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Thienopyrimidine-based $P2Y_{12}$ platelet aggregation inhibitors

Steven W. Kortum *, Rhonda M. Lachance, Barbara A. Schweitzer †, Gopichand Yalamanchili ‡, Hayat Rahman ‡, Michael D. Ennis, Rita M. Huff, Ruth E. TenBrink

Pfizer Global Research and Development, Pfizer Inc., St. Louis Laboratories, 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA

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ABSTRACT

Herein we describe the design and synthesis of a novel series of potent thienopyrimidine $P2Y_{12}$ inhibitors and the negative impact protein binding has on the inhibition of platelet aggregation.

The success of Plavix® (clopidogrel) in inhibiting platelet aggregation and the subsequent discovery of the $P2Y_{12}$ receptor as the mechanism of action validates the inhibition of the $P2Y_{12}$ receptor as a viable strategy for platelet aggregation inhibition.[1,2] Whereas clopidogrel is an irreversible inhibitor of the $P2Y_{12}$ receptor, we were interested in developing a reversible inhibitor.[3] AZD-6140, an orally active $P2Y_{12}$ reversible inhibitor in clinical evaluation for acute coronary syndrome (ACS), shown in Figure 1 has a total of six chiral centers (four contiguous).[4] One of our goals was to develop a less complex adenosine diphosphate (ADP)-stimulated $P2Y_{12}$ antagonist while retaining the hydrophilic and hydrophobic regions as in AZD6140.[5]

We, along with others, investigated the thienopyrimidine core as a potential candidate for platelet inhibition.[6] Our efforts were concentrated on the central theme of a hydrophobic northern region with a hydrophilic southern region as exemplified by compound **21k**.

The synthesis of the chloro intermediates **5** and **7** are outlined in Scheme 1. Butyraldehyde **1** and methyl cyanoacetate **2** were combined in the presence of elemental sulfur and triethylamine in the classic Gewald synthesis to give aminothiophene **3** in 70% yield.[7,8]

Aminothiophene **3** reacted with potassium cyanate in acetic acid at room temperature for 18 h to give thienopyrimidinedione **4** in 65% yield. Thienopyrimidinedione **4** was converted to the 4,6-dichlorothienopyrimidine 5 using phenylphosphonic dichloride at 150 °C and then quenched in ice water to give the desired product in >95% yield. At atmospheric pressure, thienopyrimidinone **4** and $POCl_3$ with catalytic *N*,*N*-dimethylformamide (DMF) gave yields ranging from 0% to 30%. Use of a sealed tube (150 °C) gave improved yields (80–95%), but for larger scale reactions, phenylphosphonic dichloride in place of phosphorus oxychloride allowed the elimination of sealed pressure vessels while maintaining high yields of 4,6-dichlorothienopyrimidine **5**. Thiophene **3** is reacted with formamide at 130 °C for 12 h to give thienopyrimidinone **6** in 75% yield. Conversion of thienopyrimidinone **6** to thienopyrimidine **7** was accomplished using thionyl chloride and DMF at 80 °C in 86% yield.

Scheme 2 outlines the synthesis of the C-6 hydrogen analogs. Displacement of the C-4 chloro group of **7** with boc-piperazine **8** was accomplished at room temperature in the presence of diisopropylethylamine (DIEA) to give thienopyrimidine **9** in 70–90% yield. For exploration of the substituents at C-6, the BOC group of

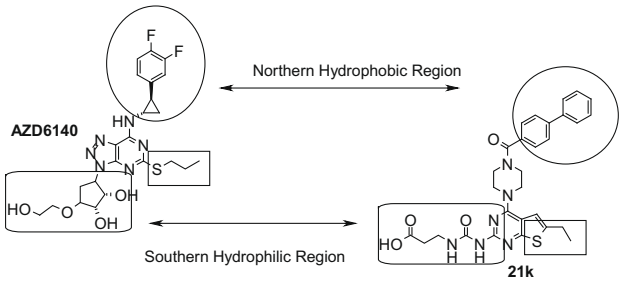

Figure 1. Comparison of AZD6140 to the thienopyrimidine compound **21k** highlighting the hydrophilic and hydrophobic regions of each molecule.

* Corresponding author. Tel.: +1 636 247 3592.
E-mail address: steve.kortum@pfizer.com (S.W. Kortum).
† Present address: Monsanto Corp., St. Louis, MO, USA.
‡ Retired.

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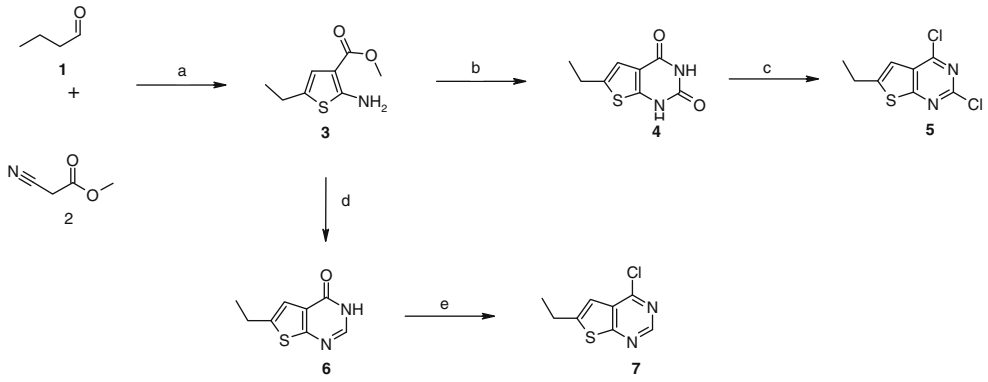
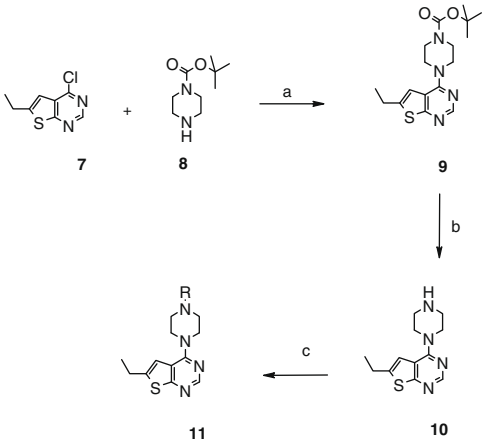
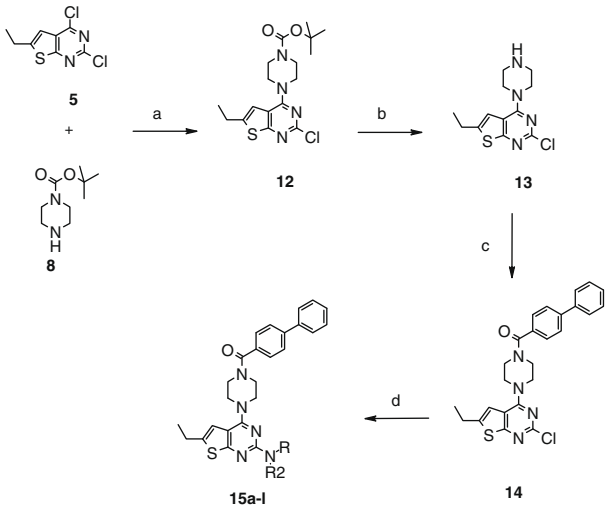


Scheme 1. Synthesis of the thienopyrimidine cores **5** and **7**. Reagents and conditions: (a) sulfur, triethylamine, DMF, rt, 18 h (70%); (b) acetic acid, H_2O, KOCN, rt, 18 h (64%); (c) phenylphosphonic dichloride, 150 °C, 3 h (95%); (d) formamide, ammonium formate, 135 °C, 12 h (75%); (e) thionyl chloride, DMF, 80 °C, 3 h (86%).



Scheme 2. Synthesis of C-6 hydrogen-substituted thienopyrimidine analogs **11a–h**. Reagents and conditions: (a) THF, DIEA, rt, 6 h (70–90%); (b) hydrochloric acid, methanol, rt, 3 h (quant); (c) DMF, DIEA, rt, 18 h (30–90%).



Scheme 3. Synthesis of the C-6 amino-substituted thienopyrimidine analogs **15a–l**. Reagents and conditions: (a) THF, DIEA, rt, 6 h (70–90%); (b) hydrochloric acid, methanol, rt, 3 h (quant); (c) DMF, DIEA, rt, 1 h (94%); (d) DIEA, NMP, 130 °C, 18 h (40–90%).

thienopyrimidine **9** was removed using HCl in methanol to give thienopyrimidine **10** in quantitative yield. Thienopyrimidine **10** was either acylated with the appropriate acid chloride using DIEA as base at room temperature to give thienopyrimidines **11a–h** in 30–60% yield or coupled with the appropriate acid using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) in comparable yields.

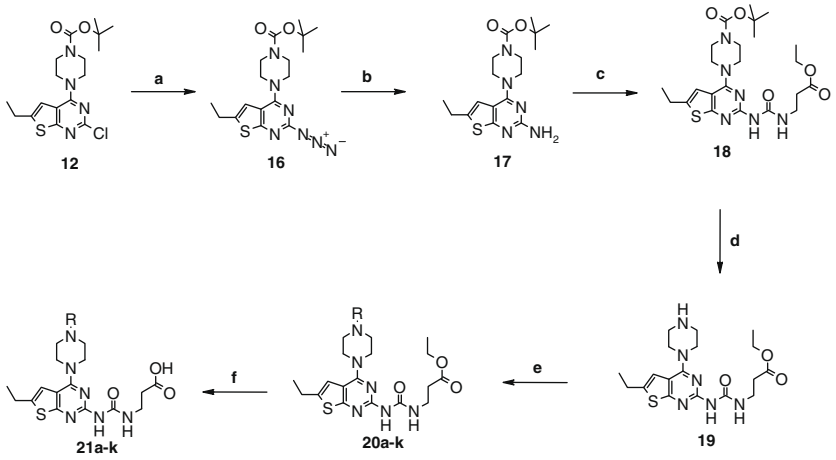
Scheme 3 outlines the synthesis of the C-6 nitrogen analogs. BOC deprotection of **12** and acylation of piperazine **13** were accomplished using the same procedures in similar yields to those of the C-6 hydrogen analogs. The C-6 chloro group of **14** was displaced using an appropriate amine with DIEA in 1-methyl-2-pyrrolidinone (NMP) at 105 °C to give the corresponding thienopyrimidines 15a–l in 40–90% yield.

Scheme 4 outlines the synthesis of urea analogs **21a–k**. The C-6 chloro group of **12** is displaced with sodium azide in NMP at 130 °C to give azide **16** in 77% yield. Azide **16** was reduced using trimethylphosphine in tetrahydrofuran (THF) to give amine **17** in 85% yield. Amine **17** was heated in pyridine with ethyl-3-isocyanatopropionate at 80 °C to give urea **18** (81%) followed by BOC deprotection to give **19** in quantitative yield. Acylation of **19** to give the intermediate esters **20a–k** in 30–60% yield and subsequent hydrolysis was accomplished in 50–90% yield using lithium hydroxide to give thienopyrimidines **21a–k**.

The $P2Y_{12}$ binding assay used for this study uses recombinant human $P2Y_{12}$ transfected Chinese Hamster Ovary (CHO) cell membranes.[9] The $P2Y_{12}$ binding assay with added protein, human serum albumin (HSA) and alpha-1 acid glycoprotein (AGP), was used to give a readout on the protein binding of our inhibitors before going into the human platelet rich plasma (hPRP) aggregation functional assay.[10,11] A large portion of the discrepancies between the $P2Y_{12}$ binding and functional assays, nM versus μM, are likely due to the increased amount of protein in the hPRP aggregation assay as compared with the $P2Y_{12}$ binding assay.

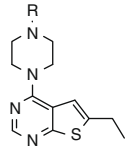
In keeping with the hydrophobic nature of the northern substituent we first looked at several hydrophobic substituents on the piperazine ring while keeping C-6 as H (Table 1). Biphenyl **11c** was the most active compound in the $P2Y_{12}$ binding assay, followed by naphthyl carbamate **11g**, indicating that larger, hydrophobic groups were preferred. All of the compounds lacking a C-6 substituent displayed poor activity in the hPRP aggregation assay.

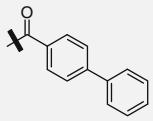
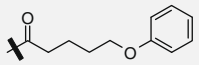
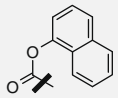
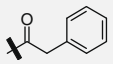
Table 2 lists analogs containing C-6 nitrogen based substituents with the 4-biphenylacetyl group as the northern piperazine substituent. Substituents with carbonyl groups directly attached to (**21k**) or one atom removed from (**15h** and **15k**) the C-6 nitrogen were the most active in both the $P2Y_{12}$ binding and hPRP aggregation assays. Moving the carbonyl even further away from the C-6 nitrogen, as in **15c** and **15g,** resulted in a 10–15-fold decrease in



Scheme 4. Synthesis of the C-6 urea-substituted analogs **21a–k**. Reagents and conditions: (a) NMP, H_2O, sodium azide, 130 °C, 18 h (77%); (b) THF, trimethylphosphine, rt, 20 h (85%); (c) pyridine, ethyl-3-isocyanatopropionate, 80 °C, 18 h (81%); (d) HCl, ethanol, rt, 1 h (quant); (e) DMF, DIEA, rt, 18 h (30–90%); (f) THF, H_2O, lithium hydroxide, rt, 6 h (50–90%).

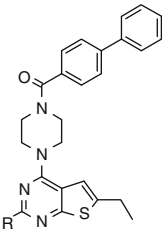
Table 1
Table of hPRP IC_{50}, %inhibition, and $P2Y_{12}$ K_i values for the C-6 hydrogen analogs

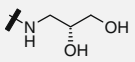
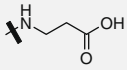
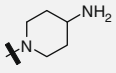
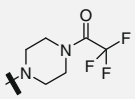
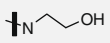
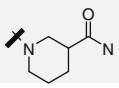


Compd	R	$P2Y_{12}$ K_i (nM)	hPRP agg. IC_{50} (μM)or %inh.[a,b]
11a		2990	28
11b		4400	0
11c		29	7
11d		2100	8
11e		NT	18
11f		NT	4
11g		130	1.25
11h		660	28

[a] %Inhibition values at 50 μM concentration (NT = not tested).
[b] Human platelet rich plasma (hPRP), see Ref. 11.

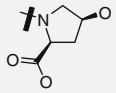
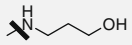
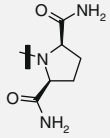
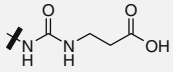
Table 2
Table of hPRP IC_{50} and $P2Y_{12}$ K_i values for the northern biphenyl analogs



Compd	R	$P2Y_{12}$ K_i (nM)	$P2Y_{12}$ + protein K_i[b] (nM)	hPRP agg. IC_{50}[a] (μM)
15a		19	75 (4X)	11
15b		35	241 (7X)	37
15c		8	122 (15X)	57
15d		240	325 (1.3X)	40
15e		839	1810 (2X)	>100
15f		37	163 (4.4X)	21
15g		31	203 (6.5X)	73

(*continued on next page*)

Table 2 (*continued*)

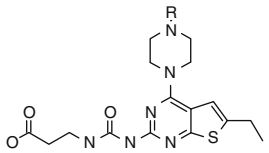
Compd	R	$P2Y_{12}$ K_i (nM)	$P2Y_{12}$ + protein K_i[b] (nM)	hPRP agg. IC_{50}[a] (μM)
15h		5	31 (6X)	4
15i		27	297 (11X)	30
15j		78	441 (5.6X)	56
15k		4	21 (5X)	3
15l		410	397 (1X)	45
21k		3	10 (3X)	5

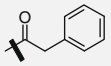
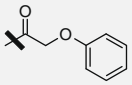
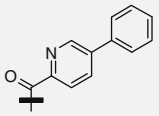
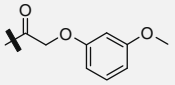
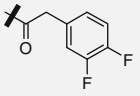
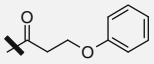
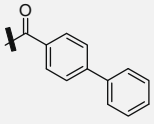
[a] Human platelet rich plasma (hPRP), see Ref. 11.
[b] Value in parenthesis is the fold shift between binding with and binding without added protein.

potency in the hPRP aggregation assay and a 2–10-fold decrease in $P2Y_{12}$ affinity. Alcohols **15a**, **15b**, **15f**, **15i**, and **15j** were 4–12-fold less potent in the $P2Y_{12}$ binding assay and 2–11-fold less potent in the hPRP aggregation assay than **21k**. For amines **15d** and **15l,** the loss in affinity was on the order of 40–80-fold in the $P2Y_{12}$ binding assay and ~8-fold less potent in the hPRP aggregation assay as compared with **21k**. Hydrophobic analogs such as trifluoroacetyl **15e** were more than 150-fold less potent in the $P2Y_{12}$ binding assay and more than 20-fold less potent in the hPRP aggregation assay than **21k**. This loss of affinity for C-6 hydrophobic substituents demonstrates the preference for hydrophilic substituents in the southern region. In general, analogs which were potent in the $P2Y_{12}$ binding assay and displayed a smaller, less than 10-fold, shift from the $P2Y_{12}$ binding assay with added protein were among the more potent analogs in the hPRP aggregation assay. Potent analogs such as **15c** (8 nM) with a 15-fold shift in potency with added protein (122 nM) were not as active in the hPRP aggregation assay as compounds such as **15h** which only had a sixfold shift upon addition of protein. Thus the right shift in affinity observed on going from no protein added to added protein in the binding assay was adequate to predict loss of potency in the hPRP aggregation assay due to protein binding.

Table 3 lists some of the analogs made where the C-6 urea was held constant while varying the northern piperazine substituent. Since most of the analogs listed in Table 1 were inactive in the hPRP aggregation assay, the C-6 urea was subsequently used to help evaluate the SAR around the northern piperazine substituent. The comparator for this series of compounds is the 4-biphenylacetyl analog **21k** (Table 2). Compounds such as **21c**, **21e**, and **21j** which have a phenyl ring appended to an alkyl chain via an ether linkage resulted in a 60–180-fold decrease in $P2Y_{12}$ affinity with only a three to sixfold decrease in hPRP aggregation affinity. Small,

Table 3
Table of hPRP IC_{50} and $P2Y_{12}$ K_i values for the southern urea analogs



Compd	R	$P2Y_{12}$ K_i (nM)	$P2Y_{12}$ + protein K_i[b] (nM)	hPRP agg. IC_{50}[a] (μM)
21a		223	231 (1X)	16
21b		36	46 (1.3X)	4
21c		539	593 (1.1X)	25
21d		37	161 (4.3X)	42
21e		191	447 (2.3X)	39
21f		177	172 (1X)	18
21g		68	140 (2X)	34
21h		295	367 (1.2X)	16
21i		460	380 (0.8X)	38
21j		213	783 (3.7X)	86
21k		3	10 (3.3X)	5

[a] Human platelet rich plasma (hPRP), see Ref. 11.
[b] Value in parenthesis is the fold shift between binding with and binding without added protein.

straight-chained alkyl substituents such as **21f**, **21h**, and **21i** also resulted in a 60–175-fold decrease in $P2Y_{12}$ binding affinity and a three to eightfold decrease in hPRP aggregation affinity.

Compounds which exhibited the greatest affinity for the $P2Y_{12}$ receptor were compounds which either contained a phenyl group directly attached to the carbonyl such as **21k** and **21d** or analogs which are small and branched immediately following the carbonyl group as in the 2-methylpropanoyl analog **21b**. Compared with the biphenyl analog **21k,** analogs such as **21a,** and **21g,** where the phenyl ring is one methylene group removed from the carbonyl were 22–77-fold less potent in the $P2Y_{12}$ binding assay respectively and 28–46-fold less potent in the hPRP aggregation assay. Only **21b** was equivalent in potency to **21k** in the hPRP aggregation assay. Here too, compounds with higher protein binding as indicated by a large shift between the $P2Y_{12}$ binding assay with and without added protein such as **21d**, **21g**, and **21j** showed weaker hPRP aggregation inhibition.

Receptor binding studies were conducted on other closely related purinergic receptors such as $P2Y_1$ and $P2Y_{13}$, which share 19% and 48% homology with $P2Y_{12}$, respectively. A subset of thienopyrimidine analogs were tested and showed a greater than 100-fold selectivity for the $P2Y_{12}$ receptor over both the $P2Y_1$ and $P2Y_{13}$ receptors. The majority of compounds made were not tested for selectivity against these receptors.

We have demonstrated that the thienopyrimidine core can be used in preparing potent $P2Y_{12}$ inhibitors. Northern hydrophobic groups combined with southern hydrophilic groups give low nanomolar $P2Y_{12}$ inhibitors with low micromolar inhibition of hPRP. Compounds such as **15h** (5 nM), **15k** (4 nM), and **21k** (3 nM) containing the biphenyl northern substituent and highly polar amide and acid substituents in the southern region exhibit the greatest potency in the $P2Y_{12}$ binding assay. These compounds also exhibit the greatest potency in the hPRP aggregation assay at 4, 3, and 5 μM, respectively. The highly polar acid and amide groups of **15k, 21k,** and **15h** help to offset protein binding attributes of the highly hydrophobic biphenyl group. A smaller northern substituent such as the isopropyl amide of **21b** with reduced protein binding improves the hPRP to $P2Y_{12}$ ratio but was 10-fold less potent than biphenyl **21k** in the $P2Y_{12}$ binding assay. As indicated by some of the differences between assay results with and without added protein, the presence of protein binding can negatively impact the hPRP aggregation activity of the thienopyrimidines.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.08.059.

References and notes

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8. *Aminothiazole synthesis of compound* **3**: to a mixture of sulfur (6.4 g, 200 mmol) in DMF (25 mL) was added methyl cyanoacetate (19.8 g, 200 mmol). The mixture was placed under nitrogen and triethylamine (15 mL, 108 mmol) was added to form a burnt orange solution. Butyraldehyde (18 mL, 200 mmol) was added dropwise at a sufficient rate as to keep the temperature of the mixture between 45 and 50 °C. Once the addition was complete the mixture was allowed to stir at room temperature for 18 h. The mixture was partitioned between brine and ethyl acetate. The layers were separated and the organic phase washed three times with brine, dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The residue was purified via flash chromatography on silica gel (100 g) using 15% ethyl acetate in hexanes. Fractions containing the desired product were concentrated under reduced pressure, slurried in hexanes and collected via vacuum filtration to give 25.74 g (70%) of the title compound: ^{1}H NMR (300 MHz, chloroform-*d*) δ ppm 1.11 - 1.31 (m, 3H) 2.61 (qd, *J* = 7.52, 1.21 Hz, 2H) 3.76–3.83 (m, 3H) 5.79 (br s., 2H) 6.62 (t, *J* = 1.31 Hz, 1H).
9. *$P2Y_{12}$ assays*: dry compounds are diluted as 10 mM dimethylsulfoxide (DMSO) stocks and are tested in a seven-point, threefold dilution series run in triplicate beginning at 10 μM. A 1 mM DMSO intermediate stock is made in a dilution plate and from this the seven dilutions are made. The highest concentration is diluted into water containing 0.02% Bovine serum albumin (BSA), and the remaining six concentrations are diluted into assay buffer containing 0.02% BSA. To a polypropylene assay plate the following are added: (a) 30 μL of assay buffer containing one protease inhibitor cocktail tablet per 50 mL; (b) 30 μL of 1 nM 33P 2-MeSADP made in assay buffer containing 0.02% BSA and 12.5 mg/mL ascorbic acid; (c) 30 μL of cold 1.5 μM 2-MeSADP for the positive control wells, or assay buffer containing 0.02% BSA and 12.5 mg/mL ascorbic acid for the negative control wells; (d) (Method a) 60 μL of 10 μg/well membranes, (Method b) 60 μL of 1 μg/well membranes, (Method c) 60 μL of 0.3 μg/well membranes.Incubate the plates at room temperature for 1 h. Stop the reaction using a cell harvester to aspirate/transfer the supernatant onto GF/B UniFilter plates, and wash three times with wash buffer, aspirating between each wash. The filter plates are dried for approximately 20 min in an oven at 50 °C. Back seals are adhered to the filter plates and 25 μL of Microscint 20 scintillation fluid is added. The filter plates are sealed, shaken for 30 min, and counted on a Top Count.
10. *$P2Y_{12}$ assays with added protein*: the same as the $P2Y_{12}$ assay without protein with the following exceptions:(Method a) 60 μL of 1 μg/well membranes made with 5X human serum albumin (HSA) (1.75%) and 5X AGP (0.075%), (Method b) 60 μL of 1 μg/well membranes made with 5X HSA (1.75%) and 5X AGP (0.075%), (Method c) 60 μL of 0.3 μg/well membranes made with 5X HSA (1.75%) and 5X AGP (0.075%).
11. *Human platelet-rich plasma (hPRP) preparation*:Fifty millilitres polypropylene centrifuge tubes were filled with whole blood and centrifuged at 910g × 10 s and then 200g × 15 min at room temperature to obtain platelet-rich plasma (PRP). PRP was removed using a wide bore transfer pipet avoiding disturbance of the buffy coat (polymorphonuclear leukocytes (PMNLs)) and red blood cells, and placed in a clean polypropylene tube. Remaining plasma, buffy coat and red cells were centrifuged at 2380g × 15 min, again at room temperature, to obtain platelet-poor plasma (PPP). PPP was removed, in the same manner as for the PRP, and placed in a clean polypropylene tube. PRP platelet counts were determined using a Z1 Coulter Particle Counter and individual PRP was diluted to 300,000 platelets/μL with autologous PPP.*hPRP aggregation assay*: the 96-well hPRP aggregation assay monitors aggregation based on the increase of light transmittance through a stirring suspension of platelets after stimulation by adenosine diphosphate (ADP) as they aggregate into large particles. Assays were performed using SpectroMax 190 Microplate Spectrophotometer with SoftMax Pro software from Molecular Devices. Platelets were tested for their sensitivity to compounds in 180 μL reaction volumes containing 144 μL PRP, 18 μL of 10X compound and 14 μL of 10X ADP in a 96-well polystyrene plate. Reactions were initiated by the addition of 20 μM ADP. Compounds were tested at a final concentration of 30 μM. Each concentration of inhibitor was tested in duplicate.