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$P2Y_1$ receptor antagonists as novel antithrombotic agents

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Abstract—The $P2Y_1$ and $P2Y_{12}$ purinergic receptors are responsible for mediating adenosine diphosphate (ADP) dependent platelet aggregation. Evidence from $P2Y_1$ knockout studies as well as from nucleotide-based small molecule $P2Y_1$ antagonists has suggested that the antagonism of this receptor may offer a novel and effective method for the treatment of thrombotic disorders. Herein, we report the identification and optimization of a series of non-nucleotide $P2Y_1$ antagonists that are potent and orally bioavailable.

$P2Y_1$ and $P2Y_{12}$ are G protein-coupled receptors that mediate the adenosine diphosphate (ADP) dependent platelet aggregation response.[1] Activation of the $P2Y_1$ receptor results in a transient increase in intracellular calcium leading to platelet shape change and rapidly reversible aggregation while the activation of $P2Y_{12}$ receptor reduces cyclic adenosine monophosphate (cAMP) levels causing an amplification of the platelet response and stabilization of the resulting aggregates. Synergistic activation of both the $P2Y_1$ and $P2Y_{12}$ receptors is necessary for the full platelet aggregation response.

$P2Y_{12}$ receptor antagonism is a well-established strategy for antithrombotic therapy. The irreversible $P2Y_{12}$ antagonist clopidogrel (Plavix®) is in widespread clinical use for the treatment of peripheral artery disease and acute coronary syndrome as well as for the secondary prevention of ischemic stroke, vascular death, and myocardial infarction.[2] Moreover, several new $P2Y_{12}$ antagonists, including prasugrel and cangrelor (Fig. 1), are currently in late stage clinical trials.[3]

By contrast, antagonism of the $P2Y_1$ receptor as a potential antithrombotic therapy has only more recently been investigated. $P2Y_1$ deficient ($P2Y_1^{-/-}$) mice have been generated and platelets from these knockout animals were shown to be resistant to ADP-induced shape change and aggregation.[4] Furthermore, the $P2Y_1^{-/-}$ mice were protected, in vivo, against ADP or collagen-induced thromboembolism relative to the wild-type animals.[4] In addition, potent nucleotide-derived $P2Y_1$ antagonists have been reported such as MRS2179 (**1**, $K_d = 109$ nM) and MRS2500 (**2**, $K_i = 0.78$ nM) as shown in Figure 2.[5] These small molecule antagonists inhibit platelet aggregation and were efficacious in preclinical arterial thrombosis models. For example, Gachet and coworkers demonstrated that intravenous administration of MRS2500 (**2**) was effective at reducing arterial

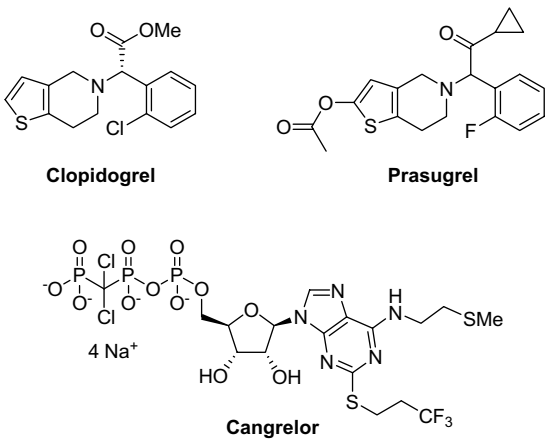

Figure 1. Structures of $P2Y_{12}$ receptor antagonists.

Keywords: P2Y1 receptor; GPCR antagonist; Antiplatelet; Antithrombotic; Cardiovascular disease.
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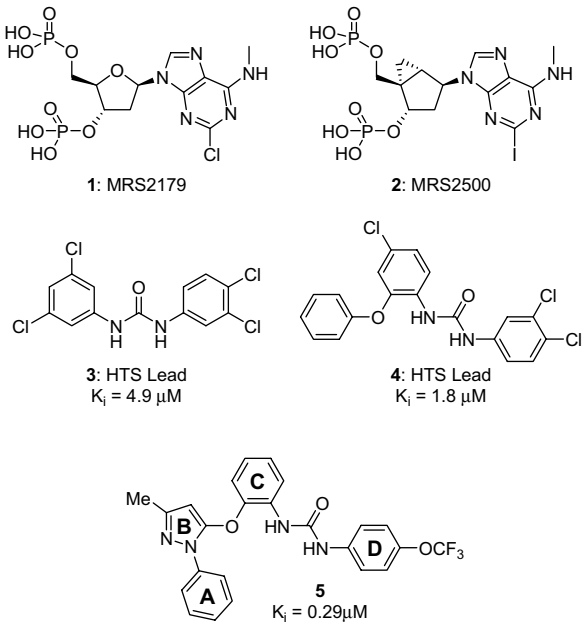
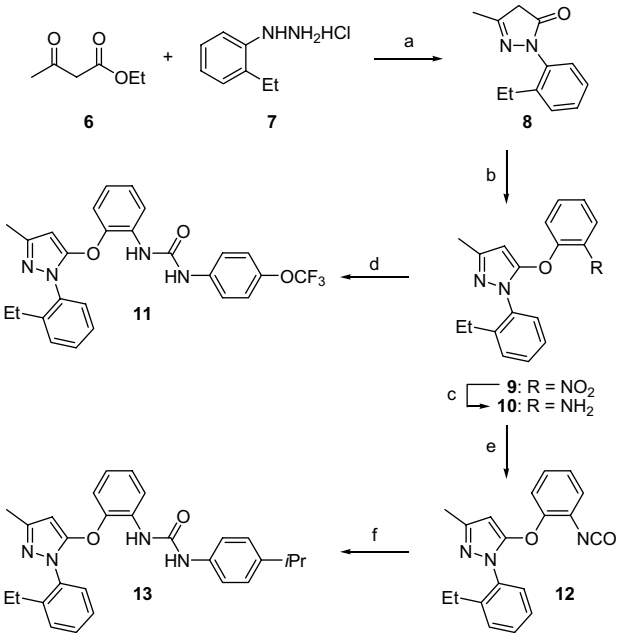


Figure 2. Structures and binding affinities of $P2Y_1$ receptor antagonists **1**–**5**.

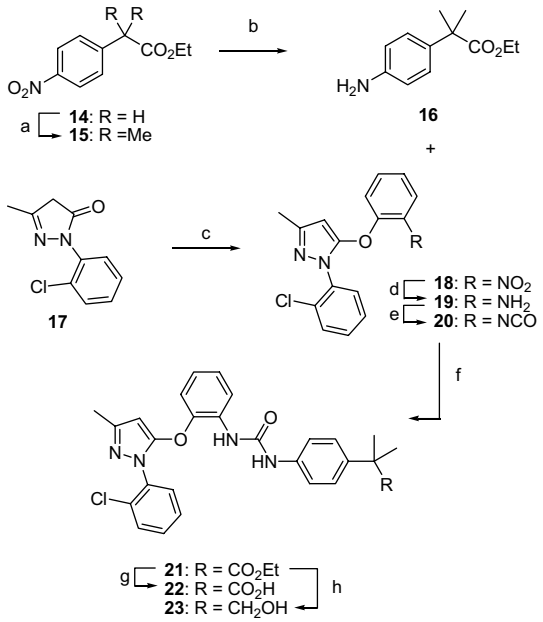
thrombosis in a laser-induced vessel wall injury mouse model.[6] In a related study, the co-administration of MRS2500 with the $P2Y_{12}$ antagonist clopidogrel in the same laser-induced vessel wall injury model revealed an additive effect of the two antagonists in preventing thromboembolism, suggesting the possible use of $P2Y_1$ and $P2Y_{12}$ antagonists as a combination treatment strategy.[6]

Given the promising evidence that $P2Y_1$ receptor antagonists may represent a novel antithrombotic strategy which might complement current therapy, we undertook a discovery effort aimed at identifying non-nucleotide, orally available $P2Y_1$ antagonists. As highlighted in Figure 2, high throughput screening (HTS) efforts against the human $P2Y_1$ receptor afforded a series of moderately potent diaryl urea hits represented by compounds **3** and **4**.[7] Preliminary lead development efforts identified aryloxypyrazole urea **5** as a suitable template for further structure activity studies. Herein, we describe these optimization efforts, which resulted in a series of potent and efficacious non-nucleotide $P2Y_1$ antagonists.

Schemes 1–5 outline the synthesis of representative $P2Y_1$ antagonists utilized in the current studies. As shown in Scheme 1, substituted 1-phenyl-3-methyl pyrazol-5-ones were prepared by the condensation of ethyl acetoacetate (**6**) and 2-ethyl phenyl hydrazine (**7**) in AcOH to afford pyrazolone **8** in good yield.[8] Reaction of **8** with 2-fluoronitrobenzene in the presence of K_2CO_3 at elevated temperature then provided aryloxypyrazole **9**. Reduction of the nitro functional group of **9** over Pd/C generated aniline **10**, which was treated with 4-trifluoromethoxyphenyl isocyanate to afford urea**11**. For analogs where the appropriately substituted phenyl isocyanate was not readily available, a complementary approach was developed as illustrated for the synthesis of compound **13**.

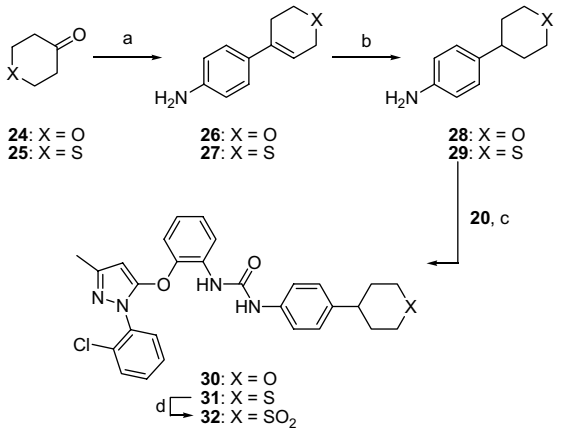


Scheme 1. Representative synthesis of ureas **11** and **13**. Reagents and conditions: (a) NaOAc, AcOH, 100 °C, 16 h, 73%; (b) 2-fluoronitrobenzene, K_2CO_3, DMF, 70 °C, 16 h, 18%; (c) Pd/C, H_2, MeOH, 25 °C, 5 h, 95%; (d) 4-(CF_3O)–C_6H_4NCO, Et_3N, THF, 60 °C, 17 h, 44%; (e) $ClC(O)OCCl_3$,1,8-bis(dimethylamino)naphthalene, CH_2Cl_2, 0 → 25 °C, 1 h, 84%; (f) 4-(*i*Pr)-$C_6H_4NH_2$, Et_3N, 60 °C, 17 h, 51%.

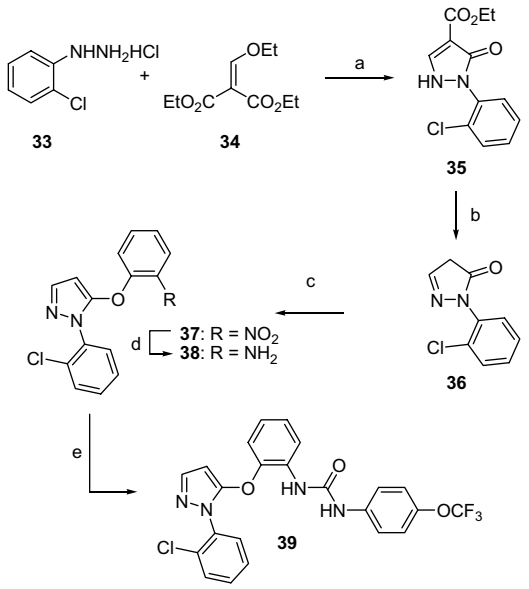


Scheme 2. Synthesis of $P2Y_1$ antagonists **22** and **23**. Reagents and conditions: (a) MeI, NaO*t*Bu, DMF, 0 °C, 1 h, 50%; (b) Pd/C, H_2, EtOH, 25 °C, 79%; (c) 2-fluoronitrobenzene, K_2CO_3, DMF, 70 °C, 28%; (d) Fe (dust), NH_4Cl, EtOH, 80 °C, 3 h, 74%; (e) $ClC(O)OCCl_3$, CH_2Cl_2, 0 → 25 °C, 1 h, 99%; (f) Et_3N, THF, 60 °C, 17 h, 68%; (g) LiOH (aq), MeOH:THF (1:1), 70 °C, 2 h, 40%; (h) $LiAlH_4$, THF, 0 → 25 °C, 3 h, 82%.

Hence, aniline **10** was treated with diphosgene and base to generate the corresponding isocyanate **12**, which was then reacted with 4-*iso*-propyl aniline to provide urea



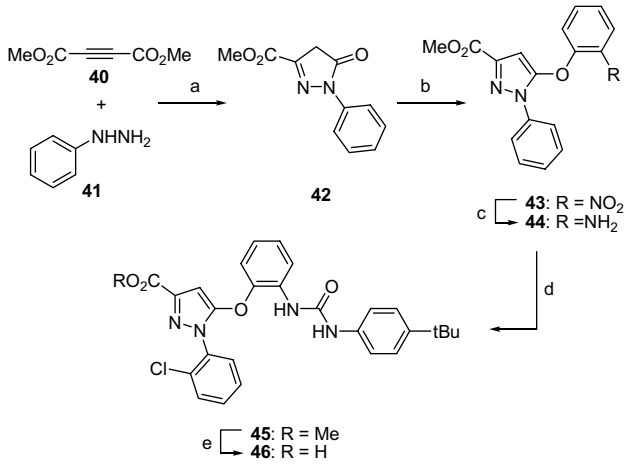
Scheme 3. Synthesis of $P2Y_1$ antagonists **30** and **32**. Reagents and conditions: (a) i—*N*-Boc-4-bromoaniline, *n*BuLi, THF, −78 → 25 °C, 6 h, 92% (X = S), 72% (X = O); ii—Et_3SiH, TFA, CH_2Cl_2, 25 °C, 1 h, 94% (X = S), 91% (X = O); (b) For X = O: Pd/C, H_2, MeOH, 4 h, 80%; For X = S: PtO_2, H_2, MeOH, 4 h, 89%; (c) **20** (Scheme 2), Et_3N, THF, 60 °C, 12 h, 91% (X = S), 88% (X = O); (d) OsO_4, NMO · H_2O, acetone: water (1:1), 25 °C, 4 h, 28%.



Scheme 4. Synthesis of $P2Y_1$ antagonist **39**. Reagents and conditions: (a) K_2CO_3, EtOH, 80 °C, 16 h, 75%; (b) i—NaOH, MeOH, 70°C, 5 h; ii—HCl (concd), 0 → 100 °C, 14 h, 49% (2 steps); (c) 2-fluoronitrobenzene, K_2CO_3, DMF, 70 °C, 16 h, 17%; (d) Fe (dust), NH_4Cl, EtOH, 80 °C, 3 h, 62%; (e) 4-(CF_3O)–C_6H_4NCO, Et_3N, THF, 65 °C, 16 h, 16%.

13. Together these two synthetic approaches provided efficient access to a variety of A-, B-, and D-ring substitutions through the use of alternative aryl hydrazines, phenyl isocyanates, anilines, and β-ketoesters.

Additional D-ring analogs **21**–**23**, bearing alcohol and carboxylic acid substituents, were prepared as outlined in Scheme 2. Initially, ethyl 2-(4-nitrophenyl)acetate (**14**, Scheme 2) was exhaustively methylated by treatment with iodomethane and KO*t*Bu to provide **15**, which was then hydrogenated over Pd/C to provide aniline **16**.[9] Its coupling partner, isocyanate **20**, was prepared from pyrazolone **17** as previously described in Scheme 1 with the exception that the nitro reduction was carried out using Fe/NH_4Cl to prevent the hydrogenolysis of the halogen substituent. Urea formation between **16** and **20** in the presence of triethylamine generated ester **21**, which was converted to carboxylic acid **22** and alcohol **23** via treatment with sodium hydroxide and lithium aluminum hydride, respectively.



Scheme 5. Synthesis of $P2Y_1$ antagonist **46**. Reagents and conditions: (a) i—MeOH, 25 °C, 18 h; ii—xylene, 130 °C, 2 h, 59% (over 2 steps); (b) 2-fluoronitrobenzene, K_2CO_3, DMSO, 75 °C, 16 h, 10%; (c) Pd/C, H_2, MeOH, 25 °C, 14 h, 57%; (d) 4-(*t*Bu)-C_6H_4NCO, Et_3N, THF, 65 °C, 16 h, 49%; (e) NaOH, MeOH:THF (1:1), 60 °C, 2 h, 56%.

Scheme 3 highlights the synthesis of D-ring analogs **30**–**32**, bearing cyclic heteroalkyl substituents. As shown, lithiation of *N*-Boc 4-bromoaniline followed by addition to tetrahydropyran-4-one (**24**) or tetrahydrothiopyran-4-one (**25**) resulted in the formation of intermediate tertiary alcohols that were treated with Et_3SiH and TFA to provide olefins **26** and **27** in good yield. Hydrogenation of these olefins gave anilines **28** and **29**, which were reacted with isocyanate **20** (from Scheme 2) to provide ureas **30** and **31**. Finally, the thioether of tetrahydrothiopyran **31** was oxidized to the corresponding sulfone **32** upon treatment with OsO_4 and 4-methylmorpholine N-oxide.

Schemes 4 and 5 illustrate the synthesis of analogs, such as **39** and **46**, with non-alkyl substitution on the pyrazole B-ring. The synthesis of **39** (Scheme 4) began with the condensation of 2-chlorophenyl hydrazine (**33**) and diethyl 2-(ethoxymethylene)malonate (**34**) to provide pyrazole-carboxylate ester **35**.[10] Treatment of **35** with NaOH followed by HCl resulted in decarboxylation to pyrazolone **36**, which was subsequently converted to urea **39** via the standard three-step sequence (nucleophilic aromatic substitution, reduction, and urea formation).

Analogs bearing a carboxylate functionality at the 3-position of the pyrazole B-ring such as **46** (Scheme 5) were prepared by the condensation of dimethyl acetylenedicarboxylate (**40**) with phenyl hydrazine (**41**) at elevated

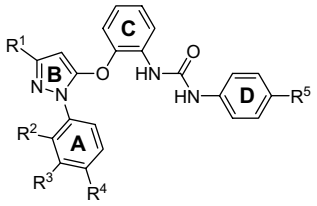
temperature to generate pyrazolone **42**, which was treated with K_2CO_3 and 2-fluoronitrobenzene to generate aryloxy pyrazole **43**.[11] Reduction of **43** to aniline **44** and treatment with 4-*tert*-butyl phenyl isocyanate provided **45**, which was then saponified with aqueous NaOH to generate the corresponding carboxylic acid **46**.

All analogs[12] were initially evaluated in a human $P2Y_1$ receptor binding assay (Tables 1–3) using [β-^{33}P]-2-thiomethyl adenosine diphosphate as the radioligand.[13] Selected analogs with promising binding affinities were then evaluated for the inhibition of agonist-induced platelet aggregation and P-selection expression (Table 4).[14]

Our SAR studies began with optimization of the A- and B-rings of the template as shown in Table 1. Evaluation of A-ring substitution revealed that the addition of a substituent at the 2-position (R^2) increased binding affinity relative to the unsubstituted case (i.e., compound **5**). Moreover, substitution at the 2-position (R^2) was preferable relative to substitution at the 3- or 4-positions (R^3 or R^4). This trend is highlighted by comparison of compounds **47** > **48** > **49** > **5**. Further evaluation of R^2-substituents (**11**, **50**–**54**) revealed that lipophilic alkyl or halogen substituents were preferred with the 2-Cl (**47**, K_i = 0.09 μM), 2-Et (**11**, K_i = 0.05 μM), and 2-*i*Pr (**50**, K_i = 0.06 μM) having the greatest binding affinities among the analogs evaluated. Limited examination of pyrazole B-ring substitution was also undertaken as illustrated in Table 1 for compounds **5**, **39**, **45**, **46**, **55** and **56**. Interestingly, the unsubstituted case (**39**, R^1 = H) and the compound bearing a methyl substituent (**5**, R^1 = Me) had reasonable activities, K_i = 0.27 and 0.29 μM, respectively; however, analogs bearing larger alkyl groups such as **55** (R^1 = Et) and **56** (R^1 = *i*Pr) were inactive. Attempts to introduce a polar functionality at the R^1-position were unsuccessful as illustrated by representative compounds **45** ($R^1 = CO_2Me$) and **46** ($R^1 = CO_2H$).

Subsequent SAR studies focused on the optimization of D-ring substitution while maintaining optimal substituents (R^2 = Et or Cl) on the A-ring as illustrated in Table

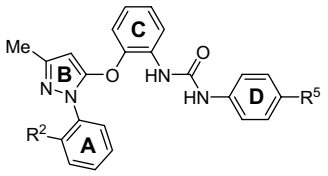
Table 1. P2Y1 receptor binding data for analogs **5**, **11**, **39**, and **45**–**56**

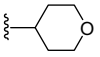
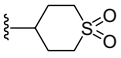
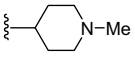
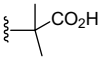
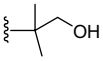


	R^1	R^2	R^3	R^4	R^5	$P2Y_1$ K_i [a] (μM)
5	Me	H	H	H	OCF_3	0.29 ± 0.03
47	Me	Cl	H	H	OCF_3	0.09 ± 0.02
48	Me	H	Cl	H	OCF_3	0.12 ± 0.02
49	Me	H	H	Cl	OCF_3	0.25 ± 0.03
11	Me	Et	H	H	OCF_3	0.05 ± 0.01
50	Me	*i*Pr	H	H	OCF_3	0.06 ± 0.01
51	Me	CF_3	H	H	OCF_3	0.13 ± 0.05
52	Me	Me	H	H	OCF_3	0.15 ± 0.04
53	Me	F	H	H	OCF_3	0.25 ± 0.07
54	Me	OMe	H	H	OCF_3	0.96 ± 0.05
39	H	H	H	H	OCF_3	0.27 ± 0.03
55	Et	H	H	H	OCF_3	>10
56	*i*Pr	H	H	H	OCF_3	>10
45	CO_2Me	H	H	H	*t*Bu	7.2 ± 0.75
46	CO_2H	H	H	H	*t*Bu	>10

[a] Assay values reported as geometric means ± SEM of n = 3 independent determinations.

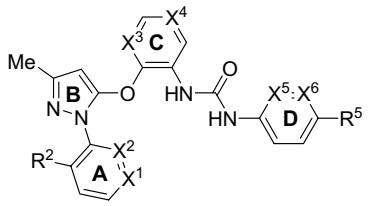
Table 2. $P2Y_1$ receptor binding data for analogs **11**, **13**, **22**, **23**, **31**, **32**, and **57**–**71**



	R^2	R^5	$P2Y_1$ K_i [a] (μM)
57	Et	H	0.70 ± 0.19
11	Et	OCF_3	0.05 ±0.01
58	Et	OMe	0.56 ± 0.07
59	Et	O*i*Pr	0.20 ± 0.03
60	Et	OPh	0.18 ± 0.04
61	Et	Cl	0.13 ± 0.04
62	H	F	2.7
13	Et	*i*Pr	0.02 ± 0.004
63	Cl	*t*Bu	0.10 ± 0.03
64	Et	CF_3	0.06 ± 0.008
65	Cl		0.12 ± 0.03
66	Cl		0.03 ± 0.01
67	Et		0.01 ± 0.003
68	Cl		0.03 ± 0.009
30	Cl		0.76 ± 0.23
32	Cl		>10
69	Cl		>10
70	Cl		1.2 ± 0.14
71	Cl		>10
22	Cl		>10
23	Cl		>10

[a] Assay values reported as geometric means ± SEM of n = 3 independent determinations.

Table 3. $P2Y_1$ receptor binding data for analogs **11** and **72–78**



	R^2	R^5	X^1	X^2	X^3	X^4	X^5	X^6	$P2Y_1$ K_i [a] (μM)
11	Et	OCF_3	CH	CH	CH	CH	CH	CH	0.05 ± 0.01
72	H	OCF_3	N	CH	CH	CH	CH	CH	>10
73	H	OCF_3	CH	N	CH	CH	CH	CH	>10
74	Et	OCF_3	CH	CH	N	CH	CH	CH	0.07 ± 0.02
75	Cl	OCF_3	CH	CH	N	CH	CH	CH	0.14 ± 0.03
76	H	OCF_3	CH	CH	CH	N	CH	CH	>10
77	Et	*i*Pr	CH	CH	CH	CH	N	CH	5.2 ± 0.4
78	Et	CF_3	CH	CH	CH	CH	CH	N	>10

[a] Assay values reported as geometric means ± SEM of $n = 3$ independent determinations.

Table 4. Inhibition of platelet aggregation and P-selectin expression for representative $P2Y_1$ antagonists

	$P2Y_1$ K_i (μM)[a]	Inhibition of platelet aggregation IC_{50} [b] (μM)	Inhibition of P-selectin expression IC_{50} [c] (μM)
11	0.05 ± 0.01	2.32 ± 0.69	0.40 ± 0.04
13	0.02 ± 0.004	6.22 ± 2.81	0.15 ± 0.05
47	0.09 ± 0.02	3.85 ± 0.87	0.58 ± 0.06
74	0.07 ± 0.02	4.11 ± 0 64	0.63 ± 0.04
75	0.14 ± 0.03	6.11 ± 2.10	0.88 ± 0.05

[a] Assay values reported as geometric means ± SEM for $n = 3$ independent determinations.

[b] Inhibition of 3 μM ADP-induced platelet aggregation using gel-filtered human platelets. IC_{50} values are reported as geometric means ± SEM for $n = 2$ independent determinations.

[c] Inhibition of 2 μM ADP-induced P-selectin expression on platelets from diluted whole blood. IC_{50} values are reported as geometric means ± SEM for $n = 2$ independent determinations.

2. Preliminary high speed parallel analoging efforts (data not shown) indicated that substitution at the para-position of the D-ring afforded optimal $P2Y_1$ binding potency. A variety of alkoxy substituents were evaluated (**11**, **58–60**) with compound **11** ($R^5 = OCF_3$, $K_i = 0.05$ μM) having the greatest $P2Y_1$ binding activity. Several alkyl substituents (**13**, **63** and **64**) were also evaluated with R^5 = *i*Pr (**13**, $K_i = 0.02$ μM) exhibiting optimal activity. Analogs bearing a cyclopropyl (**65**), cyclopentyl (**66**), or cyclohexyl (**67** and **68**) substituent at the R^5 position also had excellent affinity for the $P2Y_1$ receptor. Efforts to maintain the potency afforded by the cycloalkyl groups while introducing heteroatom functionality to modulate the physical properties of the template generally resulted in the loss of $P2Y_1$ potency as illustrated by **30** (R^5 = tetrahydropyran), **32** (R^5 = tetrahydro-oxo-thiopyran), and **69** (R^5 = *N*-methylpiperidine). Similarly, attempts to introduce alcohol or carboxylate functionality at the R^5 position was not tolerated as illustrated by analogs **22** and **23**. Finally, the limited installation of 5-membered heterocycles at the R^5 position (**70** and **71**) was also not successful.

Having identified preferred substituents for the A-, B-, and D-rings, we next investigated opportunities for the introduction of heteroatoms into the template as a way to modulate potency and/or physical properties. Specifically, we evaluated isomeric pyridyl replacements of the A-, C-, and D-rings as shown in Table 3. Whereas pyridyl A-rings (**72**, **73**) and pyridyl D-rings (**77** and **78**) were not tolerated, installation of a pyridyl nitrogen at the X^3 position of the C-ring was tolerated as evidenced by analogs **74** and **75**, which had comparable activities to their all-carbon counterparts.

Finally, while not shown, we also evaluated the addition of substituents to the C-ring; however, in all cases this resulted in a significant loss in binding activity. Similarly, efforts to modify the urea motif via *N*-alkylation of either nitrogen resulted in the complete loss $P2Y_1$ of binding affinity.

Together the preliminary SAR work outlined in Tables 1–3 afforded a collection of novel and potent, non-nucleotide, $P2Y_1$ antagonists. We subsequently selected a subset of these analogs for additional characterization to better evaluate the antithrombotic therapeutic potential of this series. First, the functional activity of selected antagonists (**11**, **13**, **47**, **74** and **75**, Table 4) was evaluated in platelet aggregation and P-selectin assays.[14] Utilizing gel-filtered human platelets, these analogs were shown to inhibit ADP-induced aggregation with micromolar potencies. These same analogs also exhibited submicromolar potencies for blocking ADP-induced P-selectin expression on human platelets thus offering evidence that the binding potency of this series of antagonists translated into functional activity. Additionally, to assess the selectivity of these $P2Y_1$ antagonists against other members of the P2Y receptor family, we evaluated analogs **11**, **47**, and **75** for binding to the $P2Y_2$ receptor, the member of the P2Y receptor family with the closest homology to $P2Y_1$. Encouragingly, **11**, **47**, and **75** were all found to have $K_i > 10$ μM for the $P2Y_2$ receptor.

Finally, to determine if the functional activity shown in Table 4 would translate into efficacy, we selected compound **47** for in vivo characterization since it offered a good balance between $P2Y_1$ potency ($K_i = 0.09$ μM) and in vitro ADME properties (human liver microsome $T_{1/2} = 42$ min, kinetic solubility = 3.5 μM, moderate Caco-2 permeability). Initial evaluation of the rat pharmacokinetic properties of **47** (Table 5) revealed the compound to be orally bioavailable ($F = 59\%$) with high volume ($V_{dss} = 15.7$ L/kg) and clearance (Cl = 81 mg/kg/mL) leading to a modest half-life ($T_{1/2} = 2.8$ h). Subsequently, the antithrombotic efficacy of analog **47** was assessed in a rat arterial injury model.[15] As highlighted in Table 6, intravenous administration of **47**

Table 5. Pharmacokinetic parameters for compound **47**

	%*F*	$T_{1/2}$ (h)	V_{dss} (mL/kg)	Cl (mL/min/kg)
47	59	2.8	15.7	81

Pharmacokinetic parameters expressed as geometric mean ($n = 2$) of Sprague–Dawley rats dosed 3 mg/kg i.v. and 10 mg/kg p.o.

Table 6. Effect of compound **47** on time to the formation of occlusive thrombus in rat ferric chloride injury model of arterial thrombosis

	IV dose			Occlusion incidence	Time to occlusion (min)
	Bolus (mg/kg)	+	Infusion (μg/kg/min)		
Vehicle	—		—	2/2	18
47	0.4		16	3/5	30
47	0.8		33	3/6	35
47	1.6		65	1/5	50

(bolus + infusion) resulted in a dose dependent decrease in both the incidence of occlusive thrombus formation and the average time to occlusion.

We have described the discovery and optimization of a series of $P2Y_1$ antagonists. These efforts resulted in the identification of analogs with nanomolar affinity for the $P2Y_1$ receptor and micromolar potency for inhibiting platelet aggregation. Evaluation of a representative analog in a rat ferric chloride arterial thrombosis model revealed it to be effective at preventing thrombus formation suggesting that the current series of antagonists may have potential as a novel antithrombotic therapy.

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