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Pyridoxine as a template for the design of antiplatelet agents

Wenlian Zhang,[a] John Yao,[a] Vinh Pham,[a] Tara Whitney,[a] Doug Froese,[a] Albert D. Friesen,[b] Linda Stang,[c] Chen Xu,[d] Ashfaq Shuaib,[d] James M. Diakur[a] and Wasim Haque[a,*]

[a]*Canam Bioresearch Inc., 6-1200 Waverley Street, Winnipeg, MB, Canada R3T 0P4*
[b]*Medicure Inc., 6-1200 Waverley Street, Winnipeg, MB, Canada R3T 0P4*
[c]*Special Coagulation Laboratory, University of Alberta Hospital, Canada T6G 2B7*
[d]*Stroke Research Laboratory, Department of Medicine, University of Alberta, Canada T6G 2B7*

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Abstract—The B_6 vitamers have been shown to display beneficial therapeutic effects in cardiovascular related disorders. The design of novel antiplatelet agents using pyridoxine as a template has led to the discovery of a class of novel cardio- and cerebro-protective agents. The present study describes the synthesis of several of these derivatives along with the antiplatelet and antiischemic activity of derivative **16**.

The activation and aggregation of platelets are key events in the pathophysiology of thrombotic diseases.[1] Interestingly, these two processes also play roles in hemostasis.[2] In the context of a pathological role, platelet adhesion and aggregation at the site of atherosclerotic lesions in coronary arteries is a primary cause of cerebral ischemia, unstable angina, and myocardial infarction. These diseases continue to be major causes of morbidity and mortality, thus, there remains a need for antiplatelet drugs that are both efficacious and act rapidly. Thrombus formation proceeds by initial platelet binding to the site of vascular injury followed by platelet–platelet cohesion. Platelets lack a nucleus and compensate by storing an array of pre-synthesized molecules inside their granules. In addition, these anucleate cells express a number of receptor types that distinguish platelets from other types of cells. These receptors govern platelet reactivity, which can be initiated by a wide range of agonists and adhesive proteins. Some of the key receptor-mediated platelet aggregation events include activation by collagen, ADP, and thrombin receptor-activating peptide (TRAP) agonists.[3] Antiplatelet drugs have found clinical application in the secondary prevention of vascular events including acute myocardial infarction, stroke, and cardiovascular death.[4] Aspirin is the most widely used antiplatelet agent, and its mechanism of action involves irreversible inactivation of cyclooxygenase 1 for the lifespan of the platelet. The newer antiplatelet alternative clopidogrel, is an irreversible ADP receptor antagonist, and there is a recent trend toward combining two mechanistically different antiplatelet agents in an effort to reduce coronary events.[5] The co-enzyme form of vitamin B6, namely pyridoxal 5′-phosphate (PLP), has been reported to be a mild (mM) inhibitor of ADP-, epinephrine-, collagen-, arachidonic acid-, and thrombin-induced platelet aggregation in in vitro experiments.[6] In addition, benzamidine, pentamidine, and PLP were found to block platelet adhesion onto artificial surfaces.[7] However, results from in vivo studies on the antiplatelet effects of vitamin B6 and its vitamers in humans are still controversial.[8] For example, in human platelet rich plasma the inhibition of ADP- and collagen-induced platelet aggregation were dramatically attenuated in the presence of albumin, presumably as a result of Schiff base formation between PLP **1** (Fig. 1) and the active lysines of the serum protein. More recently, it has been shown that pyridoxal **2** and pyridoxine **3** can suppress GPIIb promoter activity.[9] These two vitamers were more efficient at reducing GPIIb expression than **1**, presumably due to their ability to more efficiently cross the cell

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* Corresponding author. Tel.: +1-204-487-2328; e-mail: whaque@canambioresearch.com

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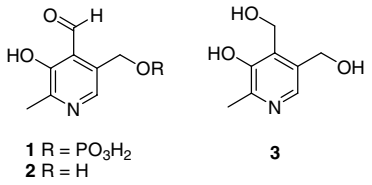
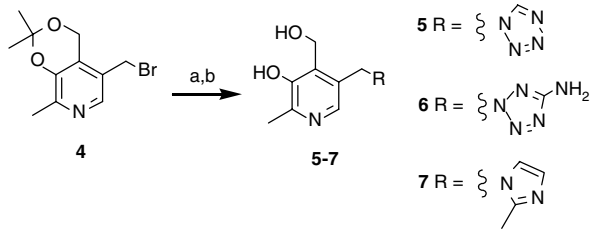


Figure 1.

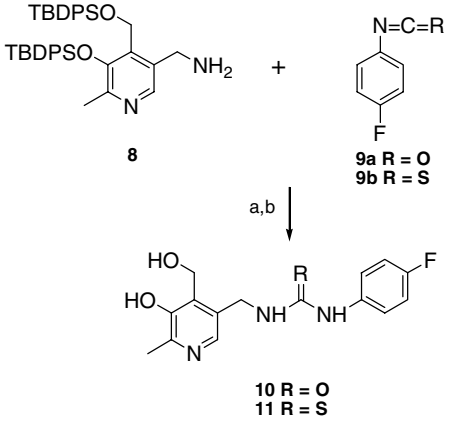
membrane. We postulated that since the B6 vitamer structures possess mild antiplatelet activity, this scaffold could serve as a basic template to design more potent platelet aggregation inhibitors. We selected the pyridoxine vitamer **3** as the template for further elaboration.

Modification of scaffold **3** was initiated by preparation of bromide **4**, which was obtained by following published protocol.[10] This intermediate was unstable, therefore, it was used without delay in the subsequent reaction. Addition of **4** to a stirred suspension of tetrazole and powdered potassium carbonate in acetonitrile at 0 °C gave the isopropylidene 5-terazole derivative in low yield,[11] as outlined in Scheme 1. Subsequent treatment with aqueous acetic acid at 80 °C gave the 5-tetrazole-pyridoxine **5**. This process was repeated with aminotetrazole and 2-methylimidazole to give compounds **6** and **7**, respectively.

The preparation of arylurea **10** and arylthiourea **11** was done according to the procedures shown in Scheme 2.



Scheme 1. Synthesis of 5-substituted pyridoxine derivatives. Reagents and conditions: (a) K_2CO_3, CH_3CN, 0 °C, tetrazole, 5-aminotetrazole or 2-methylimidazole, 30 min, (**5**: 26%, **6**: 19%, **7**: 44%); (b) HOAc/H_2O 4/1, 80 °C, 1 h.



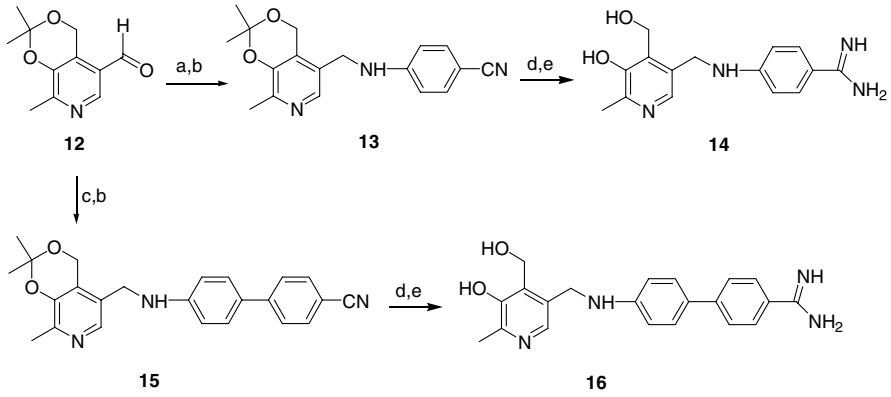
Scheme 2. Synthesis of arylurea and arylthiourea substituted pyridoxine analogs. Reagents and conditions: (a) Tol, reflux, **9a** or **9b**, 16 h, (with **9a**: 79%, with **9b**: 65%); (b) TBAF, THF, rt, 16 h, (**10**: 89%, **11**: 60%).

Thus, reaction of amino compound **8**[11] with either 4-fluorophenylisocyanate **9a** or 4-fluorophenylisothiocyanate **9b** under reflux in toluene gave the corresponding urea/thiourea derivative in reasonable yield. Subsequent removal of the silyl protecting groups was accomplished by treatment with tetrabutylammonium fluoride, yielding **10** and **11**, respectively. We also carried out the functionalization of **3** with benzamidine and biphenylamidine moieties as shown in Scheme 3. Starting from aldehyde **12**,[12] formation of the Schiff base by reaction with 4-aminobenzonitrile in the presence of a catalytic amount of 4-toluenesulfonic acid followed by reduction of the imine with sodium borohydride gave compound **13**. Subjection of this compound to the classical Pinner reaction[13] provided benzamidine **14**.[14] Repetition of this reaction sequence from **12** with 4-amino-4′-cyanobiphenyl proceeded via **15** to give the corresponding biphenylamidine **16**.[14] Compound **16** was treated with 1 equiv HCl to give the hydrochloride salt, which was then used for the subsequent in vitro biological and ex vivo studies.

For determination of the inhibition of platelet aggregation, platelet rich plasma (PRP) was obtained by drawing whole blood into sodium citrate tubes (3.2%) and centrifuging. Platelet poor plasma (PPP) was obtained by centrifuging the remainder of the sample until the platelets were removed. The PRP was adjusted to a count of 280×10^9/L with PPP. Incubations were done with 200 μL of platelets and 25 μL of the compound stock solution (4.5 mM), giving an approximate final platelet count of 250×10^9/L and compound concentration of 500 μM. This mixture was incubated for 30 min at rt, and the baseline transmittance measured with an aggregometer. Agonist (25 μL) was then added to achieve a final concentration of collagen (1 and 5 μg/mL), ADP (4 μM), and TRAP (12 μM). The percent reduction in platelet aggregation induced by the pyridoxine derivatives is shown in Table 1. Compound **16** appears to attenuate platelet aggregation by inhibiting ADP-induced events.

Ischemia-reperfusion injury is a root cause of a many of the important cardiovascular diseases including myocardial infarction and thrombotic stroke. Myocardial ischemia is a condition that occurs when the uptake of oxygen in the heart is below the level needed to maintain the rate of cellular oxidation.[15] Prolonged myocardial ischemia leads to significant tissue injury and ultimately myocyte necrosis. Myocardial infarct size is well established as an important predictor of cardiac function, and thus prognosis.[16]

Vitamer **1** has previously been reported to reduce damage induced by ischemia-reperfusion injury.[17] More recently, the poly(ADP-ribose) polymerase inhibitor HO-3089 has been reported to impede ADP-induced platelet aggregation as well as to exert protection in animal models of cardiac and brain ischemia-reperfusion injury.[18] We therefore investigated the ability of the amidine derivative **16** to reduce infarct size in the male Wistar rat model of left anterior descending coronary artery occlusion (25 min) and reperfusion (2 h) according



Scheme 3. Synthesis of amidine functionalized pyridoxines. Reagents and conditions: (a) 4-aminobenzonitrile, C_6H_6/cat *p*-TsOH, reflux, 18 h, (61%); (b) $NaBH_4$, HOAc, 4 °C–rt, 15 min, (93%); (c) 4-amino-4′-cyanobiphenyl, C_6H_6/cat *p*-TsOH, reflux, 18 h; (d) HCl/EtOH, 4 °C–rt, 16 h; (e) 2 M NH_3–MeOH, 80 °C, sealed tube, 2 h, (**14**: 82%; **16**: 63%).

Table 1. Percent reduction of platelet aggregation induced by agonists collagen, ADP, and TRAP

Compound	Concn (mM)	Collagen		ADP (4 μM)	TRAP (12 μM)
		5 (μg/mL)	1 (μg/mL)		
1	0.5	0	0	0	0
3	0.5	4	4	0	0
5	0.5	0	6	4	7
6	0.5	0	1	0	2
7	0.5	1	0	1	3
10	0.5	1	0	0	0
11	0.5	4	23	18	6
14	0.5	24	10	79	88
16	0.5	30	93	82	96
	0.25	1	41	63	93
	0.1	ND[a]	ND[a]	66	5

[a] ND = not done.

to previously published protocol.[19] A comparison of compounds **1** (positive control) and **16** (bolus injection of 0.1 and 0.07 mmol/kg, respectively) for their ability to reduce infarct size relative to saline control is shown in Table 2. Treatment of rats with the test compound for 5 min prior to ischemia led to a reduction in infarct size of 31% for compound **1** and 30% for compound **16**.

Currently, thrombolysis is the only approved treatment for stroke. Unfortunately, this approach can only be applied in less than 5% of patients presenting with an acute ischemic stroke. Thus, new drugs are urgently needed to complement thrombolysis in acute ischemic stroke.[20] We tested compound **16** in an embolic model of focal cerebral ischemia in rats. Focal ischemia was induced

Table 2. Ischemia-reperfusion study, %reduction in infarct size after bolus injection of **1** (0.1 mmol/kg) and **16** (0.07 mmol/kg)

Compound	AAR[a]	IS[b]	*n*	%Reduction in IS
Saline	52 (±3.1)	48 (±3.1)	10	
1	49 (±5.8)	34 (±2.8)[c]	10	31
16	46 (±2.7)	32 (±3.3)[c]	8	30

[a] Area at risk (AAR), standard error (SEM) is given in parentheses.
[b] Infarct size (IS) as %AAR.
[c] $P < 0.05$.

Table 3. Infarct volume reduction after treatment with compound **16**

Concentration of **16** (mg/kg)	Infarct volume	*n* (Number of animals)
0	40.0	10
10	30.5	10
20	26.5[a]	10
40	2.9[a]	10

[a] $P < 0.05$.

by embolizing a preformed clot into the middle cerebral artery.[21] 1 h After embolization, doses of 10, 20, or 40 mg/kg of **16** were intravenously infused over 60 min. Cerebral infarct volume[20] was significantly reduced when evaluated 48 h after embolization, compared to control as shown in Table 3.

Compound **16** was also evaluated in an ex vivo model of thrombogenesis[22] for its ability to inhibit platelet adhesion. This compound was administered to pigs at three doses (1, 10, and 30 mg/kg), and platelet adhesion was monitored in an ex vivo Plexiglas chamber by measuring the deposition of ^{111}In-labeled platelets as per established protocol.[23] Compound **16** reduced platelet adhesion by 66% ($n = 8$, mean and SEM, $P < 0.05$ vs baseline) at high shear rate (3397/s) only at the highest dose.[24]

ADP is a primary platelet agonist, and plays an important role in platelet aggregation. Platelet aggregation was measured in whole blood from pigs treated with 1, 10, and 30 mg/kg of **16** using ADP (40 μM) as the agonist. Impedance aggregometry measurements[25] revealed that even at the lowest administered dose of **16**, ADP-induced whole blood aggregation was completely abolished ($n = 4$, mean and SEM, $P < 0.05$ vs baseline).[24]

Our initial goal was to enhance the mild antiplatelet activity of the vitamin B6 scaffold by installing a pharmacologically active auxillary such as a phenyl- or biphenyl-amidine at the 5-position of this scaffold. The metabolite of this scaffold, P5P, is also known to possess beneficial antiischemic properties.[17] Modification of the

natural vitamer **3** with a biphenylamidine moiety has led to compound **16**. This modification has improved the vitamer scaffold in terms of its antiplatelet properties, while maintaining the scaffolds ability to attenuate ischemia-reperfusion induced damage. We were intrigued by observation that this compound retained two seemingly unrelated activities. Additional modification of this scaffold is currently underway in an effort to further improve its antiplatelet activity.

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14. Compound **14**: ^{1}H NMR (300 MHz, CD_3OD) δ 7.86 (1H, br s), 7.65 (2H, d, $J=8.9$ Hz), 6.79 (2H, d, $J=8.9$ Hz), 4.98 (2H, s), 4.47 (2H, s), 2.45 (3H, s). ES MS ($C_{15}H_{19}N_4O_2$), calcd for MH^+: 287.34; found: 287.29. Compound purity was determined by RP-HPLC (C18, Zorbax SB-C8, Agilent Technologies) using gradients of acetonitrile and 0.1% (v/v) TFA in water. The retention time for **14** was 10.5 min; purity: 95.3%. Compound **16**: ^{1}H NMR (300 MHz, CD_3OD) δ 7.90 (1H, br s), 7.84 (2H, d, $J=9.0$ Hz), 7.79 (2H, d, $J=9.0$ Hz), 7.56 (2H, d, $J=8.7$ Hz), 6.77 (2H, d, $J=8.7$ Hz), 4.99 (2H, s), 4.40 (2H, s), 2.44 (3H, s). ES MS ($C_{21}H_{23}N_4O_2$), calcd for MH^+: 363.43; found: 363.36. Compound purity was determined by RP-HPLC (C18, Zorbax SB-C8, Agilent Technologies) using gradients of methanol and 16.7 mM aqueous Na_2HPO_4, pH 3.0. The retention time for **16** was 14.3 min; purity: 99.3%.
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