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Design, synthesis, pharmacological characterization of a fluorescent agonist of the $P2Y_{14}$ receptor

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ABSTRACT

The $P2Y_{14}R$ is a $G_{i/o}$-coupled receptor of the P2Y family of purinergic receptors that is activated by extracellular UDP and UDP-glucose (UDPG). In an earlier report we described a $P2Y_{14}R$ fluorescent probe, MRS4174, based on the potent and selective antagonist PPTN, a naphthoic acid derivative. Here, we report the design, preparation, and activity of an agonist-based fluorescent probe MRS4183 (**11**) and a shorter $P2Y_{14}R$ agonist congener, which contain a UDP-glucuronic acid pharmacophore and BODIPY fluorophores conjugated through diaminoalkyl linkers. The design relied on both docking in a $P2Y_{14}R$ homology model and established structure activity relationship (SAR) of nucleotide analogs. **11** retained $P2Y_{14}R$ potency with EC_{50} value of 0.96 nM (inhibition of adenylyl cyclase), compared to parent UDPG (EC_{50} 47 nM) and served as a tracer for microscopy and flow cytometry, displaying minimal nonspecific binding. Binding saturation analysis gave an apparent binding constant for **11** in whole cells of 21.4 ± 1.1 nM, with a $t_{1/2}$ of association at 50 nM **11** of 23.9 min. Known $P2Y_{14}R$ agonists and PPTN inhibited cell binding of **11** with the expected rank order of potency. The success in the identification of a new $P2Y_{14}R$ fluorescent agonist with low nonspecific binding illustrates the advantages of rational design based on recently determined GPCR X-ray structures. Such conjugates will be useful tools in expanding the SAR of this receptor, which still lacks chemical diversity in its collective ligands.

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G protein-coupled receptors (GPCRs) that respond to extracellular uracil and adenine nucleotides (P2Y receptors, P2YRs) constitute important and ever-growing targets for pharmacological exploration. Eight nucleotide GPCRs are divided into two subfamilies of P2YRs, based on sequence and functional similarity: $P2Y_1R$-like, $P2Y_{1,2,4,6,11}$; and $P2Y_{12}R$-like, $P2Y_{12-14}$. The P2YRs are broadly distributed among many tissues and are expressed in immune cells, intestine, kidney, lung, nervous systems, and others.[1]

The $P2Y_{14}R$ is involved in inflammation, hypoxia[2] and mechanical pain hypersensitivity,[3] and its activation enhances neutrophil chemotaxis[4] and promotes the release of mediators from mast cells.[5] Studies with $P2Y_{14}R^{-/-}$ mice suggested that $P2Y_{14}R$ antagonism might be a target for diabetes therapy; another study suggested that $P2Y_{14}R$ activation enhances insulin release.[6,19] Thus, the $P2Y_{14}R$ is a potential pharmaceutical target for inflammation, hypoxia, and endocrine mis-function.

Given the diversity of expression of the $P2Y_{14}R$ and the ubiquitous nature of its endogenous activators, that is, UDP (Chart 1 and **1**, EC_{50} 160 nM[20]) and UDP-sugars (**2**, EC_{50} 261 nM[8]; **4**, EC_{50} 370 nM[8]), the development of ligands selectively targeting this receptor is a considerable challenge and an important goal for pharmacological studies and potential therapeutic applications. A limited number of chemical classes have been identified as ligands

Abbreviations: BODIPY, boron-dipyrromethene; cAMP, 3′,5′-cyclic adenosine monophosphate; CHO, Chinese hamster ovary; COMU, [[(*Z*)-(1-cyano-2-ethoxy-2-oxoethylidene)amino]oxy-morpholin-4-ylmethylidene]-dimethylazanium hexafluorophosphate; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; ECL, extracellular loop; FCM, flow cytometry; HATU, 1-[bis (dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; IBMX, 3-isobutyl-1-methylxanthine; 2-MeSADP, 2-methylthio-adenosine-5′-diphosphate; MESF, molecules of equivalent soluble fluorochrome; MRS2578, *N*,*N*″-1,4-butanediyl*bis*[*N*′-(3-isothiocyanatophenyl)]thiourea]; MRS2957, P^1-(uridine 5′-)-P^3-(N^4-methoxycytidine 5′-)triphosphate; PPTN, 4-(4-(piperidin-4-yl)-phenyl)-7-(4-(trifluoromethyl)-phenyl)-2-naphthoic acid; SAR, structure activity relationship; UDPG, uridine-5′-diphosphoglucose; TM, transmembrane helix.

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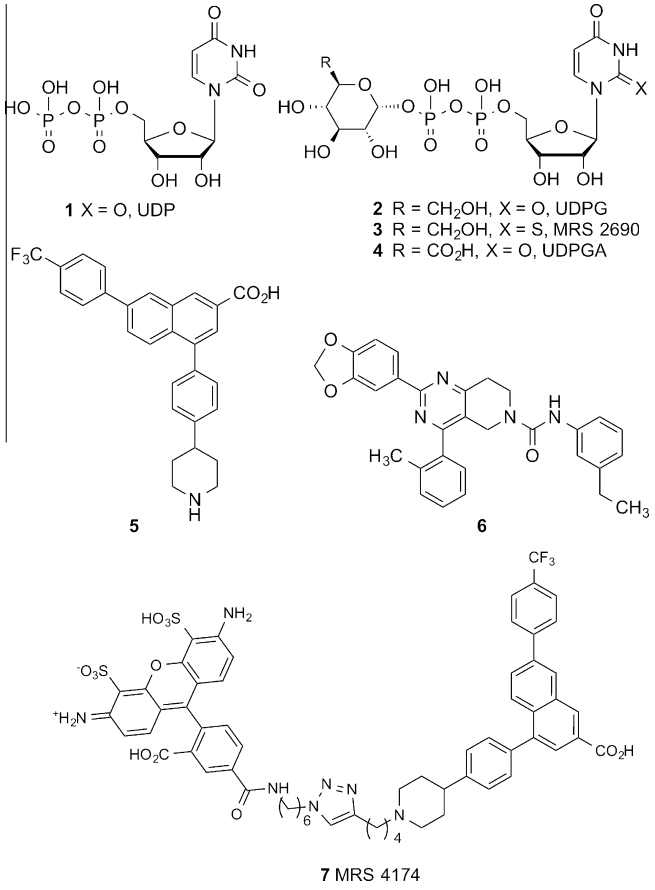

Chart 1. Representative $P2Y_{14}R$ agonists **1**–**4**, antagonists **5**, **6** and fluorescent antagonist MRS 4174 **7**.

and explored for their effects on the $P2Y_{14}R$: synthetic nucleotide analogs of **1** and **2**, such as **3** (EC_{50} 11 nM[20]),[7,8] derivatives of 2-naphthoic acid, most notably the potent and selective antagonist 4-(4-(piperidin-4-yl)-phenyl)-7-(4-(trifluoromethyl)-phenyl)-2-naphthoic acid (PPTN, **5**, K_B 0.43 nM),[9–11] and substituted pyrido [4,3-*d*]pyrimidines including potent analog **6** (IC_{50} 10 nM).[12]

Nucleotide analogs of endogenous $P2Y_{14}R$ agonist **1** would be poor drug candidates due to their low bioavailability associated with high charge and their chemical instability due to hydrolysis by endonucleotidases. Derivatives of nonnucleotide $P2Y_{14}R$ antagonist **5** also suffer from poor drug-like characteristics due to high lipophilicity.[9,13] However, the chemical stability, high affinity and selectivity of **5** toward the $P2Y_{14}R$ attracted our attention and inspired its use as a template for the development of fluorescent antagonist affinity probes.[10,14] We applied virtual docking to design analogs of **5** that retain or enhance $P2Y_{14}R$ affinity and tolerate the increase in molecular size and interactions associated with fluorophore conjugation. A human (h) $P2Y_{14}R$ homology model was constructed based on the recently reported structures of the $hP2Y_{12}R$.[15,16] This model was successfully utilized in predicting a suitable site on **5** for fluorophore conjugation and the minimum linker length required to provide adequate spacing between the pharmacophore and the fluorophore. The resulting PPTN-Alexa Fluor 488 conjugate (MRS4174, **7**) displayed subnanomolar antagonist affinity at the $hP2Y_{14}R$ and a very low level of nonspecific binding. Its binding to the $hP2Y_{14}R$ stably expressed in mammalian cells was inhibited by other $P2Y_{14}R$ ligands with the appropriate rank order of potency.

In the present study, we designed with the aid of molecular modeling and synthesized a pharmacologically complementary high affinity fluorescent agonist-based probe. The suitability of the site of attachment and the dependence of potency on the length of the spacer chain were predicted by a structure-based analysis using a homology model of the $P2Y_{14}R$.[14,21] Our previous modeling study concluded that the glucose moiety of **4** was the most structurally permissive region of this endogenous agonist since it bound in the second subpocket of the $P2Y_{14}R$ binding site, which is accessible to the extracellular medium. Therefore, two fluorescent derivatives of **4** were designed and prepared (Scheme 1) based on predictions from computational modeling of $P2Y_{14}R$ agonist binding.[21] The strategy behind the selection of these target compounds is described below. The conjugation of a fluorophore was accomplished via condensation of commercially available fluorescent amines with the carboxylic group of **4**.

Based on previous structure activity relationship (SAR) studies of **2**, two designs of fluorescent affinity probes of varying chain length were compared. The structures of **1** and **2**, now identified as endogenous $P2Y_{14}R$ agonists, have been extensively probed by chemical modification with regard to their ability to activate the $P2Y_{14}R$, as well as $P2Y_2$, $P2Y_4$ and $P2Y_6Rs$.[7,8,17] The C6 carbon of the hexose moiety of UDPGA **4** was found suitable for chain extension with retention of $P2Y_{14}R$ agonist activity. A number of amide derivatives at the glucose C6 have been constructed, and most of these derivatives retained agonist activity. It was also found that $P2Y_{14}R$ agonist activity was retained with attachment of large groups such as unprotected, as well as acetyl- and Boc-protected aminoethylamides, and polyamidoamine (PAMAM) dendrimers of generation (G) 3 and 6 attached to the C6 carbon.[17] Moreover, it was found that dendrimer conjugates possessed enhanced potency when compared to **4**.

Thus, we chose the structure of **4** as a starting point for designing and building fluorescent probes. Our objectives were to facilitate the availability of such affinity probes and to validate further the previously constructed computational models. Hence, we chose as our primary fluorophore boron-dipyrromethene (BODIPY), which has been used in other fluorescent probes of GPCRs.[18] Unlike the chemical series of hydrophobic $P2Y_{14}R$ antagonist **5**, in which a less hydrophobic fluorophore, AlexaFluor 488, in conjugate **7** was optimal, the restriction of choosing a hydrophilic fluorophore was relieved due to the inherent high polarity and hydrophilicity of **4**. Additionally, various BODIPY dyes with built-in reactive amine linkers of varying length are readily available commercially. Two amide-bound conjugates, **10** and MRS4183 **11** (Scheme 1), were docked into a homology model of the $hP2Y_{14}R$ to predict their fit prior to synthesis. The target molecules **10** and **11** contained linkers consisting of 7 and 14 (including benzene ring) atoms, respectively, between C6 of hexose and the pyrrole ring of BODIPY.

A homology model of the $hP2Y_{14}R$ was constructed as we reported, based on the high resolution X-ray crystallographic structure of the $P2Y_{12}R$ complex with 2-MeSADP.[15,16,21] Various known $P2Y_{14}R$ agonists were docked in the orthosteric binding site. The attempted docking of the fluorescent agonists **10** and **11** did not provide a satisfactory binding pose for conjugate **10**, suggesting the inability of the binding pocket to accommodate both pharmacophore and fluorophore connected by a short, 7-atom linker containing ethylene diamine. However, the hypothetical binding mode of **11** revealed that the longer, 14-atom linker containing 1,5-diaminopentane is sufficiently long to connect the pharmacophore inside the deep binding site with the fluorophore presented on the extracellular side of the receptor (Fig. 1). According to the obtained binding mode, the nucleobase binds inside the pocket formed by TMs 3–5, including aromatic stacking with $Y102^{3.33}$ (using standard numbering relative to the TMs[26]) and with carbonyl oxygen atoms forming hydrogen bond contacts with side chains of $H184^{5.35}$ and $N188^{5.39}$. The ribose hydroxyls form contacts with $N156^{4.57}$ side chain and the backbone carbonyl of