner, was inhibited by these active compounds, representative curves are shown in Figure 3. Also, compound **29** showed significant inhibition of platelet aggregation induced by convulxin with an IC_{50} of 6.70±0.41 μM. The antiplatelet mechanism of the most active compounds (**17**, **29,** and **38**) was evaluated in platelet aggregation by direct stimulation of protein kinase C (PKC) (for PMA). Thus in PMA-stimulated platelets, the inhibitory potential increased in the following order: **17** (IC_{50} 77.96±30.00 μM),

29 (IC_{50} 21.83±2.73 μM), and **38** (IC_{50} 6.79±3.02 μM). Also, the compound **38** inhibited arachidonic acid-induced platelet aggregation with an IC_{50} of 42.01±3.48 μM. Thus in compound **38**, the introduction of a chlorine atom at position 6 on the aromatic ring and a bromine atom at the terminal carbon of the acyl chain improved its platelet antiaggregant activity.

In this setting, we continued the study of the antiplatelet mechanism of compound **38**. Since the PKC family is a major regulator of platelet granule secretion [44], we showed that compound **38** at 20 μM inhibited the expression of platelet P-selectin (CD62) and surface granulophysin (CD63); two markers that represent granules release (Figure 4A and B). Also, we demonstrated that the antiplatelet activity of compound **38** was due to its pharmacological action on mitochondrial bioenergetics in human platelets. In agreement with this, compound **38** reduced resting mitochondrial membrane potential (depolarization) (Figure 4C). Decreased membrane potential, in the presence of compound **38** at 20 μM, led to lower ATP generation (Figure 4D), and caused an increase in ROS generation (Figure 4E) and intraplatelet calcium (Figure 4F). Conversely, these effects of compound **38** on mitochondrial bioenergetics did not induce phosphatidylserine exposure on platelet membrane; confirming the non-cytotoxic effects on platelets (evaluated by annexin V binding in washed platelets) (Figure 4G). Altogether, these results indicate that compound **38** decreased a central target in human platelet activation as in mitochondrial bioenergetics and this could modulate a lower response of platelets to activating agents [45], which is associated with potent non-selective inhibition of platelet aggregation induced by TRAP-6, collagen, convulxin, PMA and arachidonic acid (Figure 5). A similar mechanism has been reported for the antiplatelet activity

of vitamin C [46] and MitoQ [47].

3.4. Structure-activity relationship

The acyl hydroquinone **16** can be considered an analog of compound **I** [30], with an acyl chain of five carbons replacing the enone cycle of compound **I** (Figure 1). Hydroquinone **I** is the prototype of *ortho*-carbonyl hydroquinones whose antiplatelet activities have been recently reported by us [30]. The structural changes from **I** to **16** increased activity by five times as an inhibitor of collagen-induced platelet aggregation (Table 2). Besides, IC_{50} of compound **16** was only two times lower than the most active compound, a spirohydroquinone, reported in that study [30]. This promissory result encouraged us to test the effects of the change of the acyl chain length on the activity of a series of acylhydroquinones as inhibitors of agonist-induced platelet aggregation.

When acyl chains are shorter than in **16**, as in **12** and **14,** with two and three carbons respectively, the activities against collagen-induced aggregation were similar to **16**, but additionally, **12** showed activity against TRAP-6 activation. On the other hand, when the acyl chain was two carbons longer, as in **17**, the activity against collagen activation increased 76 times higher than **16**, with a submicromolar IC_{50}, and highly selective against collagen-induced platelet aggregation, the activity against TRAP-6 decreased to half of **12**. When a longer acyl chain was present, with 8 or 9 carbons, compounds **19** and **21** were shown to be cytotoxic, in the test of leakage of LDH, therefore the antiplatelet activity was not evaluated.

Substitution by methyl groups at positions 3 and 4 on the aromatic ring was also evaluated in the test of leakage of LDH. The results showed that the introduction of

methyl groups in 3,4-dimethylacylhydroquinones **20** and **22** abolished the cytotoxicity in comparison with compounds **19** and **21**. Although these dimethyl derivatives were less active than **17**, they were highly active against collagen-induced platelet aggregation. Moreover, the activity of **22** against TRAP-6-induced platelet aggregation was the same as exhibited by the most active *ortho*-carbonyl hydroquinone reported previously by us [30]. The effect of the methyl substitution on the activities against agonist-induced aggregation was dependent on the length of the acyl chain, increasing or decreasing the activities by variable quantities. On the other hand, these nine evaluated compounds were inactive against convulxin-induced platelet aggregation.

The effect of introducing a bromine atom at the terminal carbon of the acyl chain, hydroquinones **23** to **27**, was also evaluated. Compound **23**, showed that the bromine introduction decreased the activity twofold against collagen-induced platelet aggregation but increased TRAP-6-induced aggregation twofold, when additionally dimethyl substitution is present, compound **24**, the activity against collagen-induced aggregation increased twenty times, and twice against TRAP-6-induced platelet aggregation. Also compounds **25** and **27** were strong inhibitors of the effect of collagen on platelets, but in the former case, high activities against the three agonists in platelet aggregation were observed, with the same values against TRAP-6- and convulxin-induced platelet aggregation.

Finally, the introduction of a chlorine atom at position 6 on the aromatic ring was evaluated in compounds **28** to **38**. Among these compounds, acylchlorohydroquinones **32** to **37** showed high cytotoxicity and consequently were discarded for further studies. Interestingly, the non-cytotoxic chloro-derivatives

showed high activities against the platelet aggregation induced by TRAP-6, collagen, and convulxin. It has likewise been reported that chloro-substituted naphthoquinones inhibit platelet activation [48, 49]. The higher activities were obtained with compound **38**, which exhibited sub-micromolar IC_{50} inhibitory activities against convulxin- and collagen-induced platelet aggregation and also a high inhibitory activity against TRAP-6. In TRAP-6 induced platelet aggregation, compound **24** showed a similar structure to compound **38**, however, the absence of Cl made this compound about 14 times less active than compound **38**.

To assess the plausible role of the semiquinone radicals derived from these compounds, the monomethyl ethers obtained from compounds **12**, **13**, **17**, **25**, and **26** were also evaluated and shown to be inactive in collagen-induced platelet aggregation (Figure 6). This result suggests the involvement of semiquinone radicals in the antiplatelet activity of these compounds.

4. CONCLUSION

Due to the key role played by platelets in the pathogenesis of atherothrombotic events, the use of platelet antiaggregant drugs is essential in the prevention of thrombus-mediated events. The results of this study showed that the chemical space around *ortho*-carbonyl hydroquinone moiety is a rich source of biologically active compounds, which enabled us to obtain an acylhydroquinone derivative that decreases the mitochondrial bioenergetics, a central target in human platelet activation, generating potent non-selective activity against platelet aggregation induced by TRAP-6, collagen, convulxin, PMA, and arachidonic acid.

Conflict of interest

The authors have no conflicts of interest to disclose.

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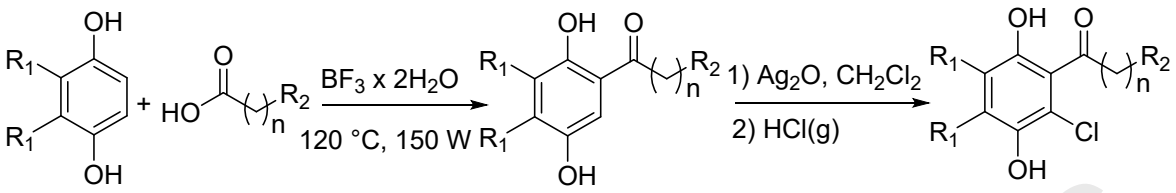
Table 1. Synthesis of acylhydroquinones by Fries rearrangement.



1: R1 = H **3 - 11** **12 - 27** **28 - 38**
2: R1 = Me

Compound	n	R_1	R_2
12	1	H	H
13	1	Me	H
14	2	H	H
15	2	Me	H
16	4	H	H
17	6	H	H
18	6	Me	H
19	7	H	H
20	7	Me	H
21	8	H	H
22	8	Me	H
23	4	H	Br
24	4	Me	Br
25	5	H	Br
26	5	Me	Br
27	9	Me	Br
28	1	H	H
29	1	Me	H
30	2	H	H
31	2	Me	H
32	6	H	H
33	6	Me	H
34	7	H	H
35	7	Me	H
36	8	H	H
37	8	Me	H
38	4	Me	Br

Table 2. Antiplatelet activity of synthesized compounds.

Compound	IC_{50} (μM) (mean ± SD)		
	TRAP-6 (5 μM)	**Collagen (1 μg/mL)**	**Convulxin (5 ng/mL)**
12	31.25±3.31*	5.95±1.10	>20
13	97.96±25.65	0.51±0.15	>20
14	131.48±11.54	3.17±1.94	>20
15	64.04±5.052	1.93±0.69	>20
16	94.14±16.57	3.03±0.64	>20
17	61.91±11.26	0.04±0.03	>20
18	53.07±5.39	0.33±0.27	>20
20	28.10±11.67	0.82±0.27	>20
22	13.45±4.41	1.14±0.21	>20
23	59.63±7.06	6.36±1.03	>20
24	59.21±16.88	0.15±0.06	>20
25	10.44±2.98	0.16±0.02	10.09± 1.17
26	61.31±5.84	7.06±2.27	>20
27	90.62±6.64 a	0.38±0.18	>20
28	34.77±11.55	8.54±2.48	14.86± 2.11
29	8.80±1.64	6.17±0.59	6.70± 0.41
30	31.41±11.94	9.79±3.24	12.79± 0.74
31	9.44 ± 0.387	4.48 ± 1.12	8.99± 0.58
38	3.98 ±0.46	1.10±0.26	0.98± 0.40
Ticagrelor	0.77±0.51	0.57±0.19	1.45±0.40

Mean ± SD (standard deviation) of three independent determinations and IC_{50} (μM) interpolated from the dose-response curves. * Maximum inhibition to 200 μM. Thrombin Receptor Activator Peptide 6: TRAP-6.

Figure legends

Figure 1. Chemical structure of avarol, arbutin, 2,5-di-(*tert-butyl*)-*p*-hydroquinone, compounds **16** (reported in this article) and **I** [30].

Figure 2. Cytotoxicity activity of acylhydroquinone and acylchlorohydroquinones derivatives. The results are shown as the mean ± SD (standard deviation) of n=3 experiments. Differences between the vehicle (DMSO 0.2%) and compounds were analyzed using ANOVA analysis and Tukey's posthoc test. **p<0.01 and ***p<0.001.

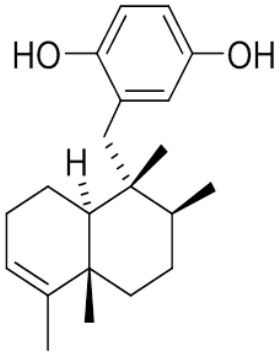
Figure 3. Representative dose-response curves of inhibition of platelet aggregation by compounds **17** and **38**. A) The half-maximal inhibitory concentration (IC_{50}) was calculated from the dose-response curve in platelet aggregation stimulated by TRAP-6, collagen, and convulxin. B) Representative curves of platelet aggregation (5 min at 37°C) pre-incubated with compounds **17** and **38** (0.02, 0.2, 2, 20 and 200 μM), and then stimulated by TRAP-6, collagen or convulxin.

Figure 4. Antiplatelet mechanism of compound **38**. A) and B) Platelet activation of α and dense granules were evaluated by P-selectin (CD62PE) and CD63PE, and analyzed by Accuri C6 flow cytometer.C) Mitochondrial membrane potential ($\Delta\Psi m$) was determined by flow cytometry, using tetramethylrhodamine, methyl ester, perchlorate (TMRM). D) Platelet ATP secretion was determined with chrono-lume reagent (firefly luciferin-luciferase) in a lumi-aggregometer. E) Intraplatelet ROS level was determined using dihydroethidium probe in a flow cytometer. F) Intraplatelet calcium levels were measured by flow cytometry with the FLUO-3 AM. G) Externalization of phosphatidylserine on the platelet membrane was detected by FITC Annexin V Apoptosis Detection Kit BD Pharmingen and activated with TRAP-

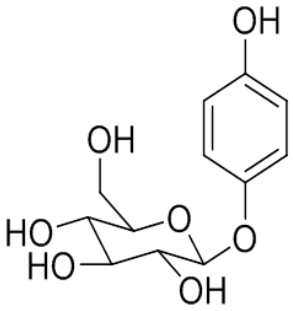
6 5 μM plus collagen 1 μg/ml. AA: antimycin A, FCCP: carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, PMA: phorbol 12-myristate 13-acetate, TRAP-6: thrombin Receptor Activator Peptide 6. The results are shown as the mean ± SD (standard deviation) of n=3 experiments. For A), B) and D) differences between absence of compound (0) and compounds (2 and 20 μM), and for C), E), F) and G) differences between control and compounds (2 and 20 μM) were analyzed using ANOVA analysis and the Tukey's post-hoc test. *p<0.05, **p<0.01 and ***p<0.001.

Figure 5. Scheme of antiplatelet mechanism of compound **38**. This compound decreased a central target in human platelet activation as the mitochondrial function and therefore exerted a potent non-selective inhibition of platelet aggregation induced by TRAP-6 (3.98±0.46), collagen (1.10±0.26), convulxin (0.98±0.40 μM), PMA (IC_{50} 6.79±3.02 μM) and arachidonic acid (IC_{50} 42.01±3.48 μM). ATP: adenosine triphosphate, GP: glycoprotein, PAR1: protease-activated receptor 1, PMA: phorbol 12-myristate 13-acetate, PKC: protein kinase C, TP: thromboxane A2 receptor, TRAP-6: thrombin Receptor Activator Peptide 6, $\Delta\Psi m$: mitochondrial membrane potential.

Figure 6. Effect of monomethyl ether of hydroquinones **12**, **13**, **17**, **25** and **26** on inhibition of platelet aggregation. A) Chemical structures of evaluated compounds. B) Platelets were stimulated with collagen 1 μg/mL and compounds at 20 μM. OMe: monomethyl ether. The results are shown as the mean ± SD (standard deviation) of n=3 experiments and were analyzed using ANOVA analysis and the Tukey's post-hoc test. ***p<0.001 vs., control, ### p<0.001 vs., structural analog (monomethyl ether).



Avarol



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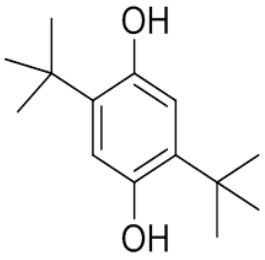

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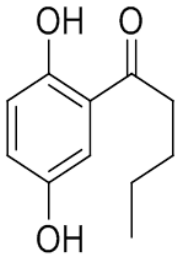

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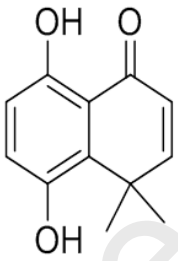

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