ACS Medicinal Chemistry Letters

Letter

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Fluorescent Human EP_3 Receptor Antagonists

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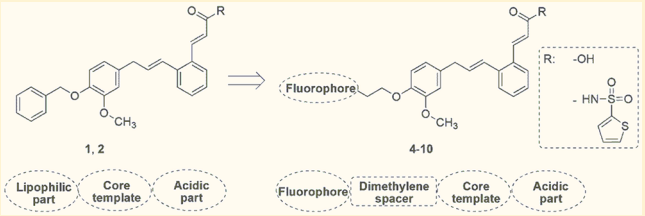
Supporting Information

ABSTRACT: Exchange of the lipophilc part of ortho-substituted cinnamic acid lead structures with different small molecule fluorophoric moieties via a dimethylene spacer resulted in hEP_3R ligands with affinities in the nanomolar concentration range. Synthesized compounds emit fluorescence in the blue, green, and red range of light and have been tested concerning their potential as a pharmacological tool. hEP_3Rs were visualized by confocal laser scanning microscopy on HT-29 cells, on murine kidney tissues, and on human brain tissues and functionally were characterized as antagonists on human platelets. Inhibition of PGE_2 and collagen-induced platelet aggregation was measured after preincubation with novel hEP_3R ligands. The pyryllium-labeled ligand **8** has been shown as one of the most promising structures, displaying a useful fluorescence and highly affine hEP_3R antagonists.



KEYWORDS: *EP_3 receptor, fluorescence, cinnamic acid derivatives, confocal laser scanning microscopy, imaging, prostanoid, GPCR, murine kidney, human brain, platelet aggregation*

The EP_3 receptor is a member of the prostanoid G-protein-coupled receptor (GPCR) family. Prostanoids are products of the arachidonic acid cascade. Arachidonic acid is released from the cell membrane and converted over prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) into the five prostanoids: prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, PGI_2, PGD_2, and thromboxane A_2. PGE_2 interacts through four GPCRs: EP_1, EP_2, EP_3, and EP_4. The EP_3 receptor is special due to its nine human splice variants.[1] Activation of the EP_3 receptor plays a crucial role in fever generation,[2] hyperalgesia, bladder hyperactivity,[3] uterine contraction, gastric acid secretion, smooth muscle contraction of the GI tract, neurotransmitter release, sodium/water reabsorption in kidney, platelet aggregation, and thrombosis.[4−6] EP_3 receptors are expressed on platelets and lower intracellular cAMP levels via G_i coupling, causing increased sensitivity of platelets to ADP or collagen-induced platelet aggregation.[7] There are many efforts to design EP_3 antagonists as novel antiplatelet agents that do not prolong bleeding since neither platelets nor healthy arterial walls produce PGE_2.[8]

Localization and distribution of the receptor are important for understanding its functions, effects, and interactions. Therefore, there is a need to label affine ligands with visualization tools. One method of choice is radiolabeling. [^{3}H]PGE_2 as a full agonist is not selective at the four EP receptor subtypes. To avoid expensive equipment and harmful exposure to radioactivity, fluorescence labeling becomes more and more important. To the best of our knowledge, no fluorescence-labeled EP_3 ligand has been synthesized so far. It was our aim to design high-affinity subtype-selective EP_3 ligands with suitable fluorescence properties. These substances should be applicable as pharmacological tools to study the corresponding EP_3 receptor subtypes in vitro on cell as well as on tissue conditions.

Literature research was found in some high-affinity and subtype-selective EP_3 antagonists among others.[9,10] Reference lead structures were synthesized as reported previously.[11] In the structural design, we considered cutting off the lipophilic residue of the ortho-substituted cinnamic acid derivatives and replacing it with a fluorophore, labeled via a short dimethyleneoxy spacer (Figure 1). Practical fluorophores should conserve the lipophilic character. To maintain the affinity, we used small fluorescent groups coupled via the short spacer to avoid sterical hindrance. Established small molecule fluorophores are, for example, cyanoisoindole,[12] Sanger's

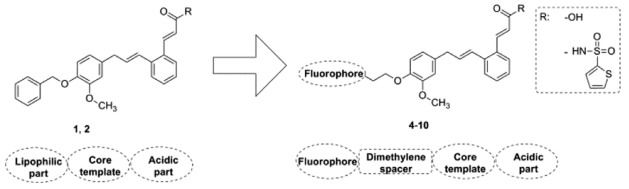
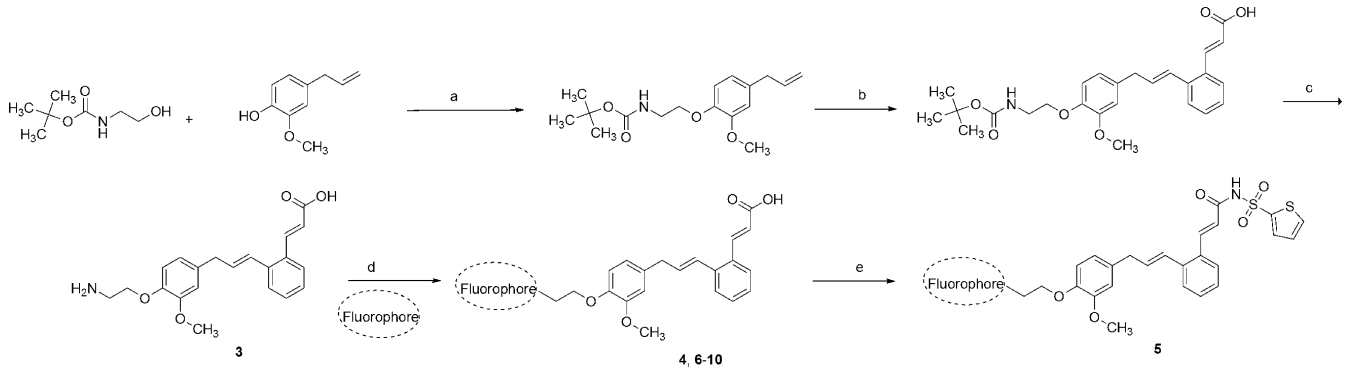


Figure 1. Design of fluorescent hEP_3R ligands.

Received: July 11, 2012
Accepted: August 8, 2012
Published: August 8, 2012

Scheme 1. Synthesis of Fluorescent-Labeled hEP$_3$R Ligands[a]



[a]Reagents and conditions: (a) PPh_3, DEAD, THF, RT, overnight. (b) *trans*-2-Bromocinnamic acid, $Pd(OAc)_2$, LiCl, Bu_4NCl, LiOAc, DMF, 120 °C, overnight. (c) HCl, THF, 60 °C, 3 h. (d) NEt_3, MeCN or DIPEA, DMF, fluorophore, RT, overnight. (e) EDC, HOBt, CH_2Cl_2, RT, overnight.

reagent,[13] pyryllium,[14,15] and fluorescein[16,17] groups. These fluorescent groups could be coupled to the reactive amino group of the dimethyleneoxy spacer of **3**.

Synthesis started with Mitsunobu reaction[18] of *N*-Boc-ethanolamine with eugenol. The resulting product was converted with *trans*-2-bromocinnamic acid via Heck reaction.[11] The crude product was purified by column chromatography and crystallization to separate resulting regioisomers. The protecting Boc group was cleaved under acidic conditions. The free amino group was coupled with appropriate fluorophore and addition of triethylamine or diisopropylethylamine.[19] The free carboxylic group was converted into an acyl sulfonamide group via coupling reactions with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)/1-hydroxybenzotriazole (HOBt).[20] All fluorescent EP$_3$R ligands were purified by column chromatography (Scheme 1). Fluorescence characterization was determined at a concentration of 10 μM in buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, and 1 mM EDTA) (Table 1).

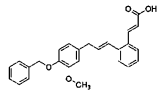
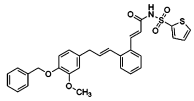
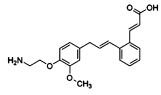
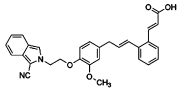
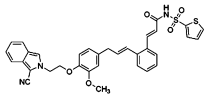
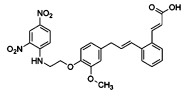
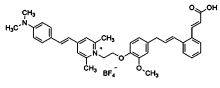
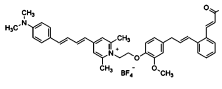
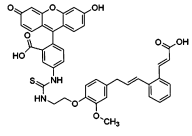
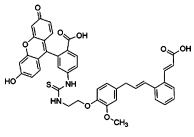
Reference structures and all novel synthesized fluorescent human EP$_3$ receptor (hEP$_3$R) ligands were tested for their affinity at the hEP$_3$R in a radioligand competition binding assay measuring [^{3}H]PGE$_2$ binding to recombinant human EP$_3$ prostanoid receptors (ChemiSCREEN membrane preparation) (Merck Millipore, Schwalbach, Germany) (Table 1). We found 5-fold up to 10-fold higher K_i values as those reported by Belley et al.[9,10] This working group used a different test assay, taking advantage of one specific splice variant of hEP$_3$R (EP$_3$−III), and used the regioisomeric mixture resulting from the Heck reaction.[9] The reference structure with the reactive amino group (**3**) showed a loss of hEP$_3$R affinity caused by the polar and basic amino group. Fluorescent compounds with the highest affinity at hEP$_3$R represent **4**−**6**. Their hEP$_3$R affinities are in the same concentration range like reference compound **2**. All three fluorescent hEP$_3$R ligands emit a blue fluorescence wavelength (300−450 nm) where interferences with tissue autofluorescence are expected. These compounds possess small Stokes shifts potentially causing self-quenching, and the fluorescence emission intensities were low in comparison with **9** and **10** (Supporting Information). Pyryllium dye-coupled ligands (**7** and **8**) exhibited moderate affinities at the hEP$_3$R. The introduction of another double bond increased the lipophilic character of the fluorophore and resulted in an increase of affinity at hEP$_3$R as well as a red shift of fluorescence emission wavelength despite a decrease of fluorescence intensity (**7** → **8**). Fluorescence characteristics of **8** with large Stokes shift and red fluorescence emission wavelengths are more favorable for use than the other fluorescent hEP$_3$R ligands. The large Stokes shift of the pyryllium-labeled hEP$_3$R ligand avoids self-quenching during laser excitation with the confocal laser scanning microscope, and the red fluorescence emission reduces interaction with mostly blue autofluorescence. Fluoresceinisothiocyanate (FITC)-labeled compounds (**9** and **10**) possess moderate Stokes shifts and are emitted brightly in the green fluorescence spectrum. However, **9** and **10** lost affinity at hEP$_3$R, most probably caused by the polar phenolic and acidic structure of the FITC fluorophore.

Reference structures (**1** and **2**) and ligands with high affinities at hEP$_3$R (**4** and **5**) or with the most promising fluorescent properties (**8**) were screened on their selectivities at all EP receptor subtypes in radioligand competition binding assays using [^{3}H]PGE$_2$ at recombinant human EP$_{1-4}$ prostanoid receptors (ChemiSCREEN membrane preparation) (Merck Millipore) (Table 2).[21] All compounds tested are hEP$_3$R selective. Merely, compound **8** shows some cross-affinity at hEP$_4$R.

To test the usefulness as pharmacological tools, all fluorescent EP$_3$ ligands were incubated on human colon adenocarcinoma grade II cells (HT-29 cells) in six-well plates. Expression of the hEP$_3$R was induced by withdrawal of fetal bovine serum (FBS) for 72 h.[22] Cells labeled with blue fluorescent hEP$_3$R ligands (**4**−**6**) were additionally stained with propidium iodide,[23] and nuclei of cells incubated with red fluorescent pyryllium-labeled **8** were stained with blue fluorescent 4′,6-diamino-2-phenylindole (DAPI).[24] All compounds turned out to be stable under assay conditions and to be appropriate for the detection of hEP$_3$Rs on starved HT-29 cells by fluorescent confocal laser scanning microscopy. hEP$_3$Rs can be detected in the cytoplasm of HT-29 cells. Blue fluorescent compounds (**4**−**6**) have to be incubated in high concentrations (3 μM) to avoid interference with autofluorescence. In comparison with control HT-29 cells, specific binding of fluorescent hEP$_3$R ligands could be increased up to 60% by media withdrawal (**4**, 58%; **5**, 55%; and **6**, 60%) (Figure 2). Consequently, hEP$_3$R expression was increased up to 60%. Specific binding of fluorescent hEP$_3$R ligands could be displaced by a 3-fold excess of **1** (Figure 3). Red fluorescent hEP$_3$R ligand **8** could be detected on HT-29 cells after media withdrawal in a concentration of 300 nM (2-fold of K_i value). Nonstarved HT-29 control cells and HT-29 cells after

dx.doi.org/10.1021/ml300191g | ACS Med. Chem. Lett. 2012, 3, 774−779

Table 1. Affinities of 1−10 at hEP$_3$R and Fluorescence Characterization

Comp.	Structure	hEP$_3$R[a] K_i± S.E.M [nM]	λ_{max}[b] Abs. Em. [nm]	Stokes shift [nm]
1		44.5 ± 6.1 (8)[c]		
2		26.6 ± 2.2 (1.6)[d]		
3		1496.8 ± 451.7		
4		18.2 ± 0.9	350 396	46
5		26.4 ± 4.5	350 397	47
6		23.4 ± 8.3	355 404	49
7		218.5 ± 55.1	467 604	137
8		135.4 ± 22.7	473 685	212
9		678.4 ± 220.2	480 574	94
10		1264.6 ± 714.5	491 571	80

[a]Competition binding assay using [^{3}H]PGE$_2$ at recombinant human EP$_3$prostanoid receptors (ChemiSCREEN membrane preparation). [b]Measured in buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, and 1 mM EDTA) at a concentration of 10 μM. [c]Ref 9. [d]Ref 10.

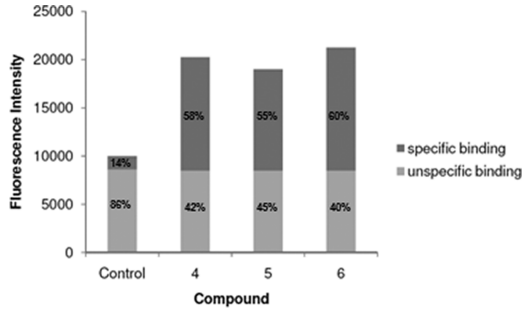


Figure 2. Induction of hEP$_3$R expression on HT-29 cells by media withdrawal. HT-29 cells were incubated with 3 μM **4**, **5**, or **6** after withdrawal of FBS for 72 h. Unspecific binding increased up to 60%. HT-29 cells cultured with FBS-containing medium were incubated with blue fluorescent hEP$_3$R ligands as a control.

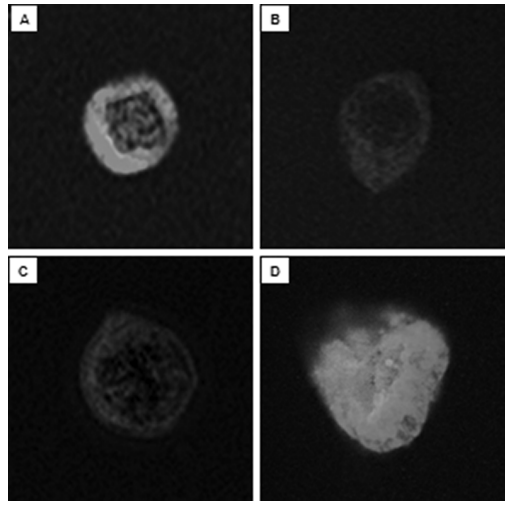


Figure 3. Fluorescence images of **4** on HT-29 cells. (A) A 3 μM concentration of **4** was incubated for 1 h on HT-29 cells cultured without FBS. HEP$_3$Rs are detectable in cytoplasm. (B) A 3 μM concentration of **4** was incubated for 1 h on HT-29 cells cultured with FBS. (C) Starved HT-29 cells were incubated 1 h with 3 μM **4** and afterward with 9 μM **1** for 1 h. (D) A 3 μM concentration of **4** was incubated for 24 h on HT-29 cells cultured without FBS. HEP$_3$Rs migrate into the inner cytoplasm.

induction of hEP$_3$R were facile to distinguish from each other. Cytoplasm and the outer membrane of the nucleus of HT-29 control cells showed fluorescence with lower intensities the starved HT-29 cells after labeling with red fluorescent pyryllium ligand **8**. Fluorescence labeling of hEP$_3$Rs with **8** could be specifically displaced by 3-fold excess of **1** (Figure 4). Elongation of the incubation time up to 24 h resulted in

Table 2. Affinities of 1, 2, 4, 5, and 8 at hEP$_1$R, hEP$_2$R, hEP$_3$R, and hEP$_4$R

	K_i ± SEM (nM)[a]			
compd	hEP$_1$R	hEP$_2$R	hEP$_3$R	hEP$_4$R
1	151350 ± 55084	1729 ± 47	45 ± 6	6313 ± 151
2	2443 ± 1551	2415 ± 342	27 ± 2	4304 ± 246
4	15990 ± 7764	4264 ± 248	18 ± 1	3183 ± 645
5	20260 ± 2800	10810 ± 198	26 ± 5	9448 ± 1417
8	68625 ± 12735	2943 ± 279	135 ± 23	902 ± 226

[a]Competition binding assay using [^{3}H]PGE$_2$ at respective recombinant hEP$_{1-4}$prostanoid receptor subtype (ChemiSCREEN membrane preparation).

dx.doi.org/10.1021/ml300191g | ACS Med. Chem. Lett. 2012, 3, 774−779

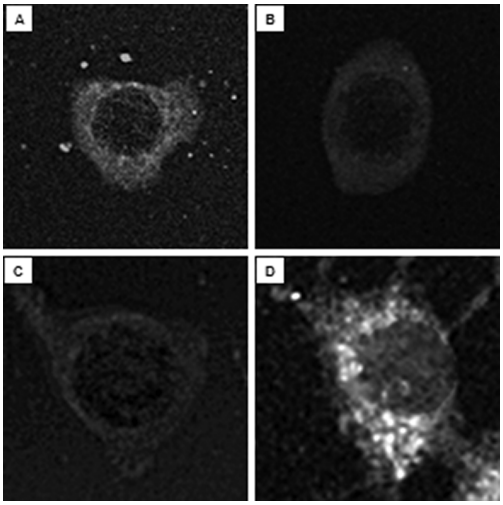


Figure 4. Fluorescence images of **8** on HT-29 cells. (A) A 300 nM concentration of **8** was incubated for 1 h on HT-29 cells cultured without FBS. HEP_3Rs are detectable in cytoplasm. (B) A 300 nM concentration of **8** was incubated for 1 h on HT-29 cells cultured with FBS. (C) Starved HT-29 cells were incubated for 1 h with 300 nM **8** and afterward with 900 nM **1** for 1 h. (D) A 300 nM concentration of **8** was incubated for 24 h on HT-29 cells cultured without FBS. HEP_3Rs migrate into the inner cytoplasm.

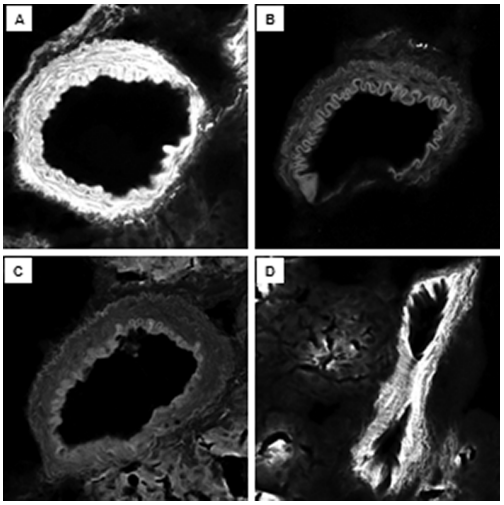


Figure 5. Fluorescence images of **8** on murine kidney tissues. (A) A 1 μM concentration of **8** was incubated for 1 h on a kidney tissue of a C57Bl6 WT mouse. EP_3Rs in smooth muscle cells of renal arteries are stained. (B) A 1 μM concentration of **8** was incubated for 1 h on a kidney tissue of an $EP_3^{-/-}$ mouse. Staining of smooth muscle cells of renal arteries is not detectable. (C) Autofluorescence of a renal artery. (D) Compound **8** gives staining of EP_3Rs in cortical collecting tubuli.

accumulation of fluorescence-labeled hEP_3Rs in inner domains of cytoplasm. Receptor internalization could not be observed (Figures 3 and 4). As a result, the red fluorescent pyryllium-labeled ligand **8** could be used in low concentrations. Fluorescent signals were facile to detect, and no interferences with autofluorescence occurred. Because of its advantages over the blue fluorescent and slightly more selective hEP_3R ligands **4**–**6**, the pyryllium-labeled **8** appeared to be the most qualified fluorescent pharmacological tool and was used for further tissue imaging studies.

EP_3 receptors abundantly occur in kidney. In situ hybridization experiments resulted in high expression of EP_3 receptors in the tubular epithelium in the outer medulla and also in the medullary thick ascending limb and cortical and medullary collecting ducts.[25−28] For fluorescence detection of EP_3R, **8** was incubated on kidney tissues of wild-type (WT) and $EP_3^{-/-}$ mice. EP_3Rs were detected in smooth muscle cells of renal arteries and in epithelilal cells of cortical collecting tubuli (Figure 5). No specific signal was observed in the EP_3 knockout mice kidney tissue. EP_4 receptors are located in the smooth muscle cells of arteries, too.[28] We could not detect any specific red fluorescence signal in EP_3 knockout mice kidney tissue. Consequently, interactions of red fluorescent pyryllium-labeled **8** with EP_4 receptors in murine tissue under these conditions could be excluded. The distribution of EP_3 receptors corresponds to PGE_2-mediated regulation of sodium/water reabsorption in kidney.

The EP_3 receptor is also present in the brain with a widespread distribution. EP_3 mRNA is expressed in neurons of the cortex, hippocampus, thalamus, hypothalamus, midbrain, and lower brain stem, causing neurotransmitter release.[29,30] HEP_4 receptors are located in hypothalamic nuclei.[30] To demonstrate the presence of hEP_3Rs in the brain, pyryllium-labeled **8** was incubated on human brain tissues of the cerebral cortex and globus pallidus where no hEP_4 receptors are located. In both brain segments, hEP_3Rs could be detected by red fluorescence signals after incubation with pyryllium-labeled hEP_3R ligand **8**. Red fluorescent labeling ligand **8** was displaceable with 100-fold excess of **1** (Figure 6).

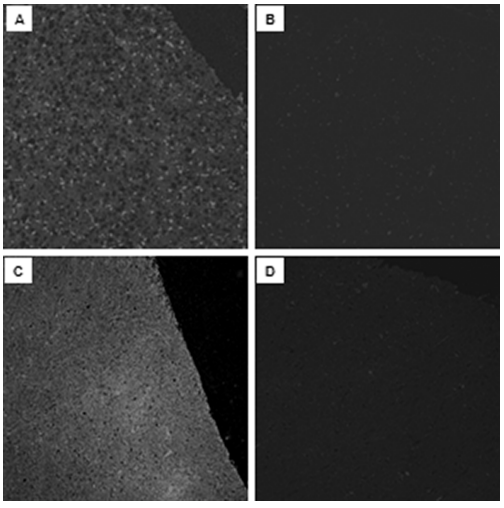


Figure 6. Fluorescence images of **8** on human brain tissues. (A) A 1 μM concentration of **8** was incubated for 1 h on a cerebral cortex tissue. HEP_3Rs are widespread labeled. (B) A 1 μM concentration of **8** was incubated for 1 h on a cerebral cortex tissue and displaced with 100 μM **1**. (C) A 1 μM concentration of **8** was incubated for 1 h on a globus pallidus tissue. hEP_3Rs are widespread labeled. (D) A 1 μM concentration of **8** was incubated for 1 h on a globuspallidus tissue and displaced with 100 μM **1**.

On the basis of the predicted role of EP_3 receptors in platelet aggregation and thrombosis, we investigated hEP_3R labeling on human platelets with pyryllium-labeled ligand **8**. After localization of the hEP_3R antagonist **8** on platelets, red fluorescent ligand was displaced with **1** (Figure 7). Application of **8** as a pharmacological tool was proved in a FACS (fluorescence activated cell sorting) assay. About 50% of the platelets could be detected as fluorescence-labeled by **8** (Figure 8). Because of

dx.doi.org/10.1021/ml300191g | ACS Med. Chem. Lett. 2012, 3, 774−779

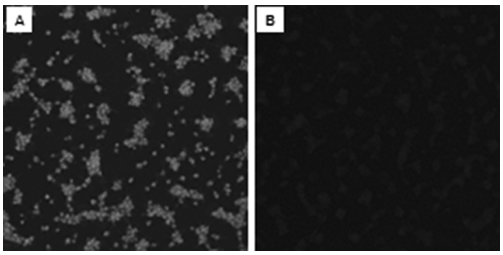


Figure 7. Fluorescence images of **8** on human platelets. (A) A 1 μM concentration of **8** was incubated for 1 h on fixed platelets on slides of flow chambers. Strong red fluorescence emission was detectable. (B) A 1 μM concentration of **8** was incubated for 1 h on fixed platelets on slides of flow chambers and displaced by 1 mM **1**. The fluorescence signal is negligible.

the concentration range for incubation, we could not exeplicitely rule out additional labeling of hEP_4R, which are simultaneously located on human platelets.

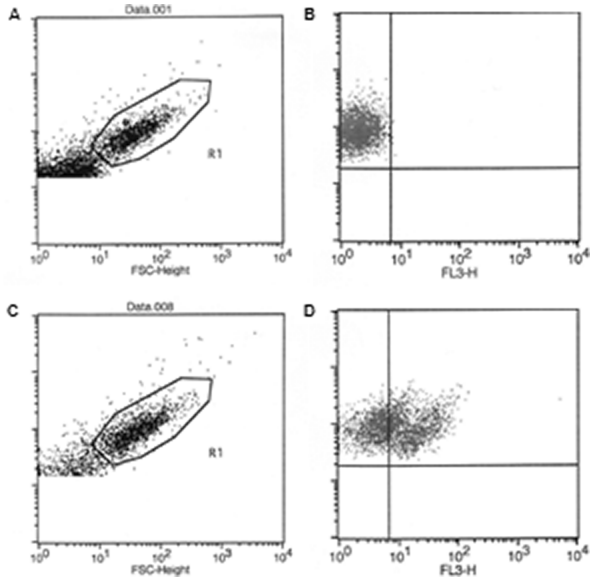


Figure 8. FACS assay with PRP preincubated with **8**. (A) Untreated PRP was measured. (B) Untreated PRP exhibits no fluorescence signal in the red fluorescence channel. (C) PRP preincubated for 10 min with 5 μM **8**. The cloud of PRP is not changed as compared to untreated PRP, indicating no platelet activation by **8**. (D) The cloud of labeled platelets is detectable in the red fluorescence channel after preincubation for 10 min with 5 μM pyryllium-labeled hEP_3R ligand **8**.

To test the antiplatelet effects of our novel fluorescent hEP_3R ligands, all compounds were analyzed in a platelet aggregation assay with collagen (0.25 μg/mL) together with PGE_2 (1 μM) as described previously.[8] After preincubation of human platelet rich plasma (PRP) with 100 nM hEP_3R ligand, PGE_2 and collagen-induced aggregation were inhibited for 40 up to 96% (Figure 9).

In conclusion, novel hEP_3R ligands labeled via dimethyleneoxy spacer with fluorophore moieties were designed. All synthesized structures possess hEP_3R affinities in the nanomolar concentration range like the parent compounds **1** and **2**. All ligands of our series inhibit PGE_2 and collagen-induced platelet aggregation in different magnitude. Optimization of fluorophore structures from lipophilic, small molecule, blue fluorescent cyanoisondole, and Sanger's reagent group, over

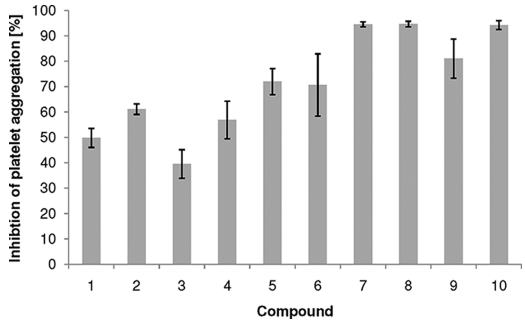


Figure 9. Inhibition of platelet aggregation induced by collagen and PGE2 after preincubation with hEP_3R antagonists. PRP was preincubated with appropriate hEP_3R ligand for 10 min. Platelet aggregation was induced with 0.25 μg/mL collagen and 1 μM PGE_2. Platelet aggregation was measured by light transmission aggregometry and compared with unspiked PRP probes.

acidic and polar, green fluorescent FITC groups resulted in red fluorescent pyryllium-labeled hEP_3R ligand **8**, which was excellent and applicable in receptor visualization by confocal laser scanning microscopy on HT-29 cells and murine kidney and human brain tissues. In addition, **8** facilitates hEP_3Rs detection on human platelets. Compound **8** is to our knowledge the first fluorescent-labeled hEP_3R ligand with potential as a pharmacological tool. Further studies and research will optimize the selectivity toward the four EP receptors, especially toward hEP_3 and hEP_4.

■ ASSOCIATED CONTENT

Supporting Information

Experimental data, chemical analysis, and further fluorescence images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Christine Elbert for sections of human brain tissues and Merck Millipore for assay support. Kind support from Hessian LOEWE programs OSF and NeFF is acknowledged.

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