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Carba-nucleosides as Potent Antagonists of the Adenosine 5′-Diphosphate (ADP) Purinergic Receptor ($P2Y_{12}$) on Human Platelets

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Whereas the activation and aggregation of blood platelets are crucial to normal hemostasis, this ensemble is also a key factor in serious cardiovascular disorders, such as myocardial infarction, unstable angina, transient ischemic attack, stroke, peripheral arterial disease, and artherosclerosis. Indeed, abnormal thrombosis is a root cause of adverse cardiovascular events that are responsible for death and disability in humans. As a consequence, platelets are targeted by clinically useful antithrombotic drugs, such as the cyclooxygenase-1 inhibitor aspirin,[1] the GPIIb/IIIa antagonist abciximab,[2] and the adenosine 5′-diphosphate (ADP) receptor antagonist clopidogrel.[3] ADP is an important agonist of platelet activation and aggregation because it induces platelet shape change accompanied by the activation of fibrinogen receptors (GPIIb/IIIa).[3] On platelets, there are three types of cell-surface receptors for ADP, which are members of the P2 purinergic class: $P2X_1$, $P2Y_1$, and $P2Y_{12}$.[4,5] $P2Y_1$ and $P2Y_{12}$ are G protein-coupled receptors (GPCRs), whereas $P2X_1$ is a ligand-gated ion channel. The G_q-coupled $P2Y_1$ receptor initiates ADP-induced platelet activation and the G_i-coupled $P2Y_{12}$ receptor amplifies activation processes, including aggregation, granule secretion, and procoagulant activity, as caused by various agonists. From this perspective, antagonists of $P2Y_{12}$ can be therapeutically effective by markedly inhibiting platelet function independent of the activating stimulus. Additionally, because of the restricted distribution of $P2Y_{12}$ in humans, this receptor is an attractive antiplatelet target for drug discovery.[6]

The widespread clinical use of clopidogrel has demonstrated the relevance of inhibiting the platelet-specific $P2Y_{12}$ receptor to prevent untoward cardiovascular events. However, clopidogrel is a prodrug that requires metabolic conversion in vivo to a highly unstable, reactive species that covalently modifies the $P2Y_{12}$ receptor.[7] On account of this property, there have been observations in humans of slow onset of pharmacological action and of high interpatient variability.[8] Thus, drug discovery efforts have been mounted to identify potent, direct acting, reversible $P2Y_{12}$ antagonists, and some promising compounds have emerged, including Cangrelor (AR-C69931MX),[9] AZD-6140,[10] and PRT-128.[11] In seeking suitable drug candidates in this area, we have been exploring carba-nucleoside derivatives that are structurally related to AZD-6140 as reversible $P2Y_{12}$ antagonists. In this paper, we report on the synthesis and biological evaluation of novel compounds, including tetrazole-containing derivatives with high receptor affinity and excellent potency for inhibiting $P2Y_{12}$-mediated effects on human platelets.

Synthesis

Commercially available acid **1** was converted to its methyl ester, **2**, by treatment with trimethylsilyldiazomethane (Scheme 1). Reaction of **2** with hydrazine afforded hydrazide **3**, which was condensed with imidate **4** (R=Me) to provide methyltriazole-containing derivative **5**. After removal of the Boc protecting group from **5** with trifluoroacetic acid, amine **6** was coupled with 4,6-dichloro-5-nitro-2-(*n*-propylthio)pyrimidine **7** (prepared from 4,6-dihydroxy-2-mercaptopyrimidine by S-alkylation, followed by nitration and conversion of the hydroxyls to chloride groups[12]) in the presence of di(isopropyl)ethylamine in THF at 40 °C to give **8**. The nitro group in **8** was reduced with Fe/HOAc to give **9**, which was treated with isoamyl nitrite to afford triazolopyrimidine **10**. Substitution of the chloride in **10** with various amines, **11**, in the presence of di(isopropyl)ethylamine in 1,4-dioxane afforded compounds **12**. Finally, removal of the acetonide in **12** with trifluoroacetic acid in water provided target compounds **13a**–**g**. Compound **13g** was a mixture of two diastereomers resulting from the use of commercially available racemic (1*RS*,2*SR*)-2-phenylcyclopropylamine (**11**) for the formation of **12**. By applying the same synthetic process, compounds **13h** and **13i**, the analogues containing a 4*H*-[1,2,4]triazol-3-yl group, were prepared by using imidate **4** (R=H) for the conversion of **3** to **5**.

Compounds **22a**–**e** were synthesized from commercially available **14**. Thus, **14** was reacted with **7** by using di(isopropyl)ethylamine as a base to obtain **15** (Scheme 2), which was treated with Fe/HOAc followed by isoamyl nitrite to afford **16**. Replacement of the Cl in **16** with amine **17**, prepared from reductive amination of 2,4-dimethoxybenzaldehyde with (1*RS*, 2*SR*)-2-phenylcyclopropylamine, provided **18**, which was converted to **19** via treatment of $(MeO)_2CMe_2$ in the presence of catalytic *p*-toluenesulfonic acid. Compound **19** was treated with MsCl/pyridine and the resulting mesylate was subjected to nucleophilic displacement with NH-containing imidazole, [1,2,3]triazole, and tetrazole (**20**; R-H) in the presence of K_2CO_3 in DMF at 100 °C to afford products (cf. **22a**–**e**). Concomitant removal of the 3,4-dimethoxybenzyl (DMB) and acetonide protecting groups with trifluoroacetic acid in water gave final products **22a**–**e**, each of which was a mixture of two diastereoisomers (due to the use of racemic amine **17**). When [1,2,3]triazole was used a nucleophile, two regioisomers,

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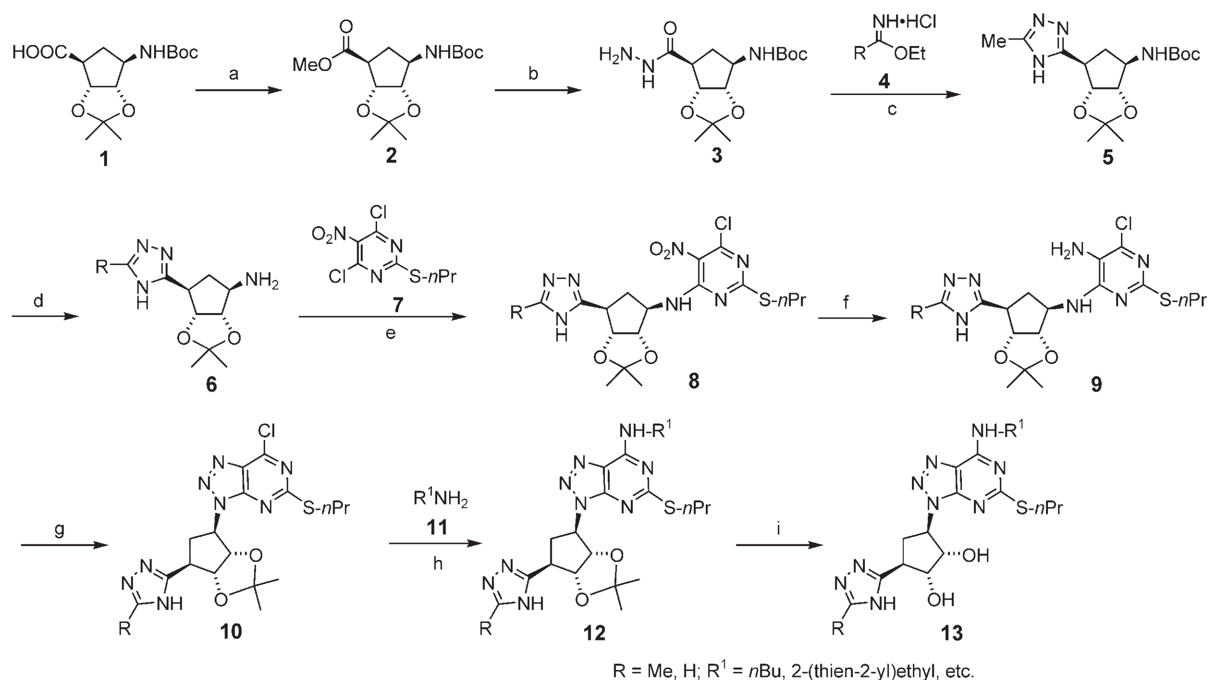
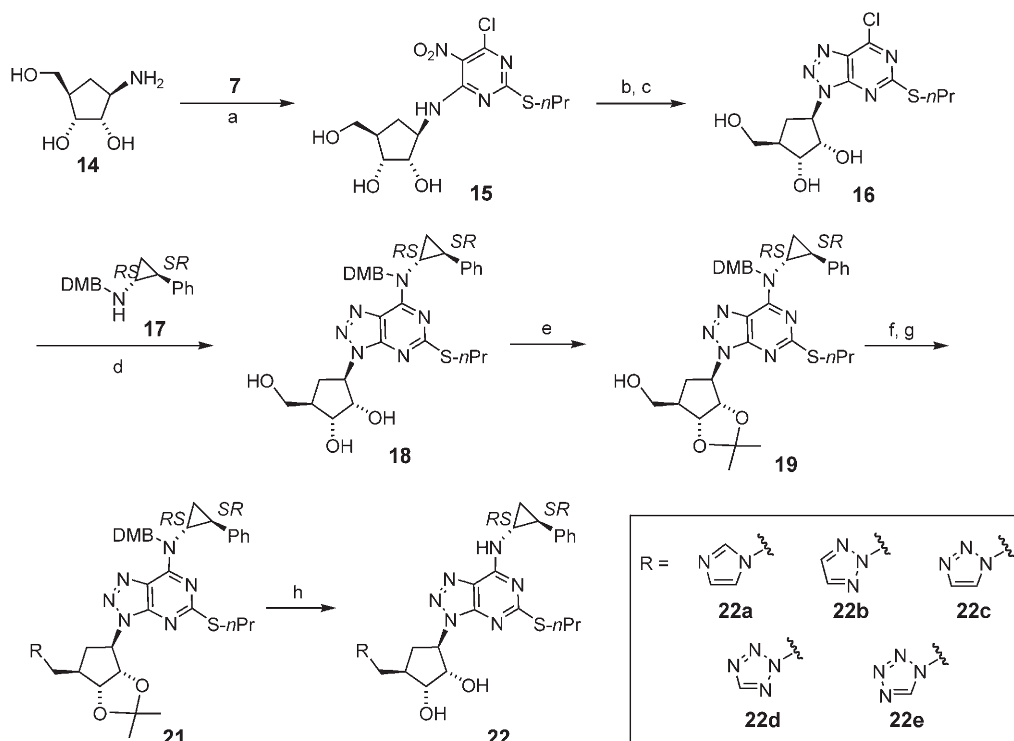


Scheme 1. a) 2 M TMS-CHN_2, CH_2Cl_2, MeOH; b) $N_2H_4 \cdot xH_2O$, EtOH; c) Et_3N, MeCN or EtOH, 80–110 °C; d) 20 % CF_3CO_2H in CH_2Cl_2; e) iPr$_2$NEt, THF, 40 °C; f) Fe, HOAc; g) isoamyl nitrite, MeCN, 70 °C; h) R^1NH_2 (**11**), iPr$_2$NEt, 1,4-dioxane; i) CF_3CO_2H, H_2O.



Scheme 2. a) iPr$_2$NEt, THF, 40 °C; b) Fe, HOAc; c) isoamyl nitrite, MeCN; d) iPr$_2$NEt, 1,4-dioxane; e) $(MeO)_2CMe_2$, TsOH; f) $MeSO_2Cl$, pyridine; g) R-H (**20**), K_2CO_3, DMF, 100 °C; h) CF_3CO_2H, H_2O. [DMB, 2,4-dimethoxybenzyl].

22 b and **22 c**, were obtained and separated by column chromatography (silica gel). The regiochemistry of **22 b** and **22 c** was determined by ^{1}H NMR studies. Similarly, two regioisomers, **22 d** and **22 e**, were obtained when tetrazole was used as a nucleophile.

During the synthesis of tetrazole-containing analogues **29**, an effort to introduce the tetrazole moiety at the early stage of the synthetic process, such as in the triazole case (Scheme 1), proved to be problematic. Thus, the tetrazole ring was introduced near the end of synthesis. Acid **1** was coupled with am-

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monium hydroxide in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) and the resulting amide was treated with trifluoroacetyl anhydride and pyridine in THF to afford cyano derivative **23** (Scheme 1).

Following a similar synthetic route to those described in Scheme 1 and Scheme 2, **23** was converted to **27**. Condensation of the cyano group in **27** with azidotrimethyltin in toluene at 110 °C formed tetrazole derivative **28**. Removal of the acetonide with trifluoroacetic acid in water gave final target compounds **29a–h** (Scheme 3). Compound **29f** was a mixture of two diastereoisomers resulting from use of racemic (1*RS*,2*SR*)-2-phenylcyclopropylamine in the conversion of **25** to **27**, whereas **29g** and **29h** were pure single isomers synthesized by using the single enantiomers (1*R*,2*S*)-2-phenylcyclopropylamine and (1*S*,2*R*)-2-phenylcyclopropylamine, respectively. These two enantiomerically pure amines were obtained from racemic (1*RS*,2*SR*)-2-phenyl-cyclopropylamine by chiral preparative HPLC separation.

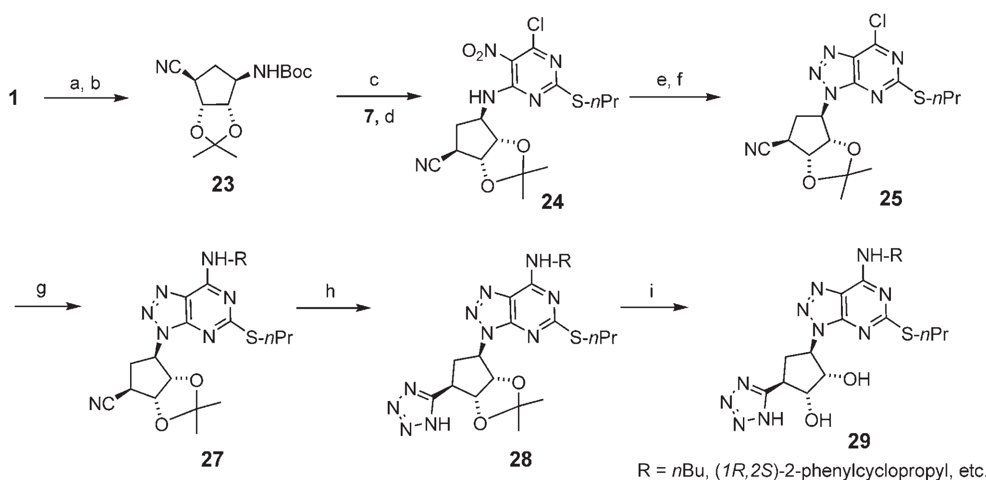
$P2Y_{12}$ receptor binding assay

The target carba-nucleosides were tested in a whole cell binding assay. Compounds and the radiolabeled ADP ligand [^{3}H]-2-MeS-ADP, custom synthesized by Amersham BioScience, were incubated for 30 min at 37 °C with cells stably expressing the human $P2Y_{12}$ receptor. The reaction was stopped by adding cold culture medium, the free and bound radioactivities were separated by filtration (Packard Filtermate), and the radioactivity was measured by using a beta-counter (Packard TOP-COUNT). The testing results are collected in Table 1.

Compounds bearing a 5-methyl-4*H*-[1,2,4]triazol-3-yl group at the R^2 position and a substituted alkyl group at the R^1 position, such as **13a**–**f**, bound to the $P2Y_{12}$ receptor with relatively weak affinity (single-digit-μM IC_{50} values). Interestingly, replacement of the (2*S*)-2-phenylpropyl group in **13e** or (2*R*)-2-phenylpropyl group in **13f** with a cyclic version in the form of the (1*RS*,2*SR*)-2-phenylcyclopropyl group (**13g**) led to a 5–13-fold increase in binding affinity. Removal of the methyl group from the triazole ring led to a further improvement in potency. For example, **13h** and **13i** exhibited IC_{50} values of 240 and 66 nM, reflecting 5-fold and 11-fold improvement over **13a** and **13g**, respectively. Replacement of the 4*H*-[1,2,4]triazol-3-yl group in **13i** with the imidazolylmethyl (**22a**), triazolylmethyl (**22b** and **22c**), and tetrazolylmethyl (**22d** and **22e**) groups generally yielded a moderate loss of affinity. However, introduction of a 1*H*-tetrazol-5-yl group at the R^2 position gave a 17-fold increase in binding affinity over the corresponding triazole derivative, **13i**. This finding led to the discovery of highly potent $P2Y_{12}$ antagonist **29f**, which had an IC_{50} value of 4.3 nM. Other analogues with a simplified R^1 substituent, such as butyl, 2,2,2-trifluoroethyl, [(2*S*)-tetrahydrofuran-2-yl]methyl, and cyclopropylmethyl, were also surveyed. Among them, butylamino derivative **29a**, showed notable affinity (IC_{50} = 14 nM). Finally, the single enantiomers of the two diastereoisomers in **29f**, namely **29g** and **29h**, were tested. Notably, (1*R*,2*S*) isomer **29g** is 30-fold more potent than (1*S*,2*R*) isomer **29h** (IC_{50} values of 2 and 61 nM, respectively), indicating that the orientation of the phenylcyclopropyl group is quite important.

FLIPR calcium assay by transducing G_i-coupled signaling to the G_q pathway

The G_i-coupled $P2Y_{12}$ mediates the inhibition of adenylate cyclase, leading to decreased intracellular levels of 3′,5′-cyclic adenosine monophosphate (cAMP). By contrast, G_q-coupled GPCRs primarily activate phospholipase C, which stimulates inositol-1,4,5-triphosphate (IP_3) formation with a subsequent increase in intracellular Ca^{2+} levels. Prior to the development of a method to convert G_i-coupled GPCR signaling to G_q-coupled GPCR signaling, there was a barrier for the functional testing and screening of G_i-coupled GPCRs. However, the discovery that a small switch in the amino acid sequence of the G_i protein could convert it to a protein for mediating a G_q-coupled response conferred a means for developing a robust, high-



Scheme 3. a) DCC, HOBt, NH_4OH, CH_2Cl_2/DMF (10:1); b) TFAA, pyridine, THF, 0–23 °C; c) CF_3CO_2H, CH_2Cl_2; d) **5**, *i*Pr_2NEt, THF, 40 °C; e) Fe, HOAc; f) isoamyl nitrite, MeCN, 70 °C; g) R-NH_2 (**26**), *i*Pr_2NEt, 1,4-dioxane; h) Me_3SnN_3, toluene, 110 °C; i) CF_3CO_2H/H_2O (4:1). [DCC, 1,3-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; TFAA, trifluoroacetic anhydride.]

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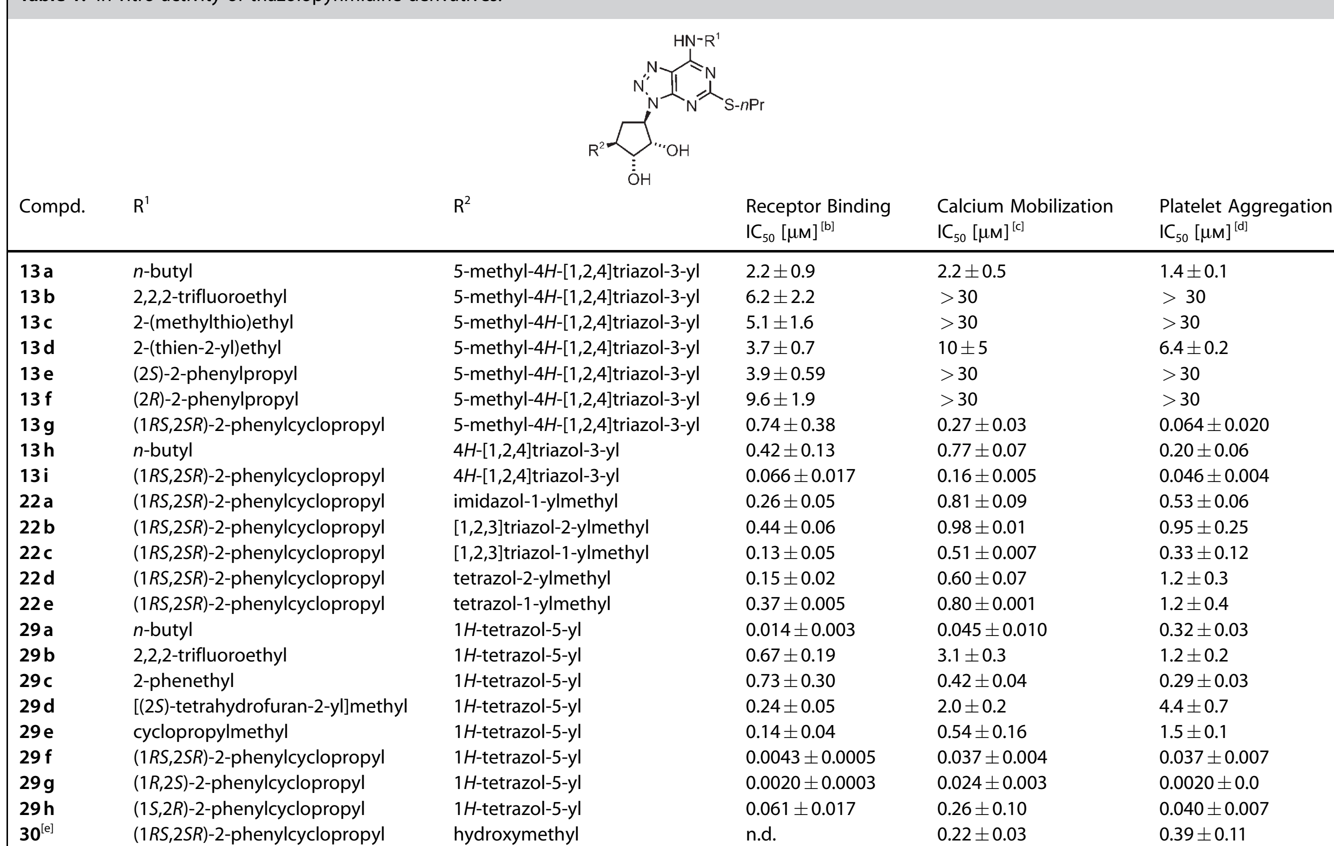
Table 1. In vitro activity of triazolopyrimidine derivatives.[a]



Compd.	R^1	R^2	Receptor Binding IC_{50} [μM][b]	Calcium Mobilization IC_{50} [μM][c]	Platelet Aggregation IC_{50} [μM][d]
13a	*n*-butyl	5-methyl-4*H*-[1,2,4]triazol-3-yl	2.2±0.9	2.2±0.5	1.4±0.1
13b	2,2,2-trifluoroethyl	5-methyl-4*H*-[1,2,4]triazol-3-yl	6.2±2.2	>30	> 30
13c	2-(methylthio)ethyl	5-methyl-4*H*-[1,2,4]triazol-3-yl	5.1±1.6	>30	>30
13d	2-(thien-2-yl)ethyl	5-methyl-4*H*-[1,2,4]triazol-3-yl	3.7±0.7	10±5	6.4±0.2
13e	(2*S*)-2-phenylpropyl	5-methyl-4*H*-[1,2,4]triazol-3-yl	3.9±0.59	>30	>30
13f	(2*R*)-2-phenylpropyl	5-methyl-4*H*-[1,2,4]triazol-3-yl	9.6±1.9	>30	>30
13g	(1*RS*,2*SR*)-2-phenylcyclopropyl	5-methyl-4*H*-[1,2,4]triazol-3-yl	0.74±0.38	0.27±0.03	0.064±0.020
13h	*n*-butyl	4*H*-[1,2,4]triazol-3-yl	0.42±0.13	0.77±0.07	0.20±0.06
13i	(1*RS*,2*SR*)-2-phenylcyclopropyl	4*H*-[1,2,4]triazol-3-yl	0.066±0.017	0.16±0.005	0.046±0.004
22a	(1*RS*,2*SR*)-2-phenylcyclopropyl	imidazol-1-ylmethyl	0.26±0.05	0.81±0.09	0.53±0.06
22b	(1*RS*,2*SR*)-2-phenylcyclopropyl	[1,2,3]triazol-2-ylmethyl	0.44±0.06	0.98±0.01	0.95±0.25
22c	(1*RS*,2*SR*)-2-phenylcyclopropyl	[1,2,3]triazol-1-ylmethyl	0.13±0.05	0.51±0.007	0.33±0.12
22d	(1*RS*,2*SR*)-2-phenylcyclopropyl	tetrazol-2-ylmethyl	0.15±0.02	0.60±0.07	1.2±0.3
22e	(1*RS*,2*SR*)-2-phenylcyclopropyl	tetrazol-1-ylmethyl	0.37±0.005	0.80±0.001	1.2±0.4
29a	*n*-butyl	1*H*-tetrazol-5-yl	0.014±0.003	0.045±0.010	0.32±0.03
29b	2,2,2-trifluoroethyl	1*H*-tetrazol-5-yl	0.67±0.19	3.1±0.3	1.2±0.2
29c	2-phenethyl	1*H*-tetrazol-5-yl	0.73±0.30	0.42±0.04	0.29±0.03
29d	[(2*S*)-tetrahydrofuran-2-yl]methyl	1*H*-tetrazol-5-yl	0.24±0.05	2.0±0.2	4.4±0.7
29e	cyclopropylmethyl	1*H*-tetrazol-5-yl	0.14±0.04	0.54±0.16	1.5±0.1
29f	(1*RS*,2*SR*)-2-phenylcyclopropyl	1*H*-tetrazol-5-yl	0.0043±0.0005	0.037±0.004	0.037±0.007
29g	(1*R*,2*S*)-2-phenylcyclopropyl	1*H*-tetrazol-5-yl	0.0020±0.0003	0.024±0.003	0.0020±0.0
29h	(1*S*,2*R*)-2-phenylcyclopropyl	1*H*-tetrazol-5-yl	0.061±0.017	0.26±0.10	0.040±0.007
30[e]	(1*RS*,2*SR*)-2-phenylcyclopropyl	hydroxymethyl	n.d.	0.22±0.03	0.39±0.11

[a] Mean IC_{50} values ($n \geq 2$). n.d. denotes not determined. [b] Whole cell binding assay for [^{3}H]-2-MeS-ADP to $P2Y_{12}$. [c] FLIPR calcium assay transduction of G_i-coupled signaling to the G_q pathway. [d] ADP-induced human washed platelet aggregation assay. [e] Reference compounds (as mixture of diastereomers).

throughput assay for G_i-coupled GPCRs.[13] In this regard, we established a stable cell line, which was cotransfected with human pcDNA3 hygroP2Y_{12} containing a hygromycin resistance gene and chimeric gene pLEC1-G_{qi5}-HA containing a neomycin resistance gene, to assess calcium mobilization by using the FLIPR Calcium Assay Kit.[13] The testing results are reported in Table 1. It is noteworthy that the test compound inhibition of $P2Y_{12}$-induced calcium influx in this cell-based FLIPR assay generally correlated well with binding affinity to $P2Y_{12}$. Thus, whereas compounds **13b** and **13c** exhibited weak activity ($IC_{50} \geq 10$ μM), tetrazole-containing derivatives **29a** and **29g** showed excellent potency in this FLIPR assay with IC_{50} values of 45 and 24 nM, respectively.

Platelet aggregation

Target compounds were further evaluated in an assay involving ADP-induced washed platelet aggregation. Washed human platelets were prepared following a standard protocol[14] and exposed to compounds in a 96-well plate assay. Platelet aggregation was initiated by adding ADP and assessed by changes in light transmittance measured by a microplate reader (650 nm) at $t=0$ and at 5 min after addition of the agonist. Aggregation was calculated as the decrease in optical density between the $t=0$ and the 5 min measurements (Table 1). The ability of compounds to inhibit ADP-induced platelet aggregation generally correlated well with activity observed in the binding and FLIPR assays, although this aggregation assay, similar to the FLIPR assay, appeared to be more sensitive than the receptor binding assay. For example, **13b**, **13c**, **13e**, and **13f** were inactive ($IC_{50} > 30$ μM) in this assay, compared with their single-digit-μM IC_{50} values in the binding assay. It is worth noting that the tetrazole moiety at the R^2 position clearly demonstrated a superior effect over the hydroxyalkyl group, which is found in reference compound **30**.[15,16] For example, tetrazole derivative **29f** was 11-fold more potent in platelet aggregation than **30** (IC_{50} values of 37 and 390 nM, respectively). Consistent with the observation in the binding and FLIPR assays, **29g** (single isomer of **29f**) was a highly potent $P2Y_{12}$ antagonist in that it inhibited ADP-induced washed platelet aggregation with an impressive IC_{50} value of 2 nM.

Conclusions

We have explored a series of novel carba-nucleosides for their usefulness as $P2Y_{12}$ antagonists. Thus, we identified several tetrazole-containing derivatives, such as **29a** and **29g**, as reversible-type antagonists with high affinity for the $P2Y_{12}$ receptor

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and with excellent potency for inhibiting $P2Y_{12}$-induced calcium influx and ADP-induced washed platelet aggregation.

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Keywords: ADP receptor · antagonist · nucleosides · $P2Y_{12}$ receptor · platelet · purinergic

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