Full Paper

Synthesis of 2,3-Disubstituted 1,4-Naphthoquinones as Antiplatelet Agents

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In continuing search for novel antiplatelet agents, the highly potent agent 2-chloro-3-methoxycarbonylethylcarboxamido-1,4-naphthoquinone **2** was selected as lead compound. Structure-activity relationships in this series were examined. Some of these compounds showed significant antiplatelet activities. Further studies on the action mechanism showed that 2-acetamido-3-chloro-1,4-naphthoquinone **4** has a direct inhibitory action on cytosolic phospholipase A_2 ($cPLA_2$) activity in platelets.

Keywords: Antiplatelet activity / $cPLA_2$ / Naphthoquinones

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Introduction

Myocardial infarction and ischemic stroke are the leading causes of morbidity and mortality in developed countries. These result from the formation of an occluding vascular thrombus caused by rupture or erosion of unstable plaque. Blood platelets play a crucial role in the development of a thrombus, a sequential event involving platelet adhesion, activation, and subsequent aggregation. Thus, many categories of antiplatelet agents have been extensively researched and developed as potential therapies for both, the treatment and prevention of cardiovascular disease [1 – 3].

In the course of our search for new antiplatelet agents [4, 5], a series of 2-alkylcarboxamido-3-chloro-1,4-naphthoquinones were synthesized and tested for their inhibitory activities on human platelet aggregation. Among these active compounds, 2-chloro-3-methoxycarbonylethylcarboxamido-1,4-naphthoquinone **2** was the most promising agent and showed potent activity in concentration-dependently inhibited thrombin (0.1 unit/mL), arachidonic acid (100 μM), collagen (10 μg/mL), and platelet-activating factor (2 ng/mL) induced aggregation in human platelet suspension. The IC_{50} values of compound **2** for inhibiting aggregation induced by the above mentioned inducers are 24.0, 2.6, 2.7, and 4.4 μM, respectively. Further studies indicate that compound **2** exerts antiplatelet effects by inhibiting phosphoinositide turnover [6]. In the present work, compound **2** was selected as the lead compound and the 3-chloro group was replaced by various 3-arylamino groups and tested for antiplatelet activity. Our findings are summarized in this report.

Results and discussion

2-Alkylcarboxamido-3-chloro-1,4-naphthoquinones **2 – 4** were synthesized using previously described methods [5]. As shown in Scheme 1, when 2-amino-3-chloro-1,4-naphthoquinone **1** was reacted with substituted acyl chlorides or acetic anhydride, in the presence of dry hydrogen chloride or concentrated sulfuric acid, the corresponding compounds **2 – 4** were obtained. As illustrated in

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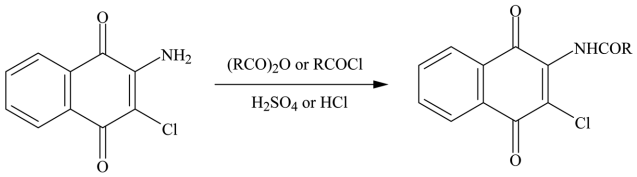
Abbreviations: cytosolic phospholipase A_2 ($cPLA_2$)

Table 1. Inhibitory Effect of 2,3-Disubstituted 1,4-Naphthoquinones on Platelet Aggregation Induced by Thrombin, Arachidonic Acid (AA), Collagen, and Platelet-activating Factor (PAF)[a].

No.	conc. (μg/mL)	Percentage aggregation			
		Thrombin	AA	Collagen	PAF
2	100	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***
	20	0.0 ± 0.0***			
	10	25.2 ± 2.3***			
	5	60.7 ± 3.3***			0.0 ± 0.0***
	2	83.8 ± 2.5***	0.0 ± 0.0***	0.0 ± 0.0***	12.2 ± 5.1***
	1		31.7 ± 13.7***	24.1 ± 12.3***	56.0 ± 6.5***
	0.5		71.0 ± 4.5***	74.9 ± 2.1***	74.7 ± 5.2**
	IC_{50}(μM)	24.0	2.6	2.7	4.4
3	100	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***
	20	0.0 ± 0.0***			0.0 ± 0.0***
	10	57.9 ± 3.9***			38.3 ± 2.8***
	5	80.6 ± 5.0***	0.0 ± 0.0***		56.7 ± 3.1***
	2		16.7 ± 10.2***	0.0 ± 0.0***	59.8 ± 2.3***
	1		88.0 ± 1.2	83.1 ± 1.4*	88.9 ± 3.1
	IC_{50}(μM)	32.7	6.3	4.2	21.8
4	100	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***
	5	43.3 ± 5.6***		10.4 ± 9.0***	0.0 ± 0.0***
	2	76.2 ± 11.5		39.3 ± 21.3**	54.9 ± 13.3**
	1		0.0 ± 0.0***	86.6 ± 3.0	90.4 ± 1.6
	0.5		7.7 ± 6.3***		
	0.2		71.0 ± 4.5***		
	IC_{50}(μM)	88.8	1.4	59.3	10.4
5	100	66.2 ± 0.9**	0.0 ± 0.0***	0.0 ± 0.0***	33.8 ± 7.4***
	50		57.4 ± 2.2***	0.0 ± 0.0***	
	20		83.0 ± 0.7*	88.4 ± 2.5	
	10		88.5 ± 1.4*		
	IC_{50}(μM)	ND	138.0	87.4	ND
6	100	91.6 ± 1.2	86.6 ± 2.7	84.1 ± 2.4**	90.2 ± 1.6
	IC_{50}(μM)	ND	ND	ND	ND
7	100	91.2 ± 1.3	89.2 ± 2.0	88.0 ± 1.4	90.3 ± 1.7
	IC_{50}(μM)	ND	ND	ND	ND
8	100	91.6 ± 0.5	81.6 ± 3.8**	82.5 ± 1.0***	90.3 ± 1.4
	IC_{50}(μM)	ND	ND	ND	ND
9	100	33.3 ± 1.7***	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***
	50		0.0 ± 0.0***	0.0 ± 0.0***	24.9 ± 10.2***
	20		70.6 ± 1.6***	74.8 ± 2.9***	83.7 ± 1.6***
	10		79.9 ± 2.4***	87.9 ± 2.6	85.6 ± 3.0**
	5		86.4 ± 1.0***		90.9 ± 1.5
	IC_{50}(μM)	ND	62.7	69.2	105.2
10	100	91.5 ± 1.8	88.6 ± 2.4	88.7 ± 1.3	90.1 ± 1.8
	IC_{50}(μM)	ND	ND	ND	ND
11	100	92.0 ± 1.5	82.4 ± 3.1	75.2 ± 7.9	79.6 ± 3.8**
	IC_{50}(μM)	ND	ND	ND	ND
12	100	90.4 ± 0.5	74.1 ± 8.5*	76.9 ± 2.3***	24.4 ± 9.8***
	IC_{50}(μM)	ND	ND	ND	ND
13	100	91.1 ± 0.4	83.2 ± 4.3*	84.0 ± 0.9***	84.6 ± 2.3
	IC_{50}(μM)	ND	ND	ND	ND
14	100	93.3 ± 1.7	82.8 ± 6.2	81.0 ± 2.9**	57.7 ± 7.7***
	IC_{50}(μM)	ND	ND	ND	ND
15	100	85.4 ± 0.5***	0.0 ± 0.0***	15.4 ± 7.7***	0.0 ± 0.0***
	50		37.8 ± 16.1**		31.2 ± 9.3***
	20		85.6 ± 2.7		82.7 ± 3.4
	IC_{50}(μM)	ND	113.8	ND	106.7
16	100	94.3 ± 1.3	85.6 ± 0.5	50.2 ± 13.6**	81.5 ± 4.5**
	IC_{50}(μM)	ND	ND	ND	ND
17	100	89.4 ± 2.4	86.1 ± 1.5	83.8 ± 2.7	87.7 ± 2.7
	IC_{50}(μM)	ND	ND	ND	ND
18	100	88.1 ± 0.8***	65.6 ± 1.6***	0.0 ± 0.0***	87.2 ± 2.6
	IC_{50}(μM)	ND	ND	ND	ND
19	100	87.7 ± 0.8***	0.0 ± 0.0***	39.8 ± 0.9***	88.6 ± 1.9
	20		0.0 ± 0.0***		
	10		77.8 ± 1.4***		
	5		85.4 ± 1.3		
	IC_{50}(μM)	ND	36.7	ND	ND
20	100	89.1 ± 2.0	82.7 ± 1.3*	66.3 ± 1.3***	87.0 ± 2.4
	IC_{50}(μM)	ND	ND	ND	ND
21	100	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***	0.0 ± 0.0***
	50				0.0 ± 0.0***
	20				21.3 ± 1.3***
	10		0.0 ± 0.0***		42.2 ± 1.3***
	5		41.7 ± 0.9***		81.2 ± 0.9***
	2		61.5 ± 1.1***		
	1		72.6 ± 0.9***		
	0.5		77.3 ± 1.0***		
	0.2		82.4 ± 2.0**		
	IC_{50}(μM)	ND	11.3	ND	41.8
22	100	90.2 ± 1.0	0.0 ± 0.0***	86.1 ± 1.1	87.9 ± 1.1*
	50		75.3 ± 1.6***		
	20		83.5 ± 1.1**		
	10		86.7 ± 1.2***		
	IC_{50}(μM)	ND	173.7	ND	ND
ASA	**IC_{50}(μM)**	>100	20.0	>100	>100

[a] Platelets were incubated with test compounds at 37°C for 1 min. Then, thrombin (0.1 unit/mL), arachidonic acid (AA, 100 μM), collagen (10 μg/mL), or platelet-activating factor (PAF, 2 ng/mL) was added to trigger the aggregation. IC_{50} values are expressed as the concentration (μM) at which 50% inhibition of platelet aggregation occurred. ASA (acetyl salicylic acid) was used as a positive control.



1 **2 - 4**

2, R = $(CH_2)_2COOCH_3$

3, R = $(CH_2)_3COOCH_3$

4, R = CH_3

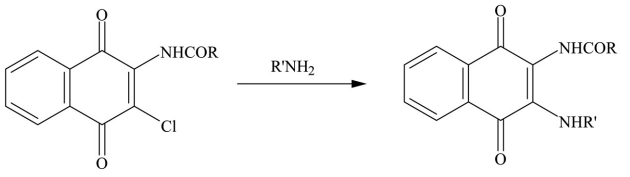
Scheme 1. Synthesis of 2-alkylcarboxamido-3-chloro-1,4-naphthoquinones **2**–**4**.

Scheme 2, reaction of compounds **2**–**4** with a variety of substituted anilines or benzylamines afforded the target compounds, 2-alkylcarboxamido-3-arylamino-1,4-naphthoquinone **5**–**22**.

The antiplatelet activities of 2,3-disubstituted 1,4-naphthoquinones **2**–**22** are summarized in Table 1. At a concentration of 10–20 μg/mL, compounds **3** and **4**, with the 2-position side chain longer and shorter, respectively, than in compound **2**, completely inhibited the platelet

Table 2. Physical and spectral data of 2,3-disubstituted 1,4-naphthoquinones.

No.	Yield (%)	Mp. (°C)	MS (M^+) (m/z)	IR (cm^{-1})	^{1}H-NMR (ppm)
2	81	174 – 175	321	1740	2.74 – 2.82 (m, 4H, -CH_2CH_2-); 3.70 (s, 3H, -OCH_3); 7.69 – 7.77 (m, 2H, H-6,7); 8.07 – 8.10 (m, 1H, H-5); 8.14 – 8.17 (m, 1H, H-8)
3	83	127 – 128	335	1730	2.00 – 2.10 (m, 2H, -$CH_2CH_2CH_2$-); 2.45 (t, *J* = 7.5 Hz, 2H, -$CH_2CH_2CH_2$-); 2.57 (t, *J* = 7.5 Hz, 2H, -$CH_2CH_2CH_2$-); 3.67 (s, 3H, -OCH_3); 7.71 – 7.75 (m, 2H, H-6,7) ; 8.04 – 8.07 (m, 1H, H-5); 8.12 – 8.15 (m, 1H, H-8)
4	98	219 – 220	249	1700	2.31 (s, 3H, -$COCH_3$); 7.71 (br, 1H, -NH-); 7.73 – 7.82 (m, 2H, H-6,7) ; 8.10 – 8.13 (m, 1H, H-5); 8.18 – 8.20 (m, 1H, H-8)
5	84	182 – 184	378	1710	1.91 – 2.03 (m, 4H, -CH_2CH_2-); 3.51 (s, 3H, -OCH_3); 6.94 (d, *J* = 8.8 Hz, 2H, H-2′,6′); 7.06 – 7.10 (m, 1H, H-4′); 7.16 – 7.24 (m, 2H, H-3′,5′); 7.77 – 7.84 (m, 2H, H-6,7); 7.98 – 8.04 (m, 2H, H-5,8); 8.90 (s, 1H, -NHCO-), 9.22 (s,1H, -NH-)
6	88	188 – 190	392	1710	2.16 (s, 4H, -CH_2CH_2-); 2.30 (s, 3H, 4′-CH_3); 3.62 (s, 3H, -OCH_3); 6.80 (d, *J* = 8.3 Hz, 2H, H-3′,5′); 7.05 (d, *J* = 8.3 Hz, 2H, H-2′,6′); 7.62 – 7.70 (m, 2H, H-6,7); 7.67 (s, 1H, -NHCO-), 7.76 (s, 1H, -NH-); 8.05 – 8.09 (m, 2H, H-5,8)
7	86	172 – 173	408	1710	1.93 (t, *J* = 7.2 Hz, 2H, -CH_2CH_2-); 2.05 (t, *J* = 7.2 Hz, 2H, -CH_2CH_2-); 3.52 (s, 3H, -OCH_3); 3.72 (s, 3H, 4′-OCH_3); 6.79 (d, *J* = 8.3 Hz, 2H, H-3′,5′); 6.90 (d, *J* = 8.3 Hz, 2H, H-2′,6′); 7.75 – 7.83 (m, 2H, H-6,7); 7.96 – 8.03 (m, 2H, H-5,8); 8.89 (s, 1H, -NHCO-), 9.03 (s, 1H, -NH-)
8	83	188 – 190	396	1730	1.98 – 2.10 (m, 4H, -CH_2CH_2-); 3.52 (s, 3H, -OCH_3); 6.95 – 7.03 (m, 4H, H-2′,3′,5′,6′); 7.75 – 7.83 (m, 2H, H-6,7) ; 7.97 – 8.03 (m, 2H, H-5,8); 8.90 (s, 1H, -NHCO-), 9.19 (s, 1H, -NH-)
9	84	182 – 184	412	1730	1.99 – 2.07 (m, 4H, -CH_2CH_2-); 3.52 (s, 3H, -OCH_3); 6.93 (d, *J* = 8.8 Hz, 2H, H-2′,6′); 7.23 (d, *J* = 8.8 Hz, 2H, H-3′,5′); 7.77 – 7.84 (m, 2H, H-6,7) ; 7.98 – 8.03 (m, 2H, H-5,8); 8.99 (s, 1H, -NHCO-), 9.31 (s, 1H, -NH-)
10	89	151 – 152	392	1730	2.48 – 2.54 (m, 4H, -CH_2CH_2-); 3.56 (s, 3H, -OCH_3); 4.59 (d, *J* = 5.8 Hz, 2H, -$NHCH_2$-); 7.22 – 7.33 (m, 5H, H-2′,3′,4′,5‘,6′); 7.72 – 7.81 (m, 2H, H-6,7); 7.92 – 7.98 (m, 2H, H-5,8); 8.97 (s, 1H, -NHCO-)
11	85	182 – 184	392	1740	1.47 – 1.53 (m, 2H, -$CH_2CH_2CH_2$-); 1.87 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 2.20 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 3.43 (s, 3H, -OCH_3); 7.09 – 7.19 (m, 1H, H-2′,4′,6′); 7.32 – 7.37 (m, 2H, H-3′,5′); 7.91 – 8.12 (m, 2H, H-6,7) ; 8.13 – 8.18 (m, 2H, H-5,8); 9.04 (s, 1H, -NHCO-), 9.19 (1H, s, -NH-)
12	87	138 – 139	406	1730	1.49 – 1.54 (m, 2H, -$CH_2CH_2CH_2$-); 1.94 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); *2.05 (t, J = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 2.29 (s, 3H, 4′-CH_3); 3.62 (s, 3H, -OCH_3); 6.80 (d, J = 8.3 Hz, 2H, H-3′,5′); 7.04 (d, J = 8.3 Hz, 2H, H-2′,6′); 7.57 (s, 1H, -NHCO-), 7.61-7.69 (m, 2H, H-6,7) ; 7.79 (s, 1H, -NH-); 8.04 – 8.07 (m, 2H, H-5,8)*
13	86	117 – 118	422	1730	1.35 – 1.40 (m, 2H, -$CH_2CH_2CH_2$-); 1.72 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 2.07 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 3.56 (s, 3H, -OCH_3); 3.72 (s, 3H, 4′-OCH_3); 6.77 (d, *J* = 8.3 Hz, 2H, H-3′,5′); 6.91 (d, *J* = 8.3 Hz, 2H, H-2′,6′); 7.74 – 7.85 (m, 2H, H-6,7); 7.96 – 8.02 (m, 2H, H-5,8); 8.81 (s, 1H, -NHCO-), 8.86 (s, 1H, -NH-)
14	86	158 – 159	410	1720	1.33 – 1.42 (m, 2H, -$CH_2CH_2CH_2$-); 1.75 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 2.09 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 3.56 (s, 3H, -OCH_3); 6.96 – 7.04 (m, 4H, H-2′,3′,5′,6′); 7.76 – 7.85 (m, 2H, H-6,7) ; 7.97 – 8.03 (m, 2H, H-5,8); 8.92 (s, 1H, -NHCO-), 9.02 (s, 1H, -NH-)
15	86	143 – 144	426	1730	1.34 – 1.39 (m, 2H, -$CH_2CH_2CH_2$-); 1.80 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 2.01 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 3.53 (s, 3H, -OCH_3); 6.94 (d, *J* = 8.8 Hz, 2H, H-2′,6′); 7.21 (d, *J* = 8.8 Hz, 2H, H-3′,5′); 7.76 – 7.84 (m, 2H, H-6,7) ; 7.97 – 8.03 (m, 2H, H-5,8); 8.99 (s, 1H, -NHCO-), 9.14 (s, 1H, -NH-)
16	87	151 – 152	406	1730	1.71 – 1.75 (m, 2H, -$CH_2CH_2CH_2$-); 2.23 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 2.34 (t, *J* = 7.2 Hz, 2H, -$CH_2CH_2CH_2$-); 3.58 (s, 3H, -OCH_3); 4.63 (d, *J* = 5.8 Hz, 2H, -$NHCH_2$-); 7.20 – 7.30 (m, 5H, H-2′,3′,4′,5′,6′); 7.70 – 7.80 (m, 2H, H-6,7); 7.92 – 7.98 (m, 2H, H-5,8); 8.85 (s, 1H, -NHCO-)
17	80	207 – 208	306	1655	1.59 (s, 3H, -$COCH_3$); 6.89 – 7.37 (m, 5H, H-2′,3′,4′,5′,6′); 7.64 – 7.92 (m, 2H, H-6,7); 7.98-8.04 (m, 2H, H-5,8)
18	89	192 – 193	320	1670	1.59 (s, 3H, -$COCH_3$); 2.33 (s, 3H, 4′-CH_3); 6.84 (d, *J* = 8.3 Hz, 2H, H-3′,5′); 7.08 (d, *J* = 8.3 Hz, 2H, H-2′,6′); 7.61 – 7.84 (m, 2H, H-6,7); 8.03 – 8.14 (m, 2H, H-5,8)
19	88	199 – 202	336	1660	1.61 (s, 3H, -$COCH_3$); 2.79 (s, 3H, 4′-OCH_3); 6.74-6.99 (m, 4H, H-2′,3′,5′,6′); 7.58 – 7.76 (m, 2H, H-6,7); 8.02 – 8.13 (m, 2H, H-5,8)
20	83	169 – 170	324	1670	1.65 (s, 3H, -$COCH_3$); 6.85 – 6.99 (m, 4H, H-2′,3′,5′,6′); 7.60 – 7.73 (m, 2H, H-6,7); 7.91-8.07 (m, 2H, H-5,8)
21	85	184 – 185	340	1660	1.72 (s, 3H, -$COCH_3$); 6.86 (d, *J* = 8.8 Hz, 2H, H-2′,6′); 7.08 (d, *J* = 8.8 Hz, 2H, H-3′,5′); 7.62 – 7.87 (m, 2H, H-6,7); 8.02 – 8.09 (m, 2H, H-5,8)
22	83	207 – 209	320	1665	2.19 (s, 3H, -$COCH_3$); 4.63 (d, *J* = 5.8 Hz, 2H, -$NHCH_2$-); 7.26 – 7.31 (m, 5H, H-2′,3′,4′,5′,6′); 7.58 – 7.72 (m, 2H, H-6,7); 7.98-8.09 (2H, m, H-5,8)



2 - 4 5 - 22

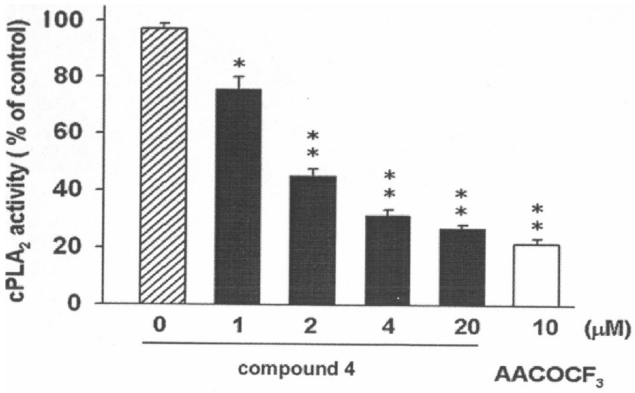
5,	R = $(CH_2)_2COOCH_3$	R' = phenyl
6,	R = $(CH_2)_2COOCH_3$	R' = *p*-CH_3-phenyl
7,	R = $(CH_2)_2COOCH_3$	R' = *p*-OCH_3-phenyl
8,	R = $(CH_2)_2COOCH_3$	R' = *p*-F-phenyl
9,	R = $(CH_2)_2COOCH_3$	R' = *p*-Cl-phenyl
10,	R = $(CH_2)_2COOCH_3$	R' = benzyl
11,	R = $(CH_2)_3COOCH_3$	R' = phenyl
12,	R = $(CH_2)_3COOCH_3$	R' = *p*-CH_3-phenyl
13,	R = $(CH_2)_3COOCH_3$	R' = *p*-OCH_3-phenyl
14,	R = $(CH_2)_3COOCH_3$	R' = *p*-F-phenyl
15,	R = $(CH_2)_3COOCH_3$	R' = *p*-Cl-phenyl
16,	R = $(CH_2)_3COOCH_3$	R' = benzyl
17,	R = CH_3	R' = phenyl
18,	R = CH_3	R' = *p*-CH_3-phenyl
19,	R = CH_3	R' = *p*-OCH_3-phenyl
20,	R = CH_3	R' = *p*-F-phenyl
21,	R = CH_3	R' = *p*-Cl-phenyl
22,	R = CH_3	R' = benzyl

Scheme 2. Synthesis of 2-alkylcarboxamido-3-arylamino-1,4-naphthoquinones **5**–**22**.

aggregation induced by thrombin, arachidonic acid (AA), collagen, and platelet-activating factor (PAF) as effectively as compound **2**.

Furthermore, compound **4** was about two times more potent than compound **2** in the inhibitory activity against platelet aggregation induced by AA. The antiplatelet activity of compound **2** decreased remarkably when the chloro group was replaced by substituted arylamino groups **5**–**10**. Compounds **11**–**16** and **17**–**22** derivatives of compound **3** and **4**, respectively, in which the chloro group was replaced by arylamino groups showed a poor inhibitory effect compared to the lead compound **2**. Among these compounds **11**–**22**, the IC_{50} value for compound **21** on platelet aggregation induced by AA was found to be 11.3 μM, which is about one-fourth less active than the lead compound **2** but more active than the positive control (acetyl salicylic acid, IC_{50} = 20.0 μM).

To sum up, the inhibitory activities of these synthetic 2,3-disubstituted 1,4-naphthoquinones proved to be more potent in the AA-induced platelet aggregation assay than other inducers. Furthermore, the antiplatelet activities of compounds **2**–**4** decreased extremely when the chloro groups were replaced by arylamino groups **5**–**22**. These results suggest that the chloro groups of compounds **2**–**4** play an important role in the antiplatelet activity, and the length of the amide chain did not affect the potency.



After the treatment of enzyme preparation with compound **4**, $AACOCF_3$, or DMSO (vehicle control) in the standard assay system at room temperature for 60 min, DTNB / EGTA reagent was added to the reaction mixture for 5 min to stop the enzyme catalysis and develop color formation. Data were calculated relative to the maximal activity from assay buffer-treated $cPLA_2$, which was taken as 100%, and are presented as the mean ± SEM (n =3). * P < 0.05 and ** P < 0.01 as compared with the vehicle control.

Figure 1. Effects of compound **4** on $cPLA_2$ activity in human platelets.

Compound **4** was selected for further studies of mechanism of action. The present study has shown that pretreatment of $cPLA_2$ (cytosolic phospholipase A_2) with compound **4** resulted in the concentration-dependent inhibition of $cPLA_2$ activity by 22.1 ± 0.6, 53.6 ± 0.3, 68.4 ± 0.1, and 72.2 ± 0.4% at concentrations of 1, 2, 4, and 20 mM, respectively, suggesting that compound **4** has a direct inhibitory action on $cPLA_2$ activity in platelets with an IC_{50} value of 1.8 ± 0.1 mM (Fig. 1). The physical and spectral data of 2,3-disubstituted 1,4-naphthoquinones are presented in Table 2.

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The authors have declared no conflict of interest.

Experimental

Chemistry

IR spectra were recorded on a Nicolet Impact 400 FT-IR spectrophotometer as KBr pellets (Nicolet, Madison, WI, USA). NMR spectra were obtained on a Bruker Avance DPX-200 FT-NMR (Bruker Bioscience, Billerica, MA, USA). MS were measured with HP 5995 GC-MS (Hewlett-Packard, USA) and VG PLATFORM II GC-MS (Varian, USA) instruments. Elemental analyses of C, H, and N were

carried out on a Perkin-Elmer 2400 Series II CHNS/O Analyser (Perkin-Elmer, Norwalk, CT, USA) and were accurate within ± 0.4% of theoretical values.

2-Anilino-3-methoxycarbonylethylcarboxamido-1,4-naphthoquinone ***5***

Aniline (3.7 g, 0.04 mol) was added to a suspension of 3-methoxycarbonylethylcarboxamido-2-chloro-1,4-naphthoquinone **2** (6.4 g, 0.02 mol) in benzene (100 mL). The reaction mixture was stirred for 30 min at room temperature, then filtered. The precipitate obtained was recrystallized from ethanol, giving **5** as dark red needles (Table 2).

2-(p-Toluidino)-3-methoxycarbonylethylcarboxamido-1,4-naphthoquinone ***6***

Compound **2** (6.4 g, 0.02 mol) was reacted with *p*-toluidine (4.3 g, 0.04 mol) as described for the preparation of **5** to afford **6** (Table 2).

2-(p-Anisidino)-3-methoxycarbonylethylcarboxamido-1,4-naphthoquinone ***7***

Compound **2** (6.4 g, 0.02 mol) was reacted with *p*-anisidine (4.9 g, 0.04 mol) as described for the preparation of **5** to afford **7** (Table 2).

2-(p-Fluoroanilino)-3-methoxycarbonylethylcarboxamido-1,4-naphthoquinone ***8***

Compound **2** (6.4 g, 0.02 mol) was reacted with *p*-fluoroaniline (4.4 g, 0.04 mol) as described for the preparation of **5** to afford **8** (Table 2).

2-(p-Chloroanilino)-3-methoxycarbonylethylcarboxamido-1,4-naphthoquinone ***9***

Compound **2** (6.4 g, 0.02 mol) was reacted with *p*-chloroaniline (5.1 g, 0.04 mol) as described for the preparation of **5** to afford **9** (Table 2).

2-Benzylamino-3-methoxycarbonylethylcarboxamido-1,4-naphthoquinone ***10***

Compound **2** (6.4 g, 0.02 mol) was reacted with benzylamine (3.7 g, 0.04 mol) as described for the preparation of **5** to afford **10** (Table 2).

2-anilino-3-methoxycarbonylpropylcarboxamido-1,4-naphthoquinone ***11***

Compound **3** (6.7 g, 0.02 mol) was reacted with aniline (3.7 g, 0.04 mol) as described for the preparation of **5** to afford **11** (Table 2).

2-(p-Toluidino)-3-methoxycarbonylpropylcarboxamido-1,4-naphthoquinone ***12***

Compound **3** (6.7 g, 0.02 mol) was reacted with *p*-toluidine (4.3 g, 0.04 mol) as described for the preparation of **5** to afford **12** (Table 2).

2-(p-Anisidino)-3-methoxycarbonylpropylcarboxamido-1,4-naphthoquinone ***13***

Compound **3** (6.7 g, 0.02 mol) was reacted with *p*-anisidine (4.9 g, 0.04 mol) as described for the preparation of **5** to afford **13** (Table 2).

2-(p-Fluoroanilino)-3-methoxycarbonylpropylcarboxamido-1,4-naphthoquinone ***14***

Compound **3** (6.7 g, 0.02 mol) was reacted with *p*-fluoroaniline (4.4 g, 0.04 mol) as described for the preparation of **5** to afford **14** (Table 2).

2-(p-Chloroanilino)-3-methoxycarbonylpropylcarboxamido-1,4-naphthoquinone ***15***

Compound **3** (6.7 g, 0.02 mol) was reacted with *p*-chloroaniline (5.1 g, 0.04 mol) as described for the preparation of **5** to afford **15** (Table 2).

2-Benzylamino-3-methoxycarbonylpropylcarboxamido-1,4-naphthoquinone ***16***

Compound **3** (6.7 g, 0.02 mol) was reacted with benzylamine (3.7 g, 0.04 mol) as described for the preparation of **5** to afford **16** (Table 2).

2-Acetamido-3-anilino-1,4-naphthoquinone ***17***

Compound **4** (5.0 g, 0.02 mol) was reacted with aniline (3.7 g, 0.04 mol) as described for the preparation of **5** to afford **17** (Table 2).

2-Acetamido-3-(p-toluidino)-1,4-naphthoquinone ***18***

Compound **4** (5.0 g, 0.02 mol) was reacted with *p*-toluidine (4.3 g, 0.04 mol) as described for the preparation of **5** to afford **18** (Table 2).

2-Acetamido-3-(p-anisidino)-1,4-naphthoquinone ***19***

Compound **4** (5.0 g, 0.02 mol) was reacted with *p*-anisidine (4.9 g, 0.04 mol) as described for the preparation of **5** to afford **19** (Table 2).

2-Acetamido-3-(p-fluoroanilino)-1,4-naphthoquinone ***20***

Compound **4** (5.0 g, 0.02 mol) was reacted with *p*-fluoroaniline (4.4 g, 0.04 mol) as described for the preparation of **5** to afford **20** (Table 2).

2-Acetamido-3-(p-chloroanilino)-1,4-naphthoquinone ***21***

Compound **4** (5.0 g, 0.02 mol) was reacted with *p*-chloroaniline (5.1 g, 0.04 mol) as described for the preparation of **5** to afford **21** (Table 2).

2-Acetamido-3-benzylamino-1,4-naphthoquinone ***22***

Compound **4** (5.0 g, 0.02 mol) was reacted with benzylamine (3.7 g, 0.04 mol) as described for the preparation of **5** to afford **22** (Table 2).

Biological assays

Evaluation of antiplatelet aggregation activity

Antiplatelet aggregation activity was determined as described previously [7, 8].

$cPLA_2$ activity assay

All procedures followed the manufacturer's instructions (Cayman Chemical). Briefly, the platelet suspension was resuspended in cold buffer (50 mM Hepes, pH = 7.4, containing 1 mM EDTA). Platelets were sonicated in ice and the lysate was centrifuged at 10 000 *g* for 15 min at 4°C. The lysate was centrifuged at 39 000 *g* for 20 min at 4°C. The supernatant was used as a source of $cPLA_2$. To avoid any residual measurement of secretory and Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) activities, the sample was processed by a membrane filter with a molecular weight cut-off of 30 000 Da and $iPLA_2$-specific inhibitor bromoenol lactone, prior to assaying. Compound **4**, $AACOCF_3$, or DMSO was incubated with the enzyme preparation in the standard assay system contained 80 mM Hepes (pH = 7.4), 150 mM NaCl, 10 mM $CaCl_2$, 4 mM Triton X-100, 30% glycerol, 1 mg/mL BSA, and 1.5 mM arachidonoyl thio-PC at room temperature for 60 min. Then, 10 µL of 25 mM 5,5′-dithiobis(2-dinitrobenzoic acid) (DTNB; Ellman's reagent) / 475 mM EGTA in 0.5 M Tris-HCl (pH = 8.0) was added to the reaction mixture for 5 min to stop enzyme catalysis and develop color formation. The absorbance at 405 nm was recorded in a microplate reader and determination of $cPLA_2$ activity was done according to the manufacturer's instructions.

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