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Synthesis of antiplatelet *ortho*-carbonyl hydroquinones with differential action on platelet aggregation stimulated by collagen or TRAP-6

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Platelet activation and secretion (P-selectin and GPIIb/IIIa activation, and CD63 and ATP secretion)

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1	SYNTHESIS OF ANTIPLATELET ORTHO-CARBONYL HYDROQUINONES
2	WITH DIFFERENTIAL ACTION ON PLATELET AGGREGATION STIMULATED
3	BY COLLAGEN OR TRAP-6
4	Running title: Hydroquinone derivatives regulate platelet function
5	Ву
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28 ABSTRACT

Cardiovascular diseases are the leading cause of death in the world. Platelets 29 have a major role in cardiovascular events as they bind to the damaged 30 31 endothelium activating and forming thrombi. Although some hydroquinone scaffoldcontaining compounds have known antiplatelet activities, currently there is a lack of 32 evidence on the antiplatelet activity of hydroquinones carrying electron attractor 33 groups. In this work, we evaluate the antiplatelet effect of a series of *ortho*-carbonyl 34 hydroquinone derivatives on cytotoxicity and function of human platelets, using 35 36 collagen and thrombin receptor activator peptide 6 (TRAP-6) as agonists. Our structure-activity relationship study shows that gem-diethyl/methyl substitutions 37 and the addition/modifications of the third ring of ortho-carbonyl hydroquinone 38 scaffold influence on the selective index (IC_{50} TRAP-6/ IC_{50} Collagen) and the 39 inhibitory capacity of platelet aggregation. Compounds 3 and 8 inhibit agonist-40 induced platelet aggregation in a non-competitive manner with IC₅₀ values of 41 1.77±2.09 µM (collagen) and 11.88±4.59 µM (TRAP-6), respectively and show no 42 cytotoxicity. Both compounds do not affect intracellular calcium levels and 43 mitochondrial bioenergetics. Consistently, they reduce the expression of P-selectin, 44 activation of glycoprotein IIb/IIIa, and release of adenosine triphosphate and CD63 45 46 from platelet. Our findings may be used for further development of new drugs in 47 platelet-related thrombosis diseases.

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Keywords: hydroquinone derivatives; *ortho*-carbonyl; platelets; thrombosis; small
molecules.

51

52 **1. INTRODUCTION**

Platelets are essentials in hemostasis and thrombosis, both of which are 53 dependent on their activation, occurring in the transition from a functional resting 54 55 state to a procoagulant and prothrombotic phenotype [1, 2]. This process is triggered by interactions of platelets with adhesive components of the extracellular 56 matrix (e.g. collagen and von Willebrand factor) or by soluble platelet agonists (e.g. 57 adenosine diphosphate [ADP], thromboxane A2 [TXA2] and thrombin, among 58 others) that bind G-protein-coupled receptors [3, 4]. Under stimulation, activated 59 60 platelets change their morphology resulting in the activation and surface expression of adhesion molecules, procoagulant phosphatidylserine (PS) exposure 61 and secretion of thrombogenic substances from platelet granules. In pathological 62 conditions, this platelet process may induce occlusive thrombosis, resulting in 63 64 ischemic events such as heart attack and stroke [1, 5], which are the leading causes of death worldwide [6]. 65

Besides their well-known antioxidant properties, polyphenols exert other beneficial biological actions [7]. Currently, antiplatelet therapy is still the mainstay in preventing platelet activation and inhibiting thrombus formation [8]. In this line, antiplatelet action both *in vitro* and *in vivo* of natural products such as flavonoids [9], curcuminoids [10], catechins [11], terpenoids [12], polyphenols [13, 14], and saponins [15] have been extensively described [16, 17].

The antiplatelet effect of several small molecules and FDA-approved drugs have been described by different mechanisms of action resulting from their antioxidant properties [7]. In addition, other mechanisms involve inhibition of oxidative phosphorylation (OXPHOS) through interaction with the electron transport chain

76 (ETC) [18]. Hydroquinone scaffold-containing compounds exert biological activities, 77 some of them by acting on mitochondrial metabolism [19-22] and activating SIRT 1 78 and AMPK [23]. In this regard, our previous results have shown that the ortho-79 carbonyl hydroguinone scaffold efficiently crosses the mitochondrial membrane reaching the ETC [19-22], owing to the strong hydrogen bond between the 80 carbonyl group and one of the phenolic hydroxyl groups [24-26]. The structure-81 activity relationship (SAR) of these hydroquinones has revealed that small 82 structural changes modify the effect on OXPHOS [22]. All these compounds 83 84 exhibited antioxidant capability, where the intramolecular hydrogen bonding is a relevant feature not only to radical-scavenging activity [27] but also on their 85 oxidation potential [28, 29]. 86

The ortho-carbonyl substituted hydroquinone **10** (Figure 1) is a protonophoric 87 uncoupling agent of OXPHOS that causes mitochondrial depolarization, 88 decreasing both intracellular adenosine triphosphate (ATP) and NAD(P)H levels, 89 the GSH/GSSG ratio, and slightly increasing reactive oxygen species (ROS) 90 formation in intact TA3/Ha cancer cells [21, 30]. Compounds 13 and 14 (Figure 1) 91 also inhibit the respiration on the same cell line. Some hydroquinone scaffold-92 containing compounds with antiplatelet activities are known and their chemical 93 94 structures are shown in Figure 2. Recently, Chang et al. [31] described that 95 hydroquinone, a natural antioxidant with dermatological uses inhibits TXA2 96 production and suppresses the arachidonic acid (AA)-induced platelet aggregation [31]. Also, arbutin the natural β -glucoside derivative of hydroquinone inhibits 97 platelet aggregation induced by different agonists (ADP, AA, collagen, and 98 thrombin) [32], and their pharmacokinetics showed that it is guickly metabolized in 99

blood in rats [33]. Besides, 2,5-di-(tert-butyl)-*p*-hydroquinone inhibits platelet
aggregation stimulated by protease-activated receptor (PAR)-1 or PAR-4 agonist
peptides (SFLLRN and AYPGKF) and, avarol inhibits platelet aggregation
stimulated by AA or A23187 (calcium ionophore) [34].

In spite of these reports, the effect of hydroquinone scaffold-containing compounds
on platelet function remains scarcely studied [18]. Thus, we decided to synthesize
and test a series of *ortho*-carbonyl hydroquinones (Figure 1) as novel antiplatelet
agents.

108 **2. RESULTS**

109 **2.1. Chemistry**

The synthetic methodology to obtain the studied compounds is depicted in Scheme 110 1. The already reported methodology of obtention of acyl hydroquinones [19, 35] 111 112 was improved using microwave irradiation at 100°C, using hydroguinone (I) or dimethyl hydroquinone (II) and the corresponding carboxylic acid as starting 113 products, with boron trifluoride dihydrate as the solvent. Compounds 1-14 were 114 synthesized using a methodology previously reported [19, 22, 36-38], involving 115 oxidation of the acylhydroquinones, reaction with enamines and finally a 116 rearrangement in acidic medium. Structural characterization and procedure to 117 118 synthesize compounds 1-3 and 8-14 were included as supplementary information.

119 **2.2. Cytotoxic activity on platelets**

In order to identify candidate compounds with potential antiplatelet activity (Figure
1), we evaluated the effect of synthesized compounds on platelet viability by LDH
leakage. The compounds 1, 2, 3, 7, 8, 9, 10, 11, 12, 13 and 14 did not exert LDH
release in platelets when incubated at the tested higher concentration (200 μM,

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Figure 3). However, under the same experimental conditions, compounds **4**, **5** and 6 showed cytotoxic activity (p<0.05) (Figure 3). Thus, the following antiplatelet experiments were performed using the non-cytotoxic compounds.

127 **2.3.** Structure-antiplatelet activity relationship for the collagen- and TRAP-6-

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stimulated platelet aggregation

Light transmission aggregometry was used to evaluate the activity of non-cytotoxic 129 compounds (1, 2, 3, 7, 8, 9, 10, 11, 12, 13 and 14) on platelet aggregation induced 130 131 by TRAP-6 or collagen, which are known platelet agonists [39]. The compounds 132 showed different potency of their platelet antiaggregant activity, being more active when inhibition was exerted on platelet aggregation induced by collagen than 133 TRAP-6 (Table 1). Among the compounds tested, compounds 3 and 8 were the 134 most active presenting IC₅₀ of 1.77±2.09 and 11.88±4.59 µM against collagen- and 135 TRAP-6-induced platelet aggregation, respectively (Table 1). Moreover, compound 136 3 exhibited high selectivity toward platelet aggregation induced by collagen 137 signaling. In addition, and according to the selectivity index (S.I., IC₅₀ TRAP-6/IC₅₀ 138 collagen), the other compounds had decreasing S.I. as follows: compounds 3 >11 139 > 7~1 >10~9 >12. In platelets stimulated with collagen, the substitution of spiro 140 group by gem-dimethyl moiety in compound 3 produced a moderate decrease 141 142 (close to 8 folds) of inhibition of platelet aggregation (compound 1). The addition of a third ring to the spiro derivative, compound **3**, produced a drop of about 14 folds 143 144 the activity (compound 9) and simultaneous additions of the third ring and replacement of the spiro moiety resulted in a decrease of about 12 folds the activity 145 146 (compound **10**). On the other hand, only three ortho-carbonyl hydroquinones (compounds 2, 8 and 12) were dual inhibitors, blocking both collagen- and TRAP-147

6-stimulated platelet aggregation. From IC₅₀ values, it is observed that a relevant 148 149 chemical requirement for dual inhibitors (i.e. S.I. values close to 1) was the gem-150 diethyl substitution (compounds 2 and 8 vs 1 and 10). The regioisomers 11 and 12 151 had evident differences in the S.I. value as well as potencies, and the blocking of the hydroxymethyl group by acylation generated the abolishment of the activity in 152 both stimuli of platelet aggregation (compounds 13 and 14), suggesting that the 153 hydroxymethyl substitution is a relevant structural requirement for inhibition of 154 155 platelet aggregation.

156 From here, we decided to continue the analysis of the antiplatelet activity by only analyzing the compounds 3 and 8. Both compounds dose-dependently inhibited 157 collagen- and TRAP-6-induced platelet aggregation (Figure 4 A-B). In order to 158 evaluate the type of inhibition exerted by compounds 3 and 8, we induced the 159 160 aggregation of platelets by using a different collagen (0.1 to 2 µg/mL) or TRAP-6 (1 to 10 μ M) concentrations in the presence of the vehicle, compound **3** or compound 161 8 (Figure 4C and E). The maximal effect induced by collagen (2 µg/mL) and TRAP-162 6 (10 µM) was fully inhibited by these compounds, suggesting a non-competitive 163 behavior of both compounds 3 and 8 (Figure 4D and F). 164

165 **2.4. Antiplatelet mechanism of compounds 3 and 8**

Activated platelets expose PS which acts as a procoagulant stimulus and a signal for phagocytic clearance of apoptotic cells [40]. In this study, we observed that PS was not modified in the presence of compounds **3** and **8** (Figure 5A). When platelets are activated with thrombin and collagen, the intracellular calcium concentration increases locally and transiently [41]. This promotes conformational changes leading to glycoprotein (GP) IIb/IIIa activation [42] in parallel with the

172 translocation to the platelet surface of P-selectin, an integral membrane GP found 173 in secretory granules [43]. Both P-selectin and GPIIb/IIIa activation were 174 significantly inhibited by compounds 3 and 8 (Figure 5 B-C) (p<0.05). In contrast, no changes in the levels of Ca²⁺ were observed in resting platelets in the presence 175 176 of compounds **3** and **8** (Figure 5D). Platelet activation is autocatalytically stimulated by platelet secretion products [44]. In this context, we shown that the secretion of 177 CD63 (Figure 5E) was decreased in TRAP-6-stimulated human platelets only by 178 179 compound 8 (p<0.05). Meanwhile, ATP secretion was decreased in collagen and 180 TRAP-6-stimulated platelets by both compounds 3 and 8 (Figure 5F).

Since it has been reported that the prevention of platelet mitochondrial dysfunction 181 through the control of mitochondrial oxidative stress production or inhibition of 182 183 respiratory complexes occurs by some compounds [45], we further studied whether compound 3 or 8 exhibited an antiaggregant activity through inhibition of 184 mitochondrial bioenergetics. As shown in Figure 6 A-E, both compounds 3 and 8 185 did not display any effects on mtNADH levels in resting platelets. Similar to the 186 above results this also occurred in maximal mtNADH oxidation upon stimulation of 187 platelets with FCCP and accumulation induced by respiratory complex III inhibition 188 with antimycin A (Figure 6F). Compound **3** lacked an effect on $\Delta \psi_m$ (Fig 6G), ROS 189 190 production (Figure 6H) and intracellular ATP levels (Figure 6I). In contrast, 191 compound **8** was able to induce a drop in $\Delta \psi_m$ (Figure 6G) in addition to an 192 increase in ROS production (Figure 6H), and a decrease in the ATP levels (Figure 6I) at the highest concentrations tested (20 μ M). 193

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196 **3. DISCUSSION**

Some hydroquinone scaffold-containing compounds are easily oxidized to quinones, which are known as pan-assay interference compounds (PAINS) with high *in vivo* toxicity [46, 47], limiting their medical potential. Notably, as previously shown [19, 22, 27, 48], the intramolecular hydrogen bonding of the *ortho*-carbonyl hydroquinone scaffold and the *gem*-dimethyl group determine the redox properties and reactivity in biological conditions of these series, reducing the ability to autooxidation or interference by interactions with fluorescent assays.

204 The effect of hydroguinone scaffold-containing compounds on platelet function remains scarcely studied, some of them exhibit an antiplatelet effect in platelets 205 stimulated with thrombin, AA, collagen, ADP, A23187 or platelet-activating factor 206 [18], whose structures are shown in Figure 2. There is a lack of evidence about 207 208 the antiplatelet activity of bi- and tricyclic hydroguinones and of hydroguinones bearing electron attractor substituents. Consequently in our study, we showed that 209 ortho-carbonyl hydroquinone derivatives had differential action on collagen- and 210 TRAP-6-stimulated platelet aggregation, determined by the type of substituent, 211 especially the gem-diethyl/methyl substitutions and the addition/modifications of a 212 213 third ring influence S.I. values and the inhibitory potencies. The observed SAR for 214 the antiplatelet effects of this series of compounds suggest the involvement of 215 radical intermediates in platelets stimulated with TRAP-6: the greater stability of 216 semiquinone radical, hypothetically formed, the higher antiplatelet activity was 217 observed. Compounds 2 and 8 exhibits closely related structure as compounds 1 218 and **10**, replacing the *gem*-dimethyl group by a *gem*-diethyl group and the alkyl substitution pattern at the aromatic ring, allowing us to evaluate the effect of 219

220 sterical hindrance and electronic effects, on the antiplatelet activity. The higher volume and the conformation of gem-diethyl groups, with their methyl moieties, 221 222 positioned at above and below the molecular plane [37, 49], overall constituting a 223 structural factor that provides more thermodynamic stability to the semiguinone radical species obtained by hydrogen abstraction atom or by an electrochemical 224 225 process [29]. These radicals are responsible for the antioxidant or prooxidant activities of hydroquinones. In the same way, the aromatic ring alkylation also 226 227 stabilizes these intermediate species facilitating their generation [27]. These pieces 228 of evidence may explain that compound 8 was more active than 2 in TRAP-6 stimulated platelets, while compounds 1 and 10 were inactive, being this 229 relationship supported by the increased ROS production by 8. On the other hand, 230 the considerable difference between the activities of the regioisomers 11 and 12, in 231 TRAP-6-stimulated platelets, highlights the importance of the hydrogen bonding in 232 the biological activities of this kind of compound. Compound **12** has two hydrogen 233 bonds in their structure, one of them between phenolic hydroxyl proton and the 234 carbonyl oxygen atom and the other, one between the other phenolic proton 235 toward the oxygen of the hydroxymethyl group. Its regioisomer, instead, displays 236 less capability to form hydrogen bonds through the hydroxymethyl group [25], a 237 238 characteristic that can diminish the interactions inside platelets, triggering a lacking activity in this platelet aggregation assay. 239

Compounds **3** and **8** exhibited lower IC_{50} against platelet aggregation induced by collagen and TRAP-6 respectively without any cytotoxic effect on platelets. Both compounds showed similar inhibitory behavior on platelet aggregation, expression of molecules at the platelet membrane as well as secretion of granules

244 components. Since oxidative stress-dependent signaling promotes mitochondrial 245 dysfunction, releasing mitochondrial components are important for both platelet 246 activation and apoptosis [45, 50, 51]. Hydroguinone scaffold-containing 247 compounds inhibit OXPHOS as we have previously described [21-23, 52]. We speculate that the target for the antiplatelet effect of our series of hydroquinone 248 249 derivatives may be the mitochondria. Notably, compound 3 was more active on activation/secretion/aggregation without 250 collagen-induced platelet affecting mitochondrial bioenergetics. In contrast, compound **8** induced $\Delta \psi m$ drop, 251 252 decreased ATP levels and increased ROS production at high concentrations. ROS production may occur at the ETC with leaks at the proton-based electrochemical 253 gradient [53], changing the $\Delta \psi_m$ which can be ascribed to compound 8. However, 254 255 compound 8 did not display any effects on mtNADH levels and its antiplatelet 256 mechanism is apparently upstream mitochondrial bioenergetic function. This 257 phenomenon is consistent with our previously reported observations in which these compounds did not exhibit effects on mitochondrial respiration and OXPHOS-258 dependent proliferation using isolated tumor mitochondria and highly oxidative 259 subpopulations of breast cancer cells, respectively [22, 23]. Taken together, our 260 results indicate that both compounds 3 and 8 lack direct effects on mitochondrial 261 262 bioenergetics and the changes in compound 8-induced mitochondrial parameters may be a consequence of some non-mitochondrial interactions, *i.e.* modulation of 263 264 NADPH oxidase activity. Further experiments are required to confirm this point.

It has been recently described that small structural changes on resveratrol confer greater oral bioavailability [54] and selective inhibitory action towards a specific platelet aggregation-inducing signaling [9]. Resveratrol inhibits platelet aggregation

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induced by agonists such as collagen, U46619, and AA but has a marginal 268 269 inhibitory effect on ADP-induced platelet activation [55, 56]. Conversely, 270 isorhapontigenin, a methoxylated analog of resveratrol of natural origin [57, 58], 271 selectively inhibits ADP-induced platelet aggregation with an IC_{50} of 1.85 μ M, lacking effects on aggregation induced by other agonists [9]. In line with this 272 observation, herein we report that compound 3, a spiro derivative of ortho-carbonyl 273 hydroguinone, is a selective inhibitor of collagen-stimulated platelet aggregation 274 $(IC_{50} = 1.77 \pm 2.09 \mu M)$ with marginal effect on TRAP-6-stimulated platelet 275 276 aggregation. Conversely, compound 8, which exhibits an inhibitory effect on platelet aggregation stimulated by both agonist collagen and TRAP-6, can be 277 considered a dual antiplatelet agent. Considering that there is one patent related to 278 279 novel spiro compounds useful as selective inhibitors of fibrinogen-dependent 280 platelet aggregation for the prevention of thrombosis (European Patent Office EP0854869B1), our results suggest that compounds 3 and 8 can contribute as 281 promising scaffolds for the design and development of novel antiplatelet agents 282 with differential action on thrombotic diseases. 283

284 4. CONCLUSION

In this work, we present a SAR study on the inhibition of platelet aggregation by an 285 ortho-carbonyl substituted hydroquinone series. Based on these findings, we 286 287 identify inhibitors with differential action on collagen- or TRAP-6-stimulated platelet 288 aggregation. Notably, gem-diethyl/methyl substitutions and the 289 addition/modifications of the third ring of ortho-carbonyl hydroguinone scaffold influence the S.I. value and inhibitory potency, respectively. A spiro derivative of an 290 ortho-carbonylbicyclic hydroguinone had selectivity toward platelet aggregation 291

dependent on collagen induced-signaling and a *gem*-diethyl derivative of tricyclic hydroquinone was a dual agent, inhibiting both collagen- and TRAP-6-stimulated platelet aggregation. These findings may be used for further development of new drugs in platelet-related thrombosis diseases.

5. EXPERIMENTAL PROCEDURES

297 **5.1. Chemistry**

General methods. ¹H and ¹³C NMR spectra were obtained from a spectrometer 298 operating at either 400.13 MHz (¹H) or 100.61 MHz (¹³C). Chemical shifts are 299 reported as ppm downfield from TMS for ¹H NMR and relative to the central CDCl₃ 300 resonance (77.0 ppm) for ¹³C NMR. All melting points are uncorrected and were 301 determined using an Electrothermal 9100 apparatus. IR spectra (KBr discs) were 302 recorded on an FT-IR spectrophotometer; wavenumbers are reported in cm⁻¹. 303 304 High-resolution mass spectra (HRMS) were obtained on an orthogonal time-offlight (ToF) mass spectrometer (QTof Micro, Micromass UK) or on a Thermo Q 305 exacting focus. Silica gel 60 (230-400 mesh ASTM) and TLC sheets silica gel 60 306 F254 were used for flash-column chromatography and analytical TLC, respectively. 307 Synthesis of acylhydroquinones III-VI. The already reported methodology of 308 obtention of acyl hydroquinones [35] was improved using microwave irradiation as 309 310 follows. To a 10 mL CEM microwave process vial, equipped with a magnetic stir bar, were added one equivalent of hydroquinone (I) or dimethyl hydroquinone (II), 311 312 1.5 equivalent of carboxylic acid and 4 mL of Boron trifluoride dihydrate. The mixture was irradiated under microwave for 25 min at 120°C. After completion of 313 314 the reaction, the mixture was allowed to cool to room temperature (RT) and extracted with ethyl acetate, the organic phase was washed with distilled water and 315

dried with anhydrous sodium sulfate, then was filtered and concentrated under
vacuum. Afterward, acylhydroquinones III-VI were purified by flash
chromatography with 6:1 hexane/ ethyl acetate as eluent.

319 Compound III (1-(2,5-dihydroxyphenyl)-1-heptanone). Hydroquinone (600 mg, 5.45 mmol), heptanoic acid (96%) (1.21 mL, 8.18 mmol) and 4 mL of boron trifluoride 320 dihydrate react yielding 547.4 mg of **III** (2.46 mmol, 45% yield). ¹H-NMR δ (CDCl₃): 321 0.90 (t, 3H, J = 6.7 Hz, 7-CH₃); 1.27-1.45 (m, 6H, 3x CH₂); 1.73 (p, 2H, J = 7.4 Hz, 322 3-CH₂): 2.93 (t, 2H, J = 7.4 Hz, CH₂CO); 4,78 (s, 1H, 5'-OH); 6.88 (d, J = 8.9 Hz, 323 1H, 3'-H); 7.02 (dd, 1H, J_1 = 8.9 Hz, J_2 = 3.0 Hz, 4'-H); 7.23 (d, 1H, J = 3.0 Hz, 6'-324 H); 11.98 (s, 1H, 2'-OH). ¹³C-NMR δ(CDCl₃): 14.04; 22.51; 24.45; 28.97; 31.59; 325 38.48; 114.98; 119.03; 119.29; 142.82; 147.59; 156.45; 206.79. M.P.: 49.7-50.3 326

327 °C. HRMS (ESI): m/z calcd for C₁₃H₁₈O₃ [M+H]⁺: 223.1289, found: 223.1286

328 Compound IV (1-(2,5-dihydroxyphenyl)-1-octanone). Hydroguinone (600 mg, 5.45 mmol), octanoic acid (98%) (1.32 mL, 8.18 mmol) and 4 mL of boron trifluoride 329 dihydrate react yielding 753.7 mg of **IV** (3.19 mmol, 58% yield). ¹H-NMR δ (CDCl₃): 330 0.90 (t, 3H, J = 6.9 Hz, 8-CH₃); 1.23-1.45 (m, 8H, 4x CH₂); 1.74 (p, 2H, J = 7,2 Hz, 331 3-CH₂); 2.93 (t, 2H, J = 7.4 Hz, CH₂CO); 4.81 (s, 1H, 5'-OH); 6.89 (d, 1H, J = 8.9 332 Hz, 3'-H); 7.03 (dd, 1H, $J_1 = 8.9$ Hz, $J_2 = 3.0$ Hz, 4'-H); 7.23 (d, 1H, J = 3.0 Hz, 6'-333 H); 11.98 (s, 1H, 2'-OH). ¹³C-NMR δ(CDCl₃): 14.08; 22.62; 24.50; 29.09; 29.26; 334 31.68; 38.48; 114.99; 119.03; 119.28; 124.83; 147.62; 156.43; 206.81. M.P.: 65.2-335 336 66.2 °C. HRMS (ESI): m/z calcd for C₁₄H₂₀O₃ [M+H]⁺: 237.1485, found: 237.1479.

337 *Compound* **V** (*1-(2,5-dihydroxyphenyl*)*-1-nonanone*). Hydroquinone (600 mg, 5.45 338 mmol), nonanoic acid (96%) (1.49 mL, 8.18 mmol) and 4 mL of boron trifluoride 339 dihydrate react yielding 627.2 mg of **V** (2.51 mmol, 46% yield). ¹H-NMR δ (CDCl₃):

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0.89 (t, 3H, J = 6.9 Hz, 9-CH₃); 1.23-1.45 (m, 10H, 5x CH₂); 1.73 (p, 2H, J = 7.4 Hz, 3-CH₂); 2.93 (t, 2H, J = 7.4 Hz, 2-CH₂CO); 4.83 (s, 1H, 5'-OH); 6.88 (d, 1H, J = 8.9 Hz, 3'-H); 7.02 (dd, 1H, $J_1 = 8.9$ Hz, $J_2 = 3.0$ Hz, 4'-H); 7.23 (d, 1H, J = 3.0 Hz,

6'-H); 11.98 (s, 1H, 2'-OH). ¹³C-NMR δ(CDCl₃): 14.11; 22.66; 24.48; 29.16; 29.31; 29.39; 31.83; 38.48; 114.93; 119.03; 119.32; 124.73; 147.47; 156.59; 206.61. M.P.: 86.2-87.5 °C. HRMS (ESI): m/z calcd for $C_{15}H_{22}O_3$ [M+H]⁺: 251.1642, found:251.1640.

Synthesis of compounds 4-7. The new naphthalenones 4-7 (Scheme 1) were 347 348 obtained following the reported method, although slight variations were introduced [19]. A mixture of acylhidroquinone (1 equiv) and Ag₂O (2.5 equiv) in 20-30 mL of 349 dichloromethane was vigorously stirred for 1-2 h. at RT, yielding the corresponding 350 quinone. This mixture was filtered through celite, and dropwise added, without 351 isolation, to a solution of the 4-(2-methyl-2-propenyl)morpholine at 0°C, allowing it 352 to reach RT in a 30-60 min time off, being monitored by thin-layer chromatography. 353 Then, the solvent was evaporated under reduced pressure. The residue was 354 dissolved in a mixture of ethanol and hydrochloric acid and refluxed by 3 h. Next 355 356 was poured on an ice/water mixture. The product was extracted with five portions of 20 mL of dichloromethane, which were dried with anhydrous sodium sulfate and 357 358 then filtered; the solvent was evaporated under reduced pressure and purified by flash chromatography using 6:1 hexane/ethyl acetate as eluent. 359

360 Compound **4** (5,8-dihydroxy-4,4-dimethyl-2-pentylnaphthalen-1(4H)-one). Two portions of 450 mg (900 mg, 4.05 mmoles) of acylhydroguinone III were oxidized 361 362 with 1.13 g of Ag₂O each one in 40 mL of dichloromethane for 2 h., the filtered 363 solutions put together dropwise added 4-(2were and over

methylpropenyl)morpholine (572 mg, 4.05 mmol) for 1.5 h. Evaporation of the 364 365 solvent and a further reflux of the residue in a mixture of ethanol (20 mL) and 366 hydrochloric acid (1 mL), following the general method, achieved 831 mg of 367 hydroquinone **4** (3.03 mmol, 75% yield). ¹H-NMR δ (CDCl₃): 0.91 (t, 3H, J = 6.7Hz, -CH₂CH₃); 1.30-1,40 (m, 4H, 2x CH₂); 1,48-1,57 (m, 2H, CH₂); 1.60 (s, 6H, 2x 4-368 CH₃); 2.38 (t, 2H, J = 6,9 Hz, O=C –C-CH₂); 4.86 (s, 1H, 5-OH); 6,62 (s, 1H, 3-H); 369 6.78 (d, 1H, J = 8.8 Hz, 6- or 7-H); 6.89 (d, 1H, J = 8.8 Hz, 6- or 7-H); 13.03 (s, 1H, 370 8-OH). ¹³C-NMR δ(CDCl₃): 14.01; 22.45; 24.96; 28.01; 28.88; 31.51; 37.80; 371 372 115.50; 123.80; 133.69; 135.05; 145.83; 156.29; 157.39; 191.51. M.P.: 114.4-115.5 °C. HRMS (ESI): m/z calcd for $C_{17}H_{23}O_3$ [M+H]⁺: 275.1647, found: 275.1645. 373 Compound **5** (5,8-dihydroxy-4,4-dimethyl-2-hexylnaphthalen-1(4H)-one). 374 Two portions of 450 mg (900 mg, 3.81 mmol) of acylhydroquinone IV were oxidized with 375 376 1.1 g of Ag₂O each one in 40 mL of dichloromethane for 2 h, the filtered solutions were put together and dropwise added over 4-(2-methylpropenyl)morpholine (565 377 mg, 4.0 mmol) by 1.5 h. Evaporation of the solvent and a further reflux of the 378 residue in a mixture of ethanol (20 mL) and hydrochloric acid (1 mL), following the 379 general method, allowed to obtain 657.2 mg of hydroquinone 5 (2.27 mmol, 60% 380 yield). ¹H-RMN δ (CDCl₃): 0.89 (t, 3H, J = 7.0 Hz, CH₂CH₃); 1.20-1.40 (m, 8H, 4x) 381 382 CH₂); 1.46-1.57 (m, 2H, CH₂); 1.60 (s, 6H, 2x 4-CH₃); 2.38 (t, 2H, J = 6.9 Hz, O=C-C-CH₂); 4.86 (s, 1H, 5-OH); 6.62 (s, 1H, 3-CH); 6.77(d, 1H, J = 8.8 Hz, 6- or 7-H); 383 6.89 (d, 1H, J = 8.8 Hz, 6- or 7-H); 13.07 (s, 1H, 8-OH). ¹³C-RMN δ(CDCl₃): 22.64; 384 25.11; 28.37; 28.96; 29.12; 29.32; 31.83; 37.52; 115.49; 115.61; 123.72; 133.81; 385 386 134.98; 145.36; 156.77; 156.96; 191.32. M.P.: 68.5-71.7 °C. HRMS (ESI): m/z calcd for C₁₈H₂₅O₃ [M+H]⁺Calc.: 289.1804, Found: 289.1800. 387

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388 Compound 6 (5,8-dihydroxy-4,4-dimethyl-2-heptylnaphthalen-1(4H)-one). Acyl hydroguinone V (500 mg, 2 mmol) was oxidized with Ag₂O (1.16 g) in accordance 389 390 with the general methodology. Then the filtrated solution was added over a solution 391 of 4-(2-etylbutenil)morpholine (337.6 mg, 2 mmol). After evaporation of the solvent, 392 the residue was refluxed in ethanol HCl mixture yielding hydroquinone 6 (284.5 mg, 48% yield). ¹H-NMR δ (CDCl₃): 0,89 (t, 3H, J = 6.9 Hz, CH₂CH₃); 1.17-1,42 (m, 393 10H, 5x CH₂); 1.46-1.57 (m, 2H, CH₂); 1.59 (s, 6H, 2x 4-CH₃); 2.38 (t, 2H, J = 6.9 394 Hz, O=C-C-CH₂); 5.29 (s, 1H, 5-OH); 6.62 (s, 1H, 3-CH); 6.67 (d, 1H, J = 8.8 Hz, 6-395 or 7-H); 6.90 (d, 1H, J = 8.8 Hz, 6- or 7-H); 13.07 (s, 1H, 8-OH). ¹³C-NMR 396 δ (CDCl₃): 14.06; 22.51; 25.16; 28.06; 28.55; 28.94; 29.17; 31.56; 31.69; 37.49; 397 115.64; 115.78; 123.67; 133.48; 134.93; 145.18; 156.75; 157.10; 191.22. M.P.: 398 74.3-76.5 °C. HRMS (ESI): m/z calcd for $C_{19}H_{64}O_3$ [M-H]⁺ : 303.1960, found: 399 400 303.4227.

Compound 7 (5,8-dihydroxy-4,4,6,7-tetramethylnaphthalen-1(4H)-one). 2',5'-401 dihydroxy-3',4'-dimethylacetophenone (400 mg, 2.2 mmol) [35], was oxidized 402 according to general methodology with Ag₂O (1.28 g). After filtering the 403 solution was added over a solution of 4-(2-methylpropenil)morfoline (314 mg, 404 405 2.2 mmol), after evaporation under vacuum a viscous residue was obtained. 406 451 mg of this residue was refluxed in a mixture of ethanol and hydrochloric 407 acid by 1 h. Following the same procedure as described before, 256 mg of 408 compound **7** (1.1 mmol, 78% yield) was obtained. ¹H-NMR δ (CDCl₃): 1,61 (s, 6H, 2x 4-CH₃); 2.23 (s, 3H, Ar-CH₃); 2.26 (s, 3H, Ar-CH₃); 4.55 (s, 1H, 5-OH); 6.23 (d, 409 1H, J = 10 Hz, 2-H), 6.82 (d, 1H, J = 10 Hz, 3-H), 13.20 (s,1H, 8-OH). ¹³C-NMR 410 δ (CDCl₃): 11.56; 13.04; 25.17; 38.03; 112.68; 123.43; 124.04; 131.51; 131.64; 411

412 143.52; 155.12; 160.73; 191.11. M.P.: 200.5-201.6 °C. HRMS (ESI): m/z calcd for
413 C₁₄H₁₆O₃ [M+1]⁺: 233.1178, found: 233.1166.

Synthesis of compounds 1-3 and 8-14. Compounds 1-3 and 8-14 were synthesized as previously reported [19, 22, 36-38]. Structural characterization and procedure to synthesize compounds 1-3 and 8-14 were included as supplementary information.

418 **5.2. Biological assays**

419 **5.2.1. Preparation of human platelets**

Platelets were obtained from the human blood of healthy volunteers (two weeks 420 drug-free) with written informed consent, as we have previously described [59]. 421 Briefly, whole blood was collected with acid-citrate-dextrose (ACD) 4:1 v/v and 422 423 centrifuged at RT for 10 min x 240 g (low acceleration and deceleration) to obtain platelet-rich plasma (PRP). PRP was then centrifuged at RT for 8 min x 800 g and 424 platelets' pellet was resuspended in calcium-free Tyrode's buffer: ACD (9:1 v/v) 425 and washed by centrifugation at RT for 8 min x 800 g. Platelets were counted with 426 hematology analyzer (Hematological counter Mindray BC-3000 Plus, Japan) and 427 428 used within 3 h.

429 **5.2.2. Cytotoxic activity**

Washed platelets $(3x10^8 \text{ platelets/mL})$ were incubated for 10 min at 37°C with the highest concentration of compounds tested (200 µM). Then, platelets were centrifuged at 800 x g for 8 min and the resulting supernatant (100 µL) was analyzed with lactate dehydrogenase (LDH) cytotoxicity assay kit (Cayman Chemical, USA). The absorbance of the reaction was measured at 490 nm in a microplate reader (Microplate Reader Thermo Scientific Multiskan Go, Finland) Journal Pre-proo

436 with 10% Triton X-100 was used as a positive control.

437 **5.2.3. Antiplatelet activity**

438 **Platelet aggregation.** Platelet aggregation was evaluated using lumiа 439 aggregometer (Chrono-Log, Haverton, PA, USA) and monitored by light transmission [59]. Washed platelets (3x 10⁸ platelets/mL) were pre-incubated for 5 440 min with CaCl₂ (2 mM) plus compounds (0.2 to 200 µM). Platelet aggregation was 441 induced with TRAP-6 (5 μ M) or collagen (1 μ g/mL) and transmittance followed for 6 442 min. DMSO 0.2% was used as a vehicle. 443

Phosphatidylserine externalization. The externalization of PS in platelets was determined by flow cytometry [60]. Washed platelets ($3x10^8$ platelets/mL) were pre-incubated for 5 min with CaCl₂ (2 mM) plus compounds. Then, 50 µL of the sample was diluted with 150 µL of annexin V binding buffer and incubated in the dark with annexin V-FITC and anti-CD61-PE for 25 min. The samples were acquired and analyzed in the Accuri C6 flow cytometer (BD, Biosciences, USA).

Platelet activation markers. The GPIIb/IIIa activation and P-selectin expression, considered as platelet activation markers [61] were evaluated by flow cytometry in washed platelets incubated with PAC-1 antibody or anti-CD62-FITC for 25 min at RT in the dark. Platelets were identified with anti-CD61-PE and the samples were acquired and analyzed in the Accuri C6 flow cytometer [62].

Platelet secretion (intracellular and extracellular ATP, and dense granules).
Extracellular ATP was determined by luminescence detection using Chrono-lume
(Chrono-Log, Haverton, PA, USA) and monitored in a lumi-aggregometer [59].
Intraplatelet ATP levels were determined with the CellTiter-Glo Luminescent Cell
Viability Assay kit (Promega, USA) according to the manufacturer's instructions.

Dense granules secretion was determined by Accuri C6 flow cytometer using anti-CD63-PE.

Intracellular calcium levels. Washed platelets (5x10⁷ platelets/mL) were mixed 462 463 with Fluo-3-AM (0.4 µM) and incubated at RT for 30 min followed by 5 min incubation with vehicle (DMSO 0.2%) or different concentrations of compounds. 464 465 After collecting data for 15 seconds, carbonylcyanide p-466 triflouromethoxyphenylhydrazone (FCCP,1 µM) was added and the data were 467 obtained for further 60 s. The effect of compounds on cytosolic calcium 468 mobilization was calculated with respect to the control.

Reactive oxygen species. ROS production was determined in washed platelets at 5×10^7 platelets/mL using 10 μ M dihydroethidium (DHE) in the presence of compounds for 20 min at 37°C. ROS formation was analyzed by the Accuri C6 flow cytometer (BD, Biosciences, USA) [63].

473 **Mitochondrial membrane potential.** Mitochondrial membrane potential ($\Delta \Psi_m$) 474 was evaluated using the cell-permeant dye tetramethylrhodamine methyl ester 475 perchlorate (TMRM) as previously described by us [23]. Washed platelets were 476 loaded with TMRM (100 nM) and then incubated with DMSO 0.2% (Control), 477 compounds or FCCP (1 µM) at 37°C for 20 min. Samples were then analyzed by 478 the Accuri C6 flow cytometer (BD, Biosciences, USA).

Intracellular NAD(P)H levels. Intracellular NAD(P)H levels were measured by
auto-fluorescence using specific excitation and emission wavelengths of 340/428
nm as described [23].

482 Statistical analysis

483 The data obtained were presented as the mean ± standard error of the mean

(SEM) of three individual experiments and analyzed using Prism 6.0 software
(GraphPad Inc., San Diego CA, USA). The half-maximal inhibitory concentration
(IC₅₀) was calculated from the dose-response curves. Differences between
samples were analyzed using a paired t-test [64]. P values <0.05 were considered
significant.

489 **Conflict of interest**

490 The authors have no conflicts of interest to disclose.

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- Table 1. Inhibitory effect of ortho-carbonyl hydroquinones on collagen- and TRAP-6-stimulated platelet aggregation.

Compounds			51
Compounds	IC ₅₀ μΜ		(IC ₅₀ TRAP-6/IC ₅₀ Collagen)
	Collagen	TRAP-6	(1930) 11 11 27 2 30 2 2 11 9 9 2 1 9
	(1 µg/mL)	(5 µM)	
	44.00 5.04	100	7.00
1	14.30±5.64	>100	7.00
2	35.34±0.96	31.95±6.31	0.90
3	1.77±2.09	>100	56.50
7	11 97+0 93	>100	8 35
1	11.57 ±0.55	>100	0.00
8	17.12±2.87	11.88±4.59	0.69
9	25.51±9.29	>100	3.92
		100	4.00
10	20.58±5.82	>100	4.86
11	4.38±3.32	>100	22.83
10	11 50 1 45	45 70 4 70	1 97
12	11.50±1.45	10.76±1.76	1.37
13	>100	>100	n.d.
14	>100	>100	n.d.
	0.0.4.00	4 0 0 40	0.00
i icagreior (positive control)	2.0±1.26	4.0±2.43	2.00
	2.02.1120		2.00

The IC_{50} values are presented as the mean \pm SEM of three independent experiments. S.I. (selectivity index) is the calculated ratio IC_{50} TRAP-6/IC₅₀ collagen. n.d.: not determined.



Figure 1. Chemical structures of *ortho*-carbonyl hydroquinones studied in thiswork.





2,5-Di-(tert-butyl)-p-hydroquinone



Figure 2. Representative struc compounds with antiplatelet activity. structures of hydroquinone scaffold-containing





Figure 4. Antiplatelet activity of compounds 3 and 8. Human washed platelet aggregation was performed in the presence or absence of compounds 3 and 8. Aggregation was stimulated with collagen (1 μ g/mL, A) or TRAP-6 (5 μ M, B). (C) The effect of compound **3** was analyzed on increasing collagen concentrations, (E) while compound 8 was tested on increasing TRAP-6 concentration. In D and F, the effect of compounds **3** and **8** on maximal agonist-induced platelet aggregation was analyzed, 2 µM collagen and 10 µM TRAP-6. Cpd: compound. The results are shown as the mean ± SEM, n=3. n.s.: not significant, **p<0.01 and *** p<0.001 vs. control (DMSO 0.2%).

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Figure 5. Effect of compounds 3 and 8 on platelet activation and secretion induced by collagen and TRAP-6. (A) Collagen/TRAP-6 (C+T)-induced externalization of PS assessed by annexin-V binding in the presence of compounds 3 and 8. (B) P-selectin expression and (C) GPIIb/IIIa activation induced by collagen and TRAP-6 were determined by flow cytometry using different compounds 3 or 8 concentrations. (D) Calcium mobilization in washed platelets in the presence of compounds 3 and 8 measured by flow cytometry. (E) Secretion of CD63 protein in the presence of compounds 3 and 8 in platelets stimulated with collagen and TRAP-6 was evaluated. (F) ATP secretion in platelet collagen and TRAP-6-stimulated aggregation was analyzed. Cpd: compound. n.s.: not significant, *p<0.05 and **p<0.01 vs. control (DMSO 0.2%).



Figure 6. Compounds 3 and 8 do not affect the mitochondrial bioenergetics of human platelets. (A-E) NADH levels in resting platelet and (F) maximal NADH oxidation induced by FCCP and mitochondrial NADH accumulation induced by Complex III inhibition. (G) Mitochondrial membrane potential. (H) Intraplatelet ROS levels and (I) intracellular ATP levels. AA: antimycin A, Cpd: compound, FCCP: carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone and Olig: oligomycin. The results are shown as the mean ± SEM of n=3 experiments. n.s.: not significant, *p<0.05, **p<0.01 and ***p<0.001 vs. control (DMSO 0.2%).



848 Scheme 1. Synthesis of the new compounds obtained.

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Highlights

- Ortho-carbonyl substituted hydroquinone derivatives have differential action • on collagen- or TRAP-6-stimulated platelet aggregation.
- Compound 3 was a selective inhibitor on collagen-stimulation and • compound **8** was a dual inhibitor on collagen- or TRAP-6-stimulation.
- These findings may be used for further development of new antiplatelet ٠ agents.

Declaration of interests

 \boxtimes The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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