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Research paper

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

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ABSTRACT

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

1. Introduction

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

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worldwide [6].

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

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

2. Results

2.1. Chemistry

The synthetic methodology to obtain the studied compounds is depicted in Scheme 1. The already reported methodology of obtention of acyl hydroquinones [19,35] was improved using

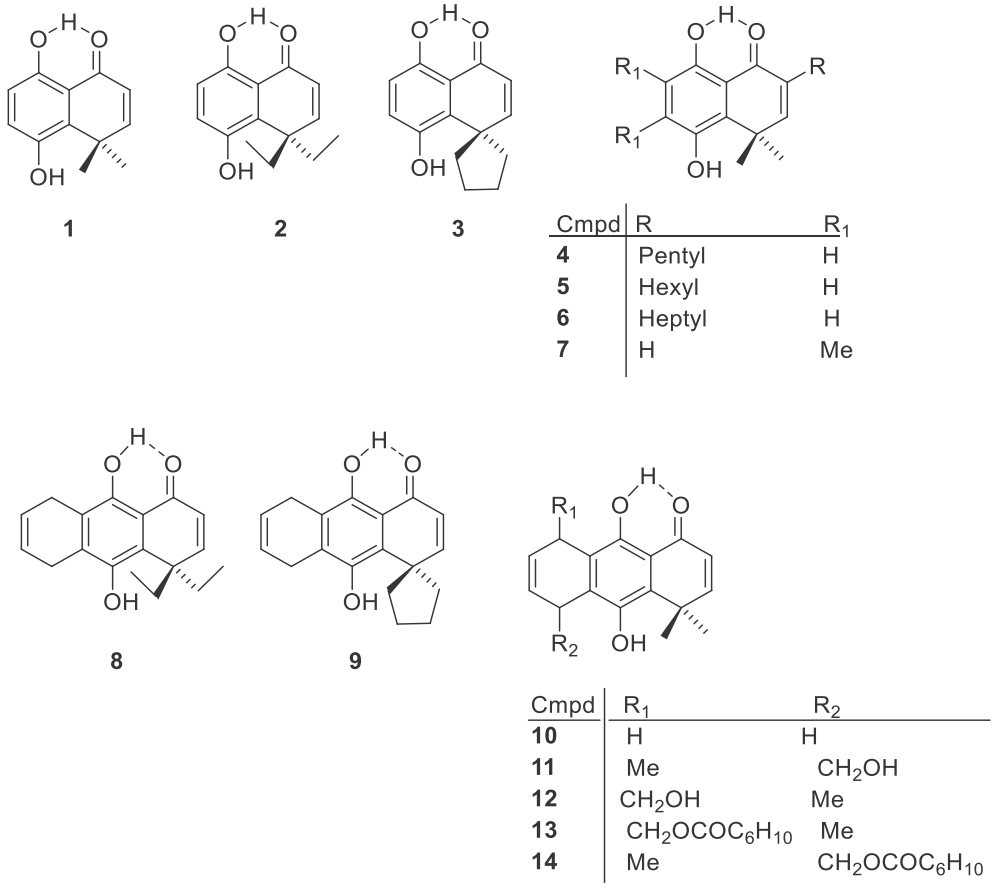


Fig. 1. Chemical structures of *ortho*-carbonyl hydroquinones studied in this work.

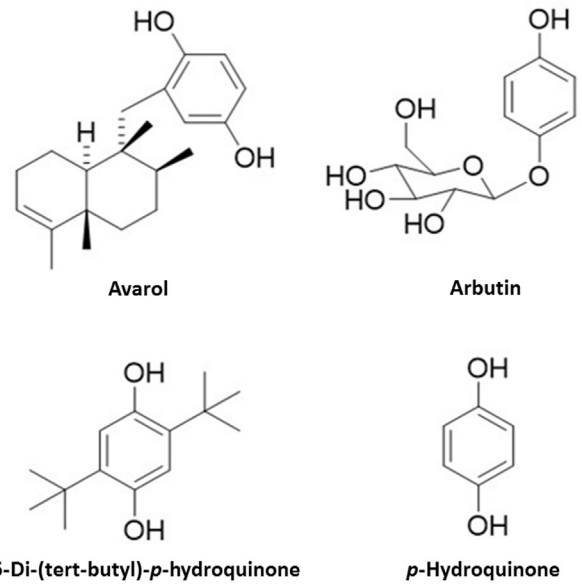


Fig. 2. Representative structures of hydroquinone scaffold-containing compounds with antiplatelet activity.

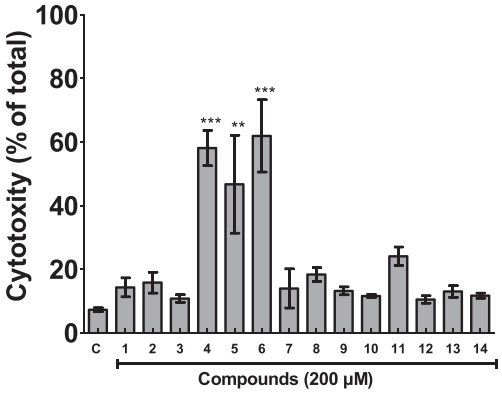


Fig. 3. Cytotoxicity of synthesized compounds on platelets. Bars correspond to the mean ± SEM, n = 5. All compounds were tested at 200 μM **p < 0.01 and ***p < 0.001 vs. control (DMSO 0.2%).

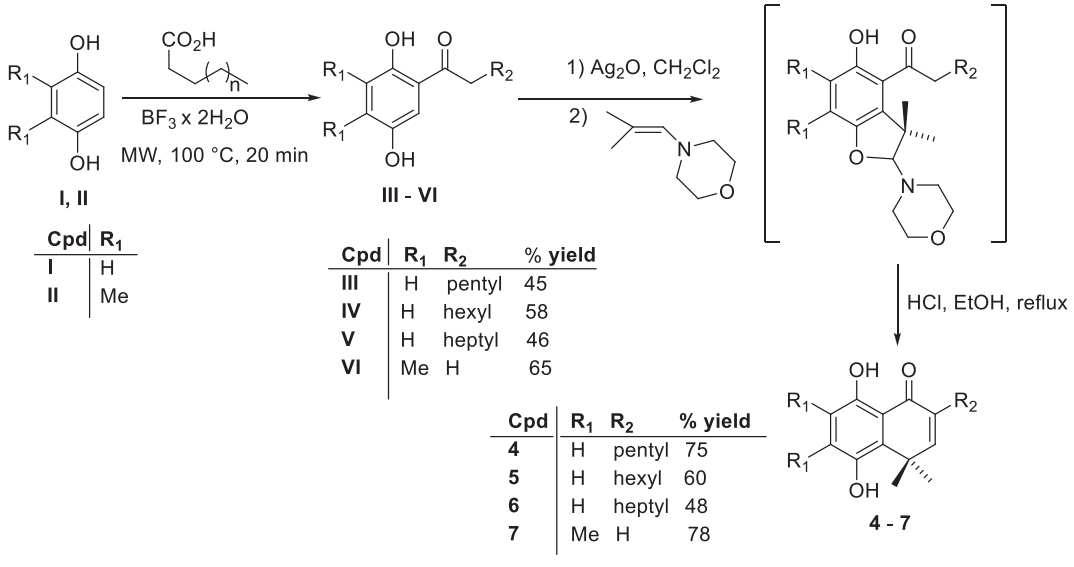
microwave irradiation at 100 °C, using hydroquinone (**I**) or dimethyl hydroquinone (**II**) and the corresponding carboxylic acid as starting products, with boron trifluoride dihydrate as the solvent. Compounds **1**–**14** were synthesized using a methodology previously reported [19,22,36–38], involving oxidation of the acylhydroquinones, reaction with enamines and finally a rearrangement in acidic medium. Structural characterization and procedure to synthesize compounds **1**–**3** and **8**–**14** were included as supplementary information.

2.2. Cytotoxic activity on platelets

In order to identify candidate compounds with potential antiplatelet activity (Fig. 1), we evaluated the effect of synthesized compounds on platelet viability by LDH leakage. 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, Fig. 3). However, under the same experimental conditions, compounds **4, 5** and **6** showed cytotoxic activity ($p < 0.05$) (Fig. 3). Thus, the following antiplatelet experiments were performed using the non-cytotoxic compounds.

2.3. Structure-antiplatelet activity relationship for the collagen- and TRAP-6-stimulated platelet aggregation

Light transmission aggregometry was used to evaluate the activity of non-cytotoxic compounds (**1, 2, 3, 7, 8, 9, 10, 11, 12, 13** and **14**) on platelet aggregation induced by TRAP-6 or collagen, which are known platelet agonists [39]. The compounds showed different potency of their platelet antiaggregant activity, being more active when inhibition was exerted on platelet aggregation induced by collagen than TRAP-6 (Table 1). Among the compounds tested, compounds **3** and **8** were the most active presenting IC_{50} of 1.77 ± 2.09 and 11.88 ± 4.59 μM against collagen- and TRAP-6-induced platelet aggregation, respectively (Table 1). Moreover, compound **3** exhibited high selectivity toward platelet aggregation induced by collagen signaling. In addition, and according to the selectivity index (S.I., IC_{50} TRAP-6/IC_{50} collagen), the other



Cpd	R_1
I	H
II	Me

Cpd	R_1	R_2	% yield
III	H	pentyl	45
IV	H	hexyl	58
V	H	heptyl	46
VI	Me	H	65

Cpd	R_1	R_2	% yield
4	H	pentyl	75
5	H	hexyl	60
6	H	heptyl	48
7	Me	H	78

Scheme 1. Synthesis of the new compounds obtained.

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

Compounds	IC_{50} μM		S.I. (IC_{50} TRAP-6/IC_{50} Collagen)
	Collagen (1 μg/mL)	TRAP-6 (5 μM)	
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
8	17.12 ± 2.87	**11.88 ± 4.59**	0.69
9	25.51 ± 9.29	>100	3.92
10	20.58 ± 5.82	>100	4.86
11	4.38 ± 3.32	>100	22.83
12	11.50 ± 1.45	15.78 ± 1.78	1.37
13	>100	>100	n.d.
14	>100	>100	n.d.
Ticagrelor (positive control)	2.0 ± 1.26	4.0 ± 2.43	2.00

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

compounds had decreasing S.I. as follows: compounds **3** > **11** > **7**~**1** > **10**~**9** > **12**. In platelets stimulated with collagen, the substitution of spiro group by *gem*-dimethyl moiety in compound **3** produced a moderate decrease (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 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 (compound **10**). On the other hand, only three *ortho*-carbonyl hydroquinones (compounds **2**, **8** and **12**) were dual inhibitors, blocking both collagen- and TRAP-6-stimulated platelet aggregation. From IC_{50} values, it is observed that a relevant chemical requirement for dual inhibitors (i.e. S.I. values close to 1) was the *gem*-diethyl substitution (compounds **2** and **8** vs **1** and **10**). The regioisomers **11** and **12** 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 both stimuli of platelet aggregation (compounds **13** and **14**), suggesting that the hydroxymethyl substitution is a relevant structural requirement for inhibition of platelet aggregation.

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

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** (Fig. 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 translocation to the platelet surface of P-selectin, an integral membrane GP found in secretory granules [43]. Both P-selectin and GPIIb/IIIa activation were significantly inhibited by compounds **3** and **8** (Fig. 5B–C) ($p < 0.05$). In contrast, no changes in the levels of Ca^{2+} were observed in resting platelets in the presence of compounds **3** and **8** (Fig. 5D). Platelet activation is autocatalytically stimulated by platelet secretion products [44]. In this context, we shown that the secretion of CD63 (Fig. 5E) was decreased in TRAP-6-stimulated human platelets only by compound **8** ($p < 0.05$). Meanwhile, ATP secretion was decreased in collagen and TRAP-6-stimulated platelets by both compounds **3** and **8** (Fig. 5F).

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

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 auto-oxidation or interference by interactions with fluorescent assays.

The effect of hydroquinone scaffold-containing compounds on platelet function remains scarcely studied, some of them exhibit an antiplatelet effect in platelets stimulated with thrombin, AA, collagen, ADP, A23187 or platelet-activating factor [18], whose structures are shown in Fig. 2. There is a lack of evidence about the antiplatelet activity of bi- and tricyclic hydroquinones and of hydroquinones bearing electron attractor substituents. Consequently in our study, we showed that *ortho*-carbonyl hydroquinone derivatives had differential action on collagen- and TRAP-6-stimulated platelet aggregation, determined by the type of substituent, especially the *gem*-diethyl/methyl substitutions and the

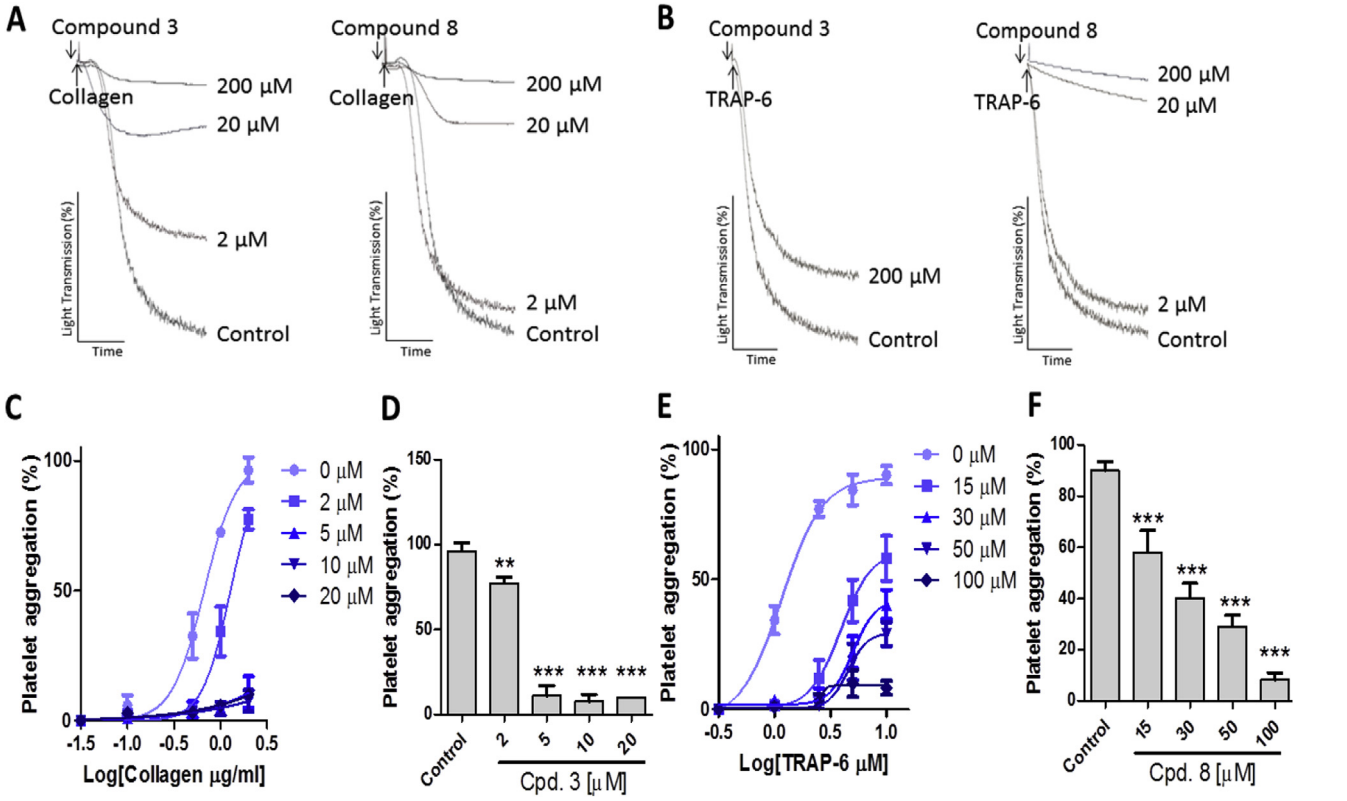


Fig. 4. Antiplatelet activity of compounds **3** and **8**. Human washed platelet aggregation was performed in the presence or absence of compounds **3** and **8**. Aggregation was stimulated with collagen (1 µg/mL, A) or TRAP-6 (5 µM, B). (C) The effect of compound **3** was analyzed on increasing collagen 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%).

addition/modifications of a third ring influence S.I. values and the inhibitory potencies. The observed SAR for the antiplatelet effects of this series of compounds suggest the involvement of radical intermediates in platelets stimulated with TRAP-6: the greater stability of semiquinone radical, hypothetically formed, the higher antiplatelet activity was observed. Compounds **2** and **8** exhibits closely related structure to compounds **1** 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 sterical hindrance and electronic effects, on the antiplatelet activity. The higher volume and the conformation of *gem*-diethyl groups, with their methyl moieties positioned at above and below the molecular plane [37,49], overall constituting a structural factor that provides more thermodynamic stability to the semiquinone radical species obtained by hydrogen abstraction atom or by an electrochemical process [29]. These radicals are responsible for the antioxidant or prooxidant activities of hydroquinones. In the same way, the aromatic ring alkylation also stabilizes these intermediate species facilitating their generation [27]. These pieces 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 relationship supported by the increased ROS production by **8**. On the other hand, the considerable difference between the activities of the regioisomers **11** and **12**, in TRAP-6-stimulated platelets, highlights the importance of the hydrogen bonding in the biological activities of this kind of compounds. Compound **12** has two hydrogen bonds in their structure, one of them between phenolic hydroxyl proton and the carbonyl oxygen atom and the other, one between the other phenolic proton toward the oxygen of the hydroxymethyl group. Its regioisomer, instead, displays less capability to form hydrogen bonds through the hydroxymethyl group [25], a characteristic that can diminish the interactions inside platelets, triggering a lacking activity in this platelet aggregation assay.

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 components. Since oxidative stress-dependent signaling promotes mitochondrial dysfunction, releasing mitochondrial components are important for both platelet activation and apoptosis [45,50,51]. Hydroquinone scaffold-containing 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 derivatives may be the mitochondria. Notably, compound **3** was more active on collagen-induced platelet activation/secretion/aggregation without affecting mitochondrial bioenergetics. In contrast, compound **8** induced $\Delta\psi m$ drop, decreased ATP levels and increased ROS production at high concentrations. ROS production may occur at the ETC with leaks at the proton-based electrochemical gradient [53], changing the $\Delta\psi_m$ which can be ascribed to compound **8**. However, compound **8** did not display any effects on mtNADH levels and its antiplatelet mechanism is apparently upstream mitochondrial bioenergetic function. This phenomenon is consistent with our previously reported observations in which these compounds did not exhibit effects on mitochondrial respiration and OXPHOS-dependent proliferation using isolated tumor mitochondria and highly oxidative subpopulations of breast cancer cells, respectively

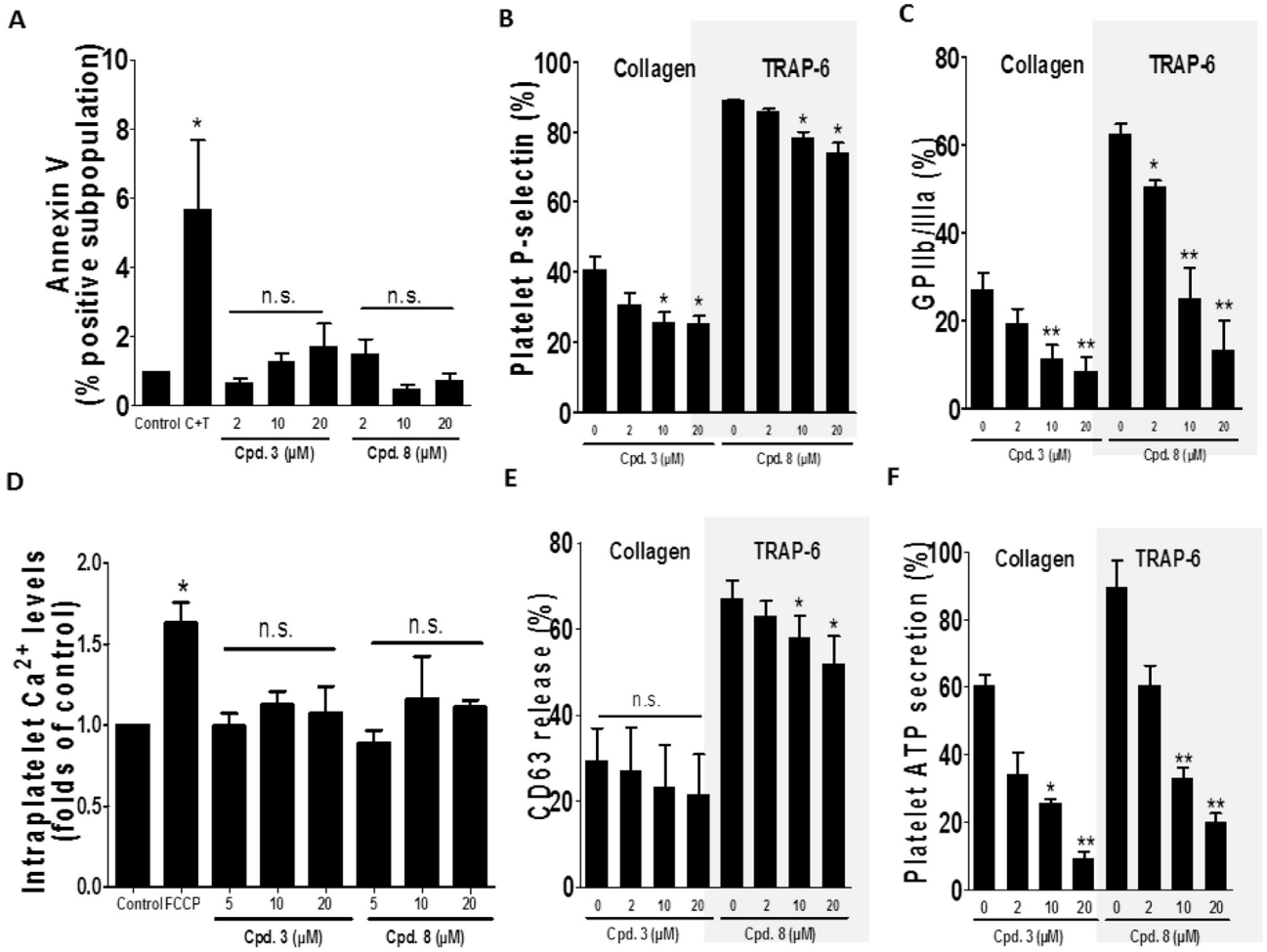


Fig. 5. Effect of compounds **3** and **8** on platelet activation and secretion induced by collagen and TRAP-6. (A) Collagen/TRAP-6 (C + T)-induced externalization of PS assessed by annexin-V binding in the presence of compounds **3** and **8**. (B) P-selectin expression and (C) GPIIb/IIIa activation 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%).

[22,23]. Taken together, our results indicate that both compounds **3** and **8** lack direct effects on mitochondrial bioenergetics and the changes in compound **8**-induced mitochondrial parameters may be a consequence of some non-mitochondrial interactions, *i.e.* modulation of 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 induced by agonists such as collagen, U46619, and AA but has a marginal inhibitory effect on ADP-induced platelet activation [55,56]. Conversely, isorhapontigenin, a methoxylated analog of resveratrol of natural origin [57,58], selectively inhibits ADP-induced platelet aggregation with an IC_{50} of 1.85 μM, lacking effects on aggregation induced by other agonists [9]. In line with this observation, herein we report that compound **3**, a spiro derivative of *ortho*-carbonyl hydroquinone, is a selective inhibitor of collagen-stimulated platelet aggregation ($IC_{50} = 1.77 \pm 2.09$ μM) with marginal effect on TRAP-6-stimulated platelet aggregation. Conversely, compound **8**, which exhibits an inhibitory effect on platelet aggregation stimulated by both agonist collagen and TRAP-6, can be considered a dual antiplatelet agent. Considering that there is one patent related to novel spiro compounds useful as selective inhibitors of fibrinogen-dependent platelet aggregation for the prevention of thrombosis (European Patent Office EP0854869B1), our results suggest that compounds **3** and **8** can contribute as promising scaffolds for the design and development of novel antiplatelet agents with differential action on thrombotic diseases.

4. Conclusion

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

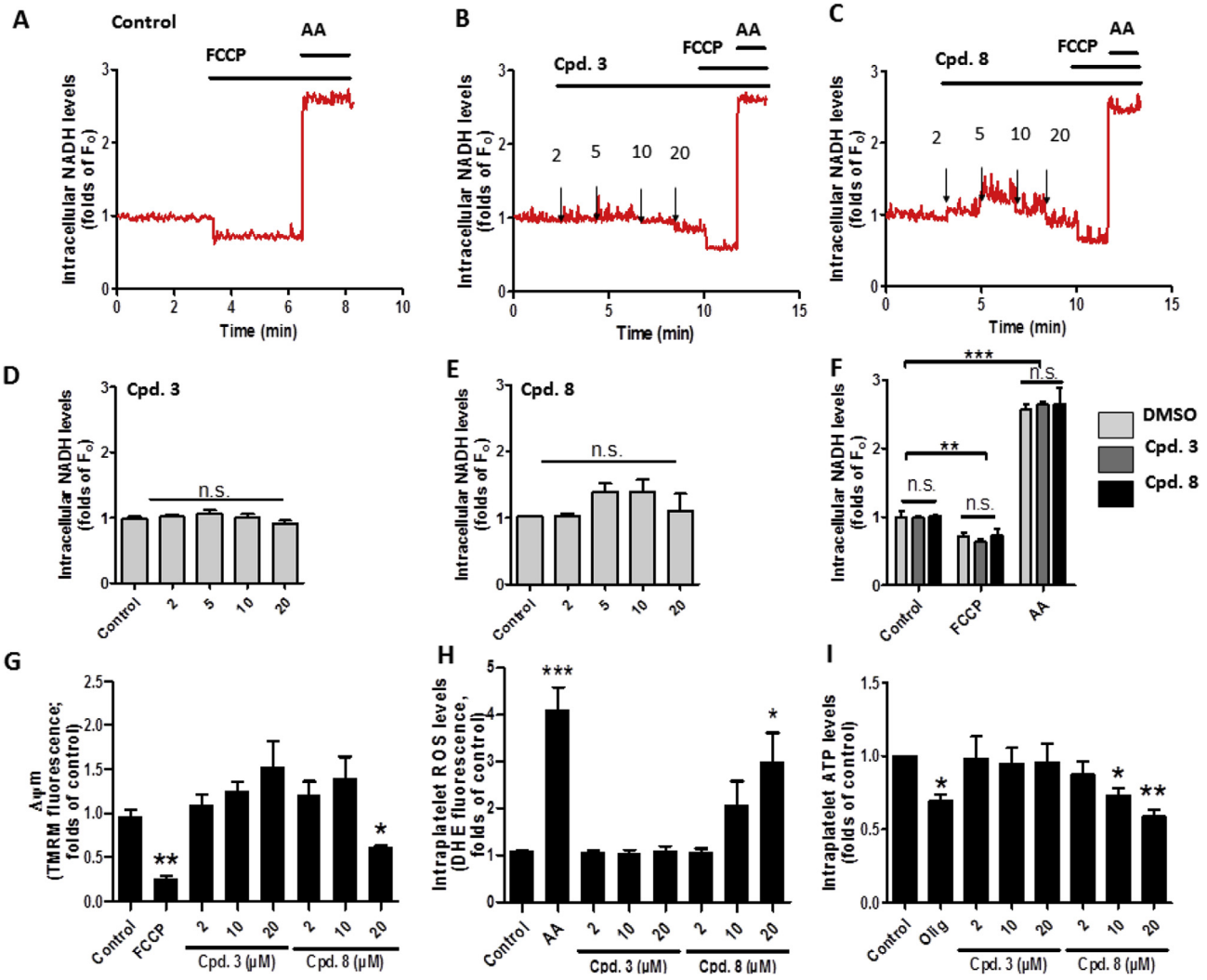


Fig. 6. Compounds 3 and 8 do not affect the mitochondrial bioenergetics of human platelets. (A–E) NADH levels in resting platelet and (F) maximal NADH oxidation induced by FCCP and mitochondrial NADH accumulation induced by Complex III inhibition. (G) Mitochondrial membrane potential. (H) Intraplatelet 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%).

5. Experimental procedures

5.1. Chemistry

General methods. ^{1}H and ^{13}C NMR spectra were obtained from a spectrometer operating at either 400.13 MHz (^{1}H) or 100.61 MHz (^{13}C). Chemical shifts are reported as ppm downfield from TMS 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; wavenumbers are reported in cm^{-1}. High-resolution mass spectra (HRMS) were obtained on an orthogonal time-of-flight (ToF) mass spectrometer (QTof Micro, Micromass UK) or on a Thermo Q exacting focus. Silica gel 60 (230–400 mesh ASTM) and TLC sheets silica gel 60 F254 were used for flash-column chromatography and analytical TLC, respectively.

Synthesis of acylhydroquinones III-VI. The already reported methodology of obtention of acyl hydroquinones [35] was improved using microwave irradiation as 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**), 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 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 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.

*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 dihydrate react yielding 547.4 mg of **III** (2.46 mmol, 45% yield). ^{1}H NMR δ($CDCl_3$): 0.90 (t, 3H, $J = 6.7$ Hz, 7-CH_3); 1.27–1.45 (m, 6H, 3x CH_2); 1.73 (p, 2H, $J = 7.4$ Hz, 3-CH_2); 2.93 (t, 2H, $J = 7.4$ Hz, CH_2CO); 4,78 (s, 1H, 5′-OH); 6.88 (d, $J = 8.9$ Hz, 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′-H); 11.98 (s, 1H, 2′-OH). ^{13}C NMR δ($CDCl_3$): 14.04; 22.51; 24.45; 28.97; 31.59; 38.48; 114.98; 119.03; 119.29; 142.82; 147.59; 156.45; 206.79. M.P.: 49.7–50.3 °C. HRMS (ESI): *m/z* calcd for $C_{13}H_{18}O_3$ $[M+H]^+$: 223.1289, found: 223.1286.

*Compound **IV** (1-(2,5-dihydroxyphenyl)-1-octanone).* Hydroquinone (600 mg, 5.45 mmol), octanoic acid (98%) (1.32 mL,

8.18 mmol) and 4 mL of boron trifluoride dihydrate react yielding 753.7 mg of **IV** (3.19 mmol, 58% yield). ^{1}H NMR δ($CDCl_3$): 0.90 (t, 3H, J = 6.9 Hz, 8-CH_3); 1.23–1.45 (m, 8H, 4x CH_2); 1.74 (p, 2H, J = 7,2 Hz, 3-CH_2); 2.93 (t, 2H, J = 7.4 Hz, CH_2CO); 4.81 (s, 1H, 5′-OH); 6.89 (d, 1H, J = 8.9 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′-H); 11.98 (s, 1H, 2′-OH). ^{13}C NMR δ($CDCl_3$): 14.08; 22.62; 24.50; 29.09; 29.26; 31.68; 38.48; 114.99; 119.03; 119.28; 124.83; 147.62; 156.43; 206.81. M.P.: 65.2–66.2 °C. HRMS (ESI): m/z calcd for $C_{14}H_{20}O_3$ $[M+H]^+$: 237.1485, found: 237.1479.

Compound ***V*** *(1-(2,5-dihydroxyphenyl)-1-nonanone).* Hydroquinone (600 mg, 5.45 mmol), nonanoic acid (96%) (1.49 mL, 8.18 mmol) and 4 mL of boron trifluoride dihydrate react yielding 627.2 mg of **V** (2.51 mmol, 46% yield). ^{1}H NMR δ($CDCl_3$): 0.89 (t, 3H, J = 6.9 Hz, 9-CH_3); 1.23–1.45 (m, 10H, 5x CH_2); 1.73 (p, 2H, J = 7.4 Hz, 3-CH_2); 2.93 (t, 2H, J = 7.4 Hz, 2-CH_2CO); 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). ^{13}C NMR δ($CDCl_3$): 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 obtained following the reported method, although slight variations were introduced [19]. A mixture of acylhidroquinone (1 equiv) and Ag_2O (2.5 equiv) in 20–30 mL of dichloromethane was vigorously stirred for 1–2 h at RT, yielding the corresponding quinone. This mixture was filtered through Celite, and dropwise added, without isolation, to a solution of the 4-(2-methyl-2-propenyl)morpholine at 0 °C, allowing it to reach RT in a 30–60 min time off, being monitored by thin-layer chromatography. Then, the solvent was evaporated under reduced pressure. The residue was dissolved in a mixture of ethanol and hydrochloric acid and refluxed by 3 h. Next 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 then filtered; the solvent was evaporated under reduced pressure and purified by flash chromatography using 6:1 hexane/ethyl acetate as eluent.

Compound ***4*** *(5,8-dihydroxy-4,4-dimethyl-2-pentylnaphthalen-1(4H)-one).* Two portions of 450 mg (900 mg, 4.05 mmol) of acylhydroquinone **III** were oxidized with 1.13 g of Ag_2O each one in 40 mL of dichloromethane for 2 h, the filtered solutions were put together and dropwise added over 4-(2-methylpropenyl)morpholine (572 mg, 4.05 mmol) for 1.5 h. Evaporation of the solvent and a further reflux of the residue in a mixture of ethanol (20 mL) and hydrochloric acid (1 mL), following the general method, achieved 831 mg of hydroquinone **4** (3.03 mmol, 75% yield). ^{1}H NMR δ($CDCl_3$): 0.91 (t, 3H, J = 6.7 Hz, -CH_2CH_3); 1.30–1,40 (m, 4H, 2x CH_2); 1,48–1,57 (m, 2H, CH_2); 1.60 (s, 6H, 2x 4-CH_3); 2.38 (t, 2H, J = 6,9 Hz, O=C–C–CH_2); 4.86 (s, 1H, 5-OH); 6,62 (s, 1H, 3-H); 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, 8-OH). ^{13}C NMR δ($CDCl_3$): 14.01; 22.45; 24.96; 28.01; 28.88; 31.51; 37.80; 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.

Compound ***5*** *(5,8-dihydroxy-4,4-dimethyl-2-hexylnaphthalen-1(4H)-one).* Two portions of 450 mg (900 mg, 3.81 mmol) of acylhydroquinone **IV** were oxidized with 1.1 g of Ag_2O 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 mg, 4.0 mmol) by 1.5 h. Evaporation of the solvent and a further reflux of the residue in a mixture of ethanol (20 mL) and hydrochloric acid (1 mL), following the general method, allowed to obtain 657.2 mg of hydroquinone **5** (2.27 mmol, 60% yield). ^{1}H-RMN δ($CDCl_3$): 0.89 (t, 3H, J = 7.0 Hz, CH_2CH_3); 1.20–1.40 (m, 8H, 4x CH_2); 1.46–1.57 (m, 2H, CH_2); 1.60 (s, 6H, 2x 4-CH_3); 2.38 (t, 2H, J = 6.9 Hz, O=C–C–CH_2); 4.86 (s, 1H, 5-OH); 6.62 (s, 1H, 3-CH); 6.77 (d, 1H, J = 8.8 Hz, 6- or 7-H); 6.89 (d, 1H, J = 8.8 Hz, 6- or 7-H); 13.07 (s, 1H, 8-OH). ^{13}C-RMN δ($CDCl_3$): 22.64; 25.11; 28.37; 28.96; 29.12; 29.32; 31.83; 37.52; 115.49; 115.61; 123.72; 133.81; 134.98; 145.36; 156.77; 156.96; 191.32. M.P.: 68.5–71.7 °C. HRMS (ESI): m/z calcd for $C_{18}H_{25}O_3$ $[M+H]^+$Calc.: 289.1804, Found: 289.1800.

Compound ***6*** *(5,8-dihydroxy-4,4-dimethyl-2-heptylnaphthalen-1(4H)-one).* Acyl hydroquinone **V** (500 mg, 2 mmol) was oxidized with Ag_2O (1.16 g) in accordance with the general methodology. Then the filtrated solution was added over a solution of 4-(2-etylbutenil)morpholine (337.6 mg, 2 mmol). After evaporation of the solvent, the residue was refluxed in ethanol HCl mixture yielding hydroquinone **6** (284.5 mg, 48% yield). ^{1}H NMR δ($CDCl_3$): 0,89 (t, 3H, J = 6.9 Hz, CH_2CH_3); 1.17–1,42 (m, 10H, 5x CH_2); 1.46–1.57 (m, 2H, CH_2); 1.59 (s, 6H, 2x 4-CH_3); 2.38 (t, 2H, J = 6.9 Hz, O=C–C–CH_2); 5.29 (s, 1H, 5-OH); 6.62 (s, 1H, 3-CH); 6.67 (d, 1H, J = 8.8 Hz, 6- or 7-H); 6.90 (d, 1H, J = 8.8 Hz, 6- or 7-H); 13.07 (s, 1H, 8-OH). ^{13}C NMR δ($CDCl_3$): 14.06; 22.51; 25.16; 28.06; 28.55; 28.94; 29.17; 31.56; 31.69; 37.49; 115.64; 115.78; 123.67; 133.48; 134.93; 145.18; 156.75; 157.10; 191.22. M.P.: 74.3–76.5 °C. HRMS (ESI): m/z calcd for $C_{19}H_{64}O_3$ $[M-H]^+$: 303.1960, found: 303.4227.

Compound ***7*** *(5,8-dihydroxy-4,4,6,7-tetramethylnaphthalen-1(4H)-one).* 2′,5′-dihydroxy-3′,4′-dimethylacetophenone (400 mg, 2.2 mmol) [35], was oxidized according to general methodology with Ag_2O (1.28 g). After filtering the solution was added over a solution of 4-(2-methylpropenil)morfoline (314 mg, 2.2 mmol), after evaporation under vacuum a viscous residue was obtained. 451 mg of this residue was refluxed in a mixture of ethanol and hydrochloric acid by 1 h. Following the same procedure as described before, 256 mg of compound **7** (1.1 mmol, 78% yield) was obtained. ^{1}H NMR δ($CDCl_3$): 1,61 (s, 6H, 2x 4-CH_3); 2.23 (s, 3H, Ar-CH_3); 2.26 (s, 3H, Ar-CH_3); 4.55 (s, 1H, 5-OH); 6.23 (d, 1H, J = 10 Hz, 2-H), 6.82 (d, 1H, J = 10 Hz, 3-H), 13.20 (s,1H, 8-OH). ^{13}C NMR δ($CDCl_3$): 11.56; 13.04; 25.17; 38.03; 112.68; 123.43; 124.04; 131.51; 131.64; 143.52; 155.12; 160.73; 191.11. M.P.: 200.5–201.6 °C. HRMS (ESI): m/z calcd for $C_{14}H_{16}O_3$ $[M+1]^+$: 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.

5.2. Biological assays

5.2.1. Preparation of human platelets

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

5.2.2. Cytotoxic activity

Washed platelets (3×10^8 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×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) with 10% Triton X-100 was used as a positive control.

5.2.3. Antiplatelet activity

Platelet aggregation. Platelet aggregation was evaluated using a lumi-aggregometer (Chrono-Log, Haverton, PA, USA) and monitored by light transmission [59]. Washed platelets (3×10^8 platelets/mL) were pre-incubated for 5 min with $CaCl_2$ (2 mM) plus compounds (0.2–200 μM). Platelet aggregation was induced with TRAP-6 (5 μM) or collagen (1 μg/mL) and transmittance followed for 6 min. DMSO 0.2% was used as a vehicle.

Phosphatidylserine externalization. The externalization of PS in platelets was determined by flow cytometry [60]. Washed platelets (3×10^8 platelets/mL) were pre-incubated for 5 min with $CaCl_2$ (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 (5×10^7 platelets/mL) were mixed 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. After collecting data for 15 s, carbonylcyanide *p*-triflouromethoxyphenylhydrazone (FCCP,1 μM) was added and the data were obtained for further 60 s. The effect of compounds on cytosolic calcium 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 μM 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].

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

5.3. Statistical analysis

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 (IC_{50}) 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.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112187.

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