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Original article

The first examples of ilexgenin A hybrids as a new class of multi-potent, anti-platelet agents

Li-Ping Lin [a,b], Fei-Hua Wu [a,*], Jing-Yu Liang [a,*]

[a] School of Traditional Chinese Pharmacy, China Pharmaceutical University, Nanjing 210009, China
[b] Jiangsu Center for Research & Development of Medicinal Plants, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing 210014, China

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ABSTRACT

Seventeen novel ilexgenin A hybrids (IA-aspirin) and (IA-NO), as donor hybrids (IA-NO will release NO *in vivo* and function as NO donor), were designed and synthesized in order to develop new multi-targeting agents for the treatment of platelet disorders. Their *in vitro* activities against ADP, AA and thrombin were evaluated. As a result, IA hybrids achieved substantial increases in the three tested pathways compared with IA. Encouragingly, the most potent hybrid compounds **6d** and **14d** displayed about 8-fold higher potency than aspirin, and 3-fold higher potency than the simultaneous administration of aspirin and IA in inhibiting ADP-induced aggregation with IC_{50} values of 0.15 mmol/L and 0.14 mmol/L, respectively. The results suggest these IA hybrids are good candidates for multi-target therapies, and especially, may be considered as promising ADP agonists.

1. Introduction

Cardiovascular disease caused by platelet-mediated thrombus formation is now a growing public health problem, which leads to an urgent need to develop more efficacious and less toxic agents for clinical use [1–4]. However, the difficulty in developing satisfactory therapies for this disorder lies in the complex pathophysiology of the disease, which includes activation of thromboxane A_2 (TXA_2) [arachidonic acid (AA) pathway], adenosine diphosphate (ADP), thrombin and even involves the inflammatory process. Thus, multi-target therapy seems to be an attractive preventive approach for platelet disorders [5,6]. Due to most of the natural compounds possessing multi-potent characteristics, many scientists have turned their interest to explore a wide variety of traditional herbal plants. As a consequence, we also have an interest in studying the plant, *Ilex pubescens* Hook. et Arn. (Aquifoliaceae), which is one of the well-known herbs widely used in China as a traditional Chinese medicine for the treatment of cardiovascular diseases. Ilexgenin A (IA) (**1**) is among the most abundant triterpenes in *I. pubescens* and has been determined to be one of its main antithrombotic ingredients (IC_{50} = 0.71 mmol/L against thrombin) [7]. We also assayed in this study for the potency of IA against ADP (IC_{50} = 1.02 mmol/L) and AA-induced (IC_{50} = 0.62 mmol/L) platelet aggregation. However, the relatively weak potency of IA may compromise its application in pharmacy industry. Therefore, we believe that our attempt in making IA hybrids to increase its effectiveness is meaningful.

On the other hand, numerous investigations in previous years, including preparation of aspirin hybrids [8] and nitric oxide-releasing derivatives [9], have been reported with some encouraging results in the inhibition of platelet aggregation. It is well-known that aspirin has been a first-line agent against cardiovascular death by preventing thrombotic events for a long time [10]. Aspirin inhibits platelet aggregation by irreversibly binding to cyclooxygenase and blocking the synthesis of thromboxane A_2 (AA pathway). Furthermore, NO also plays an important role in regulating the inhibition of platelet aggregation and thrombus formation in cerebrovascular and cardiovascular systems [11]. Herein, a series of novel IA hybrids were designed and synthesized by coupling aspirin, or NO, to the 24- and (or) the 28-position of IA through dibromoalkanes intermediates with differing carbon chain lengths. By combination of IA and aspirin, or NO, together to exert synergic action in antiplatelet activity, we hope to find new multi-potent, anti-platelet agents.

2. Experimental

Owing to the difficulty in designing aspirin ester prodrugs, the synthetic strategy was first to introduce the linker which was then used to connect the aspirin moiety. Thus, different dibromoalkanes (1,3-dibromopropane, 1,4-dibromobutane, 1,5-dibromopentane

* Corresponding authors.
E-mail addresses: fhwu2000@sina.com (F.-H. Wu), jyliang08@126.com (J.-Y. Liang).

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and 1,6-dibromohexane, respectively) were initially reacted with **1** to selectively generate major products 24,28-dibromoalkyl ilexgenin A **2a–d** (45%–81%), together with 28-bromoalkyl ilexgenin A **3a–d** as minor products (7%–12%). We synthesized compounds **2** and **3** as follows: To a stirred solution of compound **1** (502 mg, 1 mmol) in DMF (5 mL) at room temperature was added K_2CO_3 (1.105 g, 8 mmol) and stirred for a period of half an hour. Different dibromoalkanes (8 mmol) in DMF (2 mL) were then added dropwise over 10 min and stirring continued for a further 2 h. Subsequently, the click chemistry method was employed (Schemes 1 and 2). Firstly, azide-functionalized derivatives **4**, **5** and **11** were prepared through diazotization of **2**, **3** and **10** (0.58 mmol) with sodium azide (1.2 mmol) using DMF as solvent at 80–90 °C for 4 h. Then two alkyne-functionalized derivatives **9** and **12** were obtained by etherification of **1a** and **8** (5 mmol) with propargyl bromide (5 mmol) in the presence of K_2CO_3 (10 mmol) in DMF. Finally, compound **9** was converted to IA-aspirin hybrid **9b** (65%) through a reaction with azide **11**, whereas compound **12** was converted into **6a–d** (80%–85%), and **7a–d** (68%–72%) by reacting with azide **4** and **5**, respectively. The yield of **6e** was only 10%, however, as the by-product. In the process, a sample of azide (**4**, **5** or **11**, 0.47 mmol) was dissolved in CH_2Cl_2 (5 mL) at room temperature. Then the mixture of $CuSO_4{\cdot}5H_2O$ (0.61 mmol) and sodium ascorbate (1.22 mmol) in H_2O (2 mL) was added dropwise under an atmosphere of nitrogen. The reaction mixture was allowed to heat at reflux for 3 h.

Eight nitroxy-alkyl esters (**14**, **15**), on the other hand, were prepared by stirring the solution with corresponding Br-displacements **2** and **3** in the presence of $AgNO_3$. To a stirred solution of **2** (0.5 mmol) or **3** (0.5 mmol) in THF:CH_3CN (1:1, 5 mL) was added $AgNO_3$ (1.2 mmol) dropwise over 10 min. The mixture was heated at reflux for 3 h in dark flasks (Scheme 3).

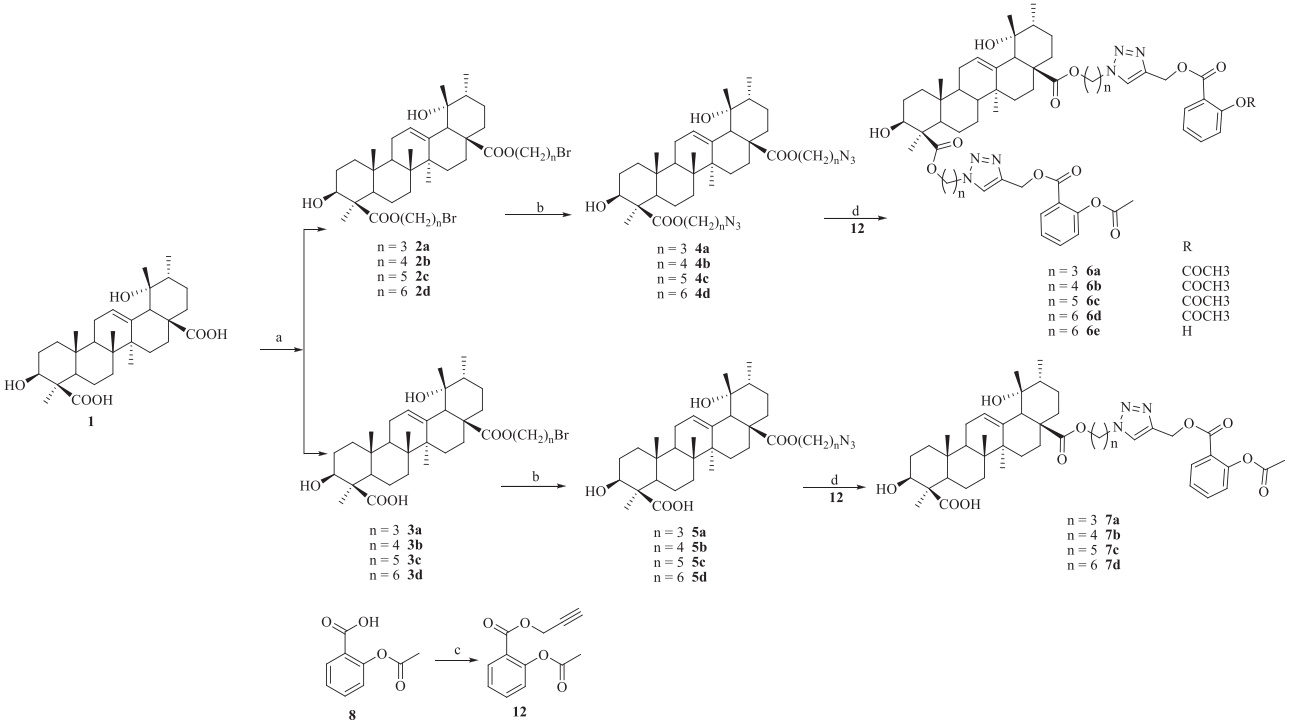
The resulting compounds were characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy. The experimental data of selected compounds were listed as follows.

Compound **6a**: ESI-MS (*m*/*z*): 1127.5 $[M+Na]^+$. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.97 (dd, 2H, *J* = 1.5, 8.0 Hz), 7.61 (s, 2H), 7.51 (ddd, 2H, *J* = 2.0, 8.0 Hz), 7.25 (ddd, 2H, *J* = 1.5, 7.5 Hz), 7.04 (dd, 2H, *J* = 1.0, 8.0 Hz), 5.38 (s, 4H), 5.30 (br s, 1H), 4.27–4.34 (m, 4H), 3.86–4.10 (m, 4H), 3.03 (dd, 1H, *J* = 4.5, 12 Hz), 2.54 (s, 1H), 2.22 (s, 6H), 2.11 (m, 4H), 1.35 (s, 3H), 1.22 (s, 3H), 1.16 (s, 3H), 0.90 (d, 3H, *J* = 7.0 Hz), 0.72 (s, 3H), 0.66 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.9, 177.2, 169.6 × 2, 164.4 × 2, 150.6 × 2, 142.8 × 2, 138.7, 134.0 × 2, 131.8 × 2, 128.3, 125.9 × 2, 124.0, 123.7 × 4, 123.0, 78.2, 73.9, 60.8 × 2, 60.4 × 2, 56.4, 54.2, 49.9, 47.8, 47.4, 47.3, 47.2, 42.2, 42.1, 39.7, 38.0, 37.6, 36.7, 32.7, 28.2, 27.3, 27.1 × 3, 26.9, 26.1, 24.4, 24.0, 20.9 × 2, 20.2, 17.2, 16.1 × 2, 13.2.

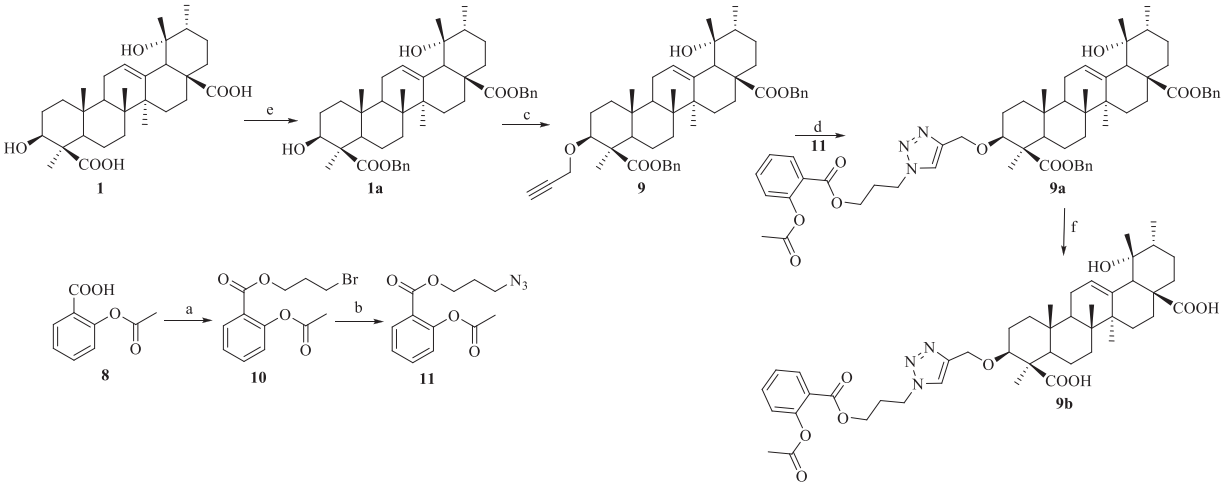
Compound **6b**: ESI-MS (*m*/*z*): 1155.7 $[M+Na]^+$. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.01, 8.00 (dd, 2H, *J* = 1.5, 8.0 Hz), 7.68 (s, 2H), 7.56, 7.55 (dd, 2H, *J* = 1.7, 8.0 Hz), 7.29 (dd, 2H, *J* = 1.1, 5.5 Hz), 7.09, 7.08 (d, 2H, *J* = 8.0 Hz), 5.31 (br s, 1H), 5.29 (s, 4H), 4.35 (m, 4H), 3.99–4.17 (m, 4H), 3.03 (dd, 1H, *J* = 4.5, 12 Hz), 2.26 (s, 6H), 1.55–1.98 (m, 8H), 1.37 (s, 3H), 1.26 (s, 3H), 1.20 (s, 3H), 0.94(d, 3H, *J* = 7.0 Hz), 0.76 (s, 3H), 0.69 (s, 3H).

Compound **6c**: ESI-MS (*m*/*z*): 1183.7 $[M+Na]^+$. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.00 (d, 2H, *J* = 8.0 Hz), 7.64 (s, 2H), 7.56 (d, 2H, *J* = 1.5, 7.5 Hz), 7.29 (tt, 2H, *J* = 1.5, 7.5 Hz), 7.08 (d, 2H, *J* = 8.0 Hz), 5.32 (br s, 1H), 5.29 (s, 4H), 4.36–4.40 (m, 4H), 3.92–4.13 (m, 4H), 3.01 (dd, 1H, *J* = 4.5, 12 Hz), 2.26 (s, 6H), 1.56–2.03 (m, 12H), 1.38 (s, 3H), 1.24 (s, 3H), 1.20 (s, 3H), 0.93 (d, 3H, *J* = 6.7 Hz), 0.74 (s, 3H), 0.67 (s, 3H).

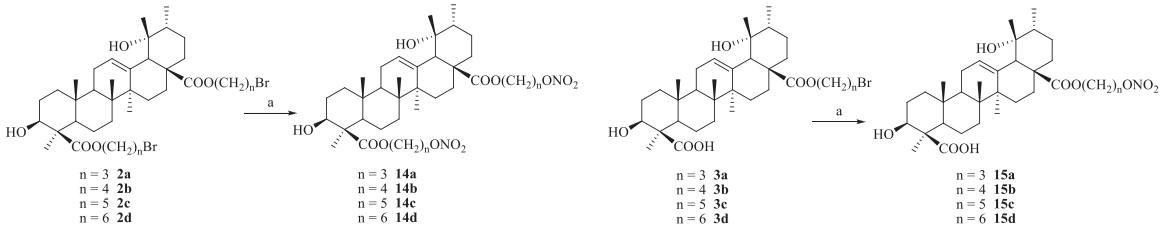
Compound **6d**: ESI-MS (*m*/*z*): 1211.7 $[M+Na]^+$; HR-MS ($[M+H]^+$): Calcd: 1189.6434, Found: 1189.6431. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.97 (dd, 2H, *J* = 1.5, 8.0 Hz), 7.61 × 2 (s, 2H), 7.52 (ddd, 2H, *J* = 2.0, 8.0 Hz), 7.25 (ddd, 2H, *J* = 1.0, 7.5 Hz), 7.04 (dd, 2H, *J* = 1.0, 8.0 Hz), 5.38 (s, 4H), 5.30 (br s, 1H), 4.27–4.33 (m, 4H), 3.86–4.10 (m, 4H), 3.03 (dd, 1H, *J* = 4.5, 12 Hz), 2.22 (s, 3H), 2.11 (s, 3H), 1.52–1.74 (m, 16H), 1.35 (s, 3H), 1.22 (s, 3H), 1.16 (s, 3H), 0.90 (d, 3H, *J* = 7.0 Hz), 0.72 (s, 3H), 0.66 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.8, 177.6, 169.5 × 2, 164.3 × 2, 150.5 × 2, 142.4 × 2, 138.0, 134.0 × 2, 131.8 × 2, 128.8, 125.9 × 2, 123.7 × 2, 123.6 × 2, 123.0 × 2, 78.2, 73.0, 63.9, 63.8, 58.1 × 2, 56.4, 53.2, 50.2, 50.1, 48.9, 47.8, 46.4, 41.2, 41.1, 39.7, 39.0, 37.3, 37.2, 30.7, 30.0 × 3, 28.2, 28.1, 28.0, 27.3, 26.0 × 2, 25.9, 25.4 × 3, 24.0, 23.7, 23.6, 20.8 × 2, 20.2, 16.6, 16.0, 13.2.



Scheme 1. The synthesis of 24- and (or) 28-substituted IA-aspirin hybrids. Reagents and conditions: (a) 1,3-dibromopropane, 1,4-dibromobutane, 1,5-dibromopentane and 1,6-dibromohexane, dry K_2CO_3, DMF, r.t.; (b) NaN_3, DMF, 80–90 °C; (c) propargyl bromide, dry K_2CO_3, DMF, r.t.; (d) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, CH_2Cl_2–H_2O, reflux.



Scheme 2. The synthesis of 3-substituted IA-aspirin hybrids. Reagents and conditions: (a) 1,3-dibromopropane, dry K_2CO_3, DMF, r.t.; (b) NaN_3, DMF, 80–90 °C; (c) propargyl bromide, dry K_2CO_3, DMF, r.t.; (d) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, CH_2Cl_2–H_2O, reflux; (e) BnCl, dry K_2CO_3, DMF, r.t.; (f) NaH, THF, 0 °C to reflux, 65% (total yield of 29%).



Scheme 3. The synthesis of 24- and (or) 28-substituted IA-NO hybrids. Reagents and conditions: (a) $AgNO_3$, avoid light, reflux, THF/CH_3CN, yield in 56%–68%.

Table 1
Antiplatelet aggregation activity of target compounds and intermediates *in vitro*.[a,c]

Compound	Inhibition rate (%) of AA (40 μmol/L)		Inhibition rate (%) of ADP (10 μmol/L)		Inhibition rate (%) of thrombin (5 U/mL)	
	0.1 mmol/L	0.25 mmol/L	0.1 mmol/L	0.25 mmol/L	0.1 mmol/L	0.25 mmol/L
2a	NA[b]	6.2 ± 2.0	4.8 ± 2.3	26.2 ± 2.5**	NA	NA
2b	2.2 ± 0.3	9.1 ± 2.4	1.2 ± 2.3	32.1 ± 4.4**	NA	0.9 ± 4.5
2c	4.7 ± 2.2	15.5 ± 4.3*	19.7 ± 3.2**	37.7 ± 8.5**	3.1 ± 3.2	10.3 ± 3.5
2d	4.0 ± 0.8	17.1 ± 3.2*	20.0 ± 0.8**	46.1 ± 4.4**	8.5 ± 3.2	11.4 ± 4.4
3a	NA	NA	NA	6.1 ± 2.0	NA	NA
3b	0.3 ± 0.1	2.1 ± 1.5	0.2 ± 1.1	12.1 ± 4.4	NA	NA
3c	1.7 ± 3.1	9.5 ± 2.1	9.7 ± 3.2	15.7 ± 5.0	1.1 ± 2.4	6.3 ± 2.8
3d	2.0 ± 0.3	14.1 ± 2.6*	13.0 ± 0.4*	24.1 ± 5.2**	4.5 ± 2.6	8.4 ± 3.0
4a	2.1 ± 1.8	4.4 ± 4.0	8.1 ± 2.8	14.4 ± 4.0**	NA	NA
4b	1.1 ± 0.8	8.0 ± 2.7	1.1 ± 0.8	8.0 ± 2.7	NA	3.7 ± 3.7
4c	3.9 ± 4.1	10.9 ± 1.5	13.9 ± 4.3*	35.9 ± 7.5**	7.4 ± 2.5	11.5 ± 2.9
4d	4.6 ± 2.3	11.0 ± 1.7	21.7 ± 2.3**	41.0 ± 12.7**	4.2 ± 2.6	6.0 ± 0.4
6a	13.9 ± 2.9**	21.3 ± 3.8**	13.9 ± 2.9*	41.3 ± 3.8**	15.2 ± 3.1**	20.1 ± 3.0**
6b	16.1 ± 2.7**	29.1 ± 2.2**	19.1 ± 4.7**	59.9 ± 2.2**	11.3 ± 4.7**	23.1 ± 2.7**
6c	16.1 ± 3.1**	30.9 ± 4.0**	18.3 ± 3.5**	69.9 ± 6.0**	10.5 ± 4.5*	31.7 ± 3.7**
6d	18.0 ± 2.3**	37.2 ± 2.1**	29.0 ± 5.3**	77.2 ± 12.5**	15.5 ± 2.5**	37.0 ± 2.1**
6e	NA	NA	NA	NA	NA	NA
7a	8.9 ± 2.4	16.3 ± 2.4**	10.9 ± 4.3*	34.3 ± 3.7**	11.3 ± 2.5*	18.1 ± 1.0*
7b	10.1 ± 1.5*	21.1 ± 3.4**	12.1 ± 4.5**	48.9 ± 4.2**	9.3 ± 2.5*	19.1 ± 1.4**
7c	12.1 ± 4.1**	25.9 ± 4.0**	13.3 ± 2.3**	56.9 ± 4.0**	9.5 ± 1.5*	26.7 ± 6.7**
7d	14.0 ± 2.3**	27.2 ± 2.1**	21.0 ± 2.3**	65.2 ± 6.5**	12.5 ± 2.5*	31.0 ± 2.1**
9b	NA	NA	NA	NA	NA	NA
14a	11.3 ± 0.8*	21.3 ± 7.0**	16.3 ± 0.6**	74.3 ± 10.2**	9.6 ± 3.2*	18.6 ± 3.8*
14b	15.2 ± 1.2**	24.6 ± 4.2**	21.2 ± 3.2**	54.8 ± 6.2**	8.1 ± 3.9*	20.0 ± 2.5**
14c	16.7 ± 2.3**	30.2 ± 5.1**	26.7 ± 1.3**	60.2 ± 6.6**	6.2 ± 3.3	20.8 ± 4.3**
14d	16.9 ± 3.0**	35.1 ± 2.3**	28.3 ± 2.0**	78.7 ± 10.3**	7.7 ± 0.6	22.3 ± 5.6*
1	2.1 ± 0.2	15.1 ± 2.3	NA	5.2 ± 1.8	6.7 ± 0.2	12.8 ± 2.9
Aspirin	25.1 ± 3.2**	44.1 ± 0.2**	NA	9.3 ± 2.4	NA	17.8 ± 0.9**
1 + aspirin	27.3 ± 0.6*	49.3.1 ± 5.0**	10.1 ± 0.1**	24.3 ± 0.2**	9.6 ± 3.0*	19.2 ± 1.8*

[a] Data are expressed as mean ± SD of three experiments. Aggregation rate of ADP was 54.7% ± 2.9%, AA was 56.2% ± 1.3% and thrombin was 58.6% ± 5.4%.
[b] NA: not active.
[c] Ref. [13].
* $p < 0.05$ *vs* vehicle.
** $p < 0.01$ *vs* vehicle.

Compound **14a**: ESI-MS (*m*/*z*): 709.4 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$): δ 5.35 (br s, 1H), 4.54 (m, 4H), 4.08–4.25 (m, 4H), 3.10 (dd, 1H, *J* = 4.1, 11.5 Hz), 2.51 (s, 1H), 1.40 (s, 3H), 1.25 (s, 3H), 1.20 (s, 3H), 0.97 (d, 3H, *J* = 5.6 Hz), 0.77 (s, 3H), 0.76 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.8, 177.2, 139.0, 128.3, 78.0, 74.1, 61.9 × 2, 61.4 × 2, 56.5, 54.2, 49.9, 48.2, 47.4, 42.7, 41.8, 39.9, 38.2, 37.2, 36.7, 32.5, 28.8, 27.3, 27.1, 26.9, 26.6 × 2, 26.4, 24.4, 23.5, 21.4, 17.3, 16.2, 15.2, 13.6.

Compound **14b**: ESI-MS (*m*/*z*): 737.4 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$): δ 5.34 (br s, 1H), 4.48 (m, 4H), 4.00–4.17 (m, 4H), 3.13 (dd, 1H, *J* = 4.2, 12.2 Hz), 2.52 (s, 1H), 1.56–1.87 (m, 8H), 1.40 (s, 3H), 1.27 (s, 3H), 1.21 (s, 3H), 0.94 (d, 3H, *J* = 6.8 Hz), 0.78 (s, 3H), 0.70 (s, 3H).

Compound **14c**: ESI-MS (*m*/*z*): 765.5 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$): δ 5.24 (br s, 1H), 4.46 (t, 4H, *J* = 6.2, 12.5 Hz), 4.10–4.19 (m, 4H), 3.10 (dd, 1H, *J* = 3.5, 11.2 Hz), 2.50 (s, 1H), 1.66–1.82 (m, 12H), 1.40 (s, 3H), 1.26 (s, 3H), 1.21 (s, 3H), 0.93 (d, 3H, *J* = 6.6 Hz), 0.77 (s, 3H), 0.71 (s, 3 H).

Compound **14d**: ESI-MS (*m*/*z*): 793.2 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$): δ 5.35 (br s, 1H), 4.48 (m, 4H), 4.00–4.17 (m, 4H), 3.09 (dd, 1H, *J* = 4.2, 12.0 Hz), 2.58 (s, 1H), 1.41 (3H, s), 1.27 (3H, s), 1.21 (3H, s), 0.94 (d, 3H, *J* = 6.6 Hz), 0.77 (s, 3H), 0.70 (s, 3H).

3. Results and discussion

Seventeen novel IA-aspirin and IA-NO donor hybrids were designed and synthesized. Both target hybrids and their intermediates were evaluated for the anti-platelet potency by Born's turbidimetric method [12] employing AA, ADP and thrombin as platelet aggregation inductors, respectively. Aspirin and IA were used as positive controls. The assay results are summarized in Table 1. Although the anti-AA activities were less than aspirin, the disubstituted target hybrid compounds (Inhibition rate for **6a**–**d** in 0.25 mmol/L ranged from 20.1% to 37.0% and for **14a**–**d** ranged from 18.6% to 22.3%), however, were more potent against thrombin than aspirin (17.8%). Furthermore, the inhibitory activities against ADP (10 μmol/L) of the target hybrids (inhibition rate for **6a**–**d** in 0.25 mmol/L ranged from 41.3% to 77.2% and for **14a**–**d** ranged from 54.8% to 78.7%) were significantly increased compared with IA (5.2%) and aspirin (9.3%), either alone or in combination. Encouragingly, the most potent compounds **6d** (77.2%) and **14d** (78.7%) displayed about an 8-fold higher potency than aspirin (9.3%), and 3-fold higher than the simultaneous administration (24.3%) of aspirin and IA with IC_{50} values of 0.15 mmol/L and 0.14 mmol/L, respectively. In addition, the potency of intermediates was far lower than the corresponding target hybrid compounds, which was consistent with our expectations that hybrids would exert synergy for anti-platelet activities.

The above results on anti-platelet activity of IA hybrids also, from the structure–activity relationship (SAR), prompted us to suspect that the anti-platelet effect was related to the carbon chain length and number of the substituents from 24 or (and) 28-COOH. The disubstituted hybrids were, *in vitro*, more potent antithrombotics than mono-substituted ones (*e.g.*, **2** *vs*. **3**, **6** *vs*. **7**). In addition, with a longer carbon-chain linker, IA disubstituted derivatives seem to show stronger inhibitory activities (*e.g.*, **6d** *vs*. **6a**–**c**, **14d** *vs*. **14b**–**c**). Comparison of IA-aspirin hybrids **9b** and **6a**–**d**, on the other hand, showed that the free 3-hydroxy seemed critical for the anti-platelet activity. It is also interesting to note that replacement of aspirin with salicylic acid, the by-product hybrid **6e** exhibited no inhibitory effect on platelet aggregation.

4. Conclusion

In summary, the IA hybrids demonstrated significant increases in anti-platelet activity both against thrombin and ADP compared with aspirin and IA. It is well-known that platelets can be activated by several physiological agonists, but the most important seems to be thrombin and ADP [14,15]. Therefore, the design and synthesis of IA hybrids to increase their potency against the two platelet aggregation inductors was a necessary and meaningful attempt for developing multi-target antiplatelet agents. Furthermore, to the best of our knowledge, this letter is among the first to report the synthesis and all-round biological evaluation of IA derivatives in AA, ADP and thrombin. Thus, the synthesis and evaluation of the anti-platelet effect of IA hybrids could be an innovative way to develop effective new anti-platelet agents. Finally, due to the key role ADP plays in hemostasis and thrombosis and the development of antiplatelet drugs targeting the $P2Y_{12}$ receptor, further investigation aimed at evaluating the activities of IA hybrids as a new class of $P2Y_{12}$ receptor antagonists is warranted.

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