The Orphan Receptor GPR17 Is Unresponsive to Uracil Nucleotides and Cysteinyl Leukotrienes

Katharina Simon, Nicole Merten, Ralf Schröder, Stephanie Hennen, Philip Preis, Nina-Katharina Schmitt, Lucas Peters, Ramona Schrage, Celine Vermeiren, Michel Gillard, Klaus Mohr, Jesus Gomez, and Evi Kostenis


Received December 16, 2016; accepted March 1, 2017

ABSTRACT

Pairing orphan G protein–coupled receptors (GPCRs) with their cognate endogenous ligands is expected to have a major impact on our understanding of GPCR biology. It follows that the reproducibility of orphan receptor ligand pairs should be of fundamental importance to guide meaningful investigations into the pharmacology and function of individual receptors. GPR17 is an orphan receptor characterized by some as a dualistic uracil nucleotide/cysteinyl leukotriene receptor and by others as inactive toward these stimuli altogether. Whereas regulation of central nervous system myelination by GPR17 is well established, verification of activity of its putative endogenous ligands has proven elusive so far. Herein, we report that uracil nucleotides and cysteinyl leukotrienes do not activate human, mouse, or rat GPR17 in various cellular backgrounds, including primary cells, using eight distinct functional assay platforms based on label-free pathway-unbiased biosensor technologies, as well as canonical second-messenger or biochemical assays. Appraisal of GPR17 activity can be accomplished with neither the coapplication of both ligand classes nor the exogenous transfection of partner receptors nucleotide purinergic G protein–coupled receptor, cysteinyl leukotriene 1, to reconstitute the elusive pharmacology. Moreover, our study does not support the inhibition of GPR17 by the marketed antiplatelet drugs cangrelor and ticagrelor, previously suggested to antagonize GPR17. Whereas our data do not disagree with a role of GPR17 per se as an orchestrator of central nervous system functions, they challenge the utility of the proposed (ant)agonists as tools to imply direct contribution of GPR17 in complex biologic settings.

Introduction

G protein–coupled receptors (GPCRs) constitute the largest family of membrane receptors in the cell. By functioning as sensors for extracellular stimuli, they are involved in a broad variety of physiologic phenomena and, therefore, belong to the most common targets of pharmaceutical drugs, although only a small percentage of GPCRs are targeted by current therapies (Rask-Andersen et al., 2011). For about 25% of the more than 400 nonolfactory GPCRs, a defined physiologically relevant ligand is still lacking (Chung et al., 2008). These receptors are known as orphan GPCRs. Deorphanization and identification of their in vivo roles are essential to clarify novel regulatory mechanisms and, consequently, to disclose novel drug targets. Whether GPR17 is such an orphan receptor is still a matter of debate (Davenport et al., 2013; Harden, 2013; Qi et al., 2013) (http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=88).

The original deorphaning report introduced the possibility that GPR17 might represent a dualistic receptor responding to two classes of endogenous signaling molecules: cysteinyl leukotrienes and uracil nucleotides (Ciana et al., 2006). Whereas this receptor ligand assignment agrees well with the phylogenetic classification of GPR17, which is located intermediate between the nucleotide purinergic G protein–coupled receptors (P2Y) and the cysteinyl leukotriene receptors, it is not yet accepted by the scientific community (Blasius et al., 1998; Heise et al., 2000; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Davenport et al., 2013; Harden, 2013; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014). More precisely, discordant findings have been reported on the nature of its cognate endogenous ligands.

---

**ABBRVIATIONS:**
- BRET, bioluminescence resonance energy transfer
- CysLT1, cysteinyl leukotriene 1
- CysLT1R, cysteinyl leukotriene 1 receptor
- DMEM, Dulbecco’s modified Eagle’s medium
- DMR, dynamic mass redistribution
- DMSO, dimethyl sulfoxide
- ERK1/2, extracellular signal-regulated kinase 1 and 2
- GPCR or GPR, G protein–coupled receptor
- IP, inositol phosphate
- LTC4, leukotriene C4
- LTD4, leukotriene D4
- MDL29,951, 2-carboxy-4,6-dichloro-1H-indole-3-propionic acid
- P2Y receptors, purinergic G protein–coupled receptors

Downloaded from molpharm.aspetjournals.org at ASIET Journals on June 29, 2021
with an ongoing controversy over their true identity (Bläsius et al., 1998; Heise et al., 2000; Ciana et al., 2006; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014). To date, 10 years after the original deorphaning report (Ciana et al., 2006), only a single independent study has confirmed the activation of GPR17 by uracil nucleotides and yet failed to recapitulate activation by cysteinyl leukotrienes (Benned-Jensen and Rosenkilde, 2010). Although these authors did indeed note statistically significant signaling upon challenge of GPR17 with uracil nucleotides, they questioned the biologic relevance of this receptor-ligand pairing because of the low-efficacy responses observed in their functional assays (Benned-Jensen and Rosenkilde, 2010). Regardless, GPR17 deorphanization must be considered tentative at this point because the number of contradictory reports (Bläsius et al., 1998; Heise et al., 2000; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014) clearly and continuously outnumbers the independent follow-up studies (Benned-Jensen and Rosenkilde, 2010) and, therefore, leaves the original deorphaning report unconfirmed.

In principle, pairings of orphan GPR17 with its endogenous agonists are findings that should be considered particularly significant because they supply the scientific community with tools to explore the pharmacology and function of this receptor. Endogenous agonists may also be used to identify antagonist ligands that provide starting points for studying receptor pharmacology and physiology. With endogenous and surrogate (ant)agonist ligands at hand, fundamental questions related to GPR17 biology may in principle be addressed in various in vitro/in vivo paradigms; however, interpretation is connected with such studies if important conclusions are based on the application of pharmacologic tools that do not reliably activate or inhibit GPR17, with the disadvantage of misleading an entire research field.

Given these apparent ambiguities, we investigated the potential interaction between orphan GPR17 and its putative endogenous ligands, cysteinyl leukotrienes and uracil nucleotides, along with a small-molecule surrogate agonist, 2-carboxy-4,6-dichloro-1H-indole-3-propionic acid (MDL29,951), in great detail (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016). Moreover, as purported endogenous ligands were used to identify GPR17 inhibitors (Ciana et al., 2006; Gelosa et al., 2014), we included these in our investigations. We undertook a highly systematic approach involving multitiered functional analysis of human, rat, and mouse GPR17 in receptor pharmacology and physiology. With endogenous and antagonist (ant)agonist ligands at hand, fundamental questions related to GPR17 biology may in principle be addressed in various in vitro/in vivo paradigms; however, interpretation is connected with such studies if important conclusions are based on the application of pharmacologic tools that do not reliably activate or inhibit GPR17, with the disadvantage of misleading an entire research field.

Given these apparent ambiguities, we investigated the potential interaction between orphan GPR17 and its putative endogenous ligands, cysteinyl leukotrienes and uracil nucleotides, along with a small-molecule surrogate agonist, 2-carboxy-4,6-dichloro-1H-indole-3-propionic acid (MDL29,951), in great detail (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016). Moreover, as purported endogenous ligands were used to identify GPR17 inhibitors (Ciana et al., 2006; Gelosa et al., 2014), we included these in our investigations. We undertook a highly systematic approach involving multitiered functional analysis of human, rat, and mouse GPR17 in receptor pharmacology and physiology. With endogenous and antagonist (ant)agonist ligands at hand, fundamental questions related to GPR17 biology may in principle be addressed in various in vitro/in vivo paradigms; however, interpretation is connected with such studies if important conclusions are based on the application of pharmacologic tools that do not reliably activate or inhibit GPR17, with the disadvantage of misleading an entire research field.

Given these apparent ambiguities, we investigated the potential interaction between orphan GPR17 and its putative endogenous ligands, cysteinyl leukotrienes and uracil nucleotides, along with a small-molecule surrogate agonist, 2-carboxy-4,6-dichloro-1H-indole-3-propionic acid (MDL29,951), in great detail (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016). Moreover, as purported endogenous ligands were used to identify GPR17 inhibitors (Ciana et al., 2006; Gelosa et al., 2014), we included these in our investigations. We undertook a highly systematic approach involving multitiered functional analysis of human, rat, and mouse GPR17 in receptor pharmacology and physiology. With endogenous and antagonist (ant)agonist ligands at hand, fundamental questions related to GPR17 biology may in principle be addressed in various in vitro/in vivo paradigms; however, interpretation is connected with such studies if important conclusions are based on the application of pharmacologic tools that do not reliably activate or inhibit GPR17, with the disadvantage of misleading an entire research field.
MgCl₂, 100 mM NaCl, 3.25°C in 0.2 ml of a 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM inhibitors, cells were preincubated with antagonists for 30 minutes.

Phosphorylated extracellular signal regulated kinases 1 and 2 (ERK1/2) were second-messenger inositol phosphate 1 (IP₁) and levels of phosphor-

1 and 2 Phosphorylation Assays.

Riss, Germany). For analysis of receptor inhibitors, these were loaded with calcium 5 indicator dye for 45 minutes. Twenty-four hours postseeding, cells were hM1-CHO, hGPR17-CHO) per well into black 96-well tissue culture plates with clear bottom. Twenty-four hours postseeding, cells were a density of 60,000 cells (hGPR17-HEK293, mGPR17-HEK293, rGPR17-HEK293, native HEK293) or 50,000 cells (hGPR17-1321N1, hM1-CHO, hGPR17-CHO) per well into black 96-well tissue culture plates with clear bottom. Twenty-four hours postseeding, cells were loaded with calcium 5 indicator dye for 45–50 minutes, stimulated and intracellular calcium flux was detected with the FlexStation 3 Multimode Benchtop Reader (Molecular Devices, Biberach an der Riss, Germany). For analysis of receptor inhibitors, these were injected 30 minutes before agonist application.

Homogeneous Time-Resolved Fluorescence-Based cAMP Accumulation and Extracellular Signal Regulated Kinases 1 and 2 Phosphorylation Assays. Changes in the intracellular second-messenger inositol phosphate 1 (IP₁) and levels of phosphorylated extracellular signal regulated kinases 1 and 2 (ERK1/2) were quantified using a Mithras LB 940 multimode reader (Berthold Technologies, Bad Wildbad, Germany) using the homogeneous time-resolved fluorescence–IP₁ kit and the homogeneous time resolved fluorescence HTRF Cellular Kit (Ciba International, Codoret, France), respectively, according to the manufacturers’ instructions and as described previously in detail. To estimate the potency of inhibitors, cells were preincubated with antagonists for 30 minutes (Bock et al., 2012).

BRET² Assays. To estimate β-arrestin2 recruitment to hGPR17, hGPR17-BRET-HEK293 cells stably expressing hGPR17-Rluc as energy donor and β-arrestin2–GFP as energy acceptor were preincubated with compounds for different time points (1.5 minutes, 5 minutes, 10 minutes, 15 minutes, 30 minutes, and 60 minutes) before the addition of the Renilla luciferase substrate (DeepBlueC coelenterazine; Gold Biotechnology, St. Louis, MO) and measurement of BRET signal using the Mithras LB 940 multimode reader. For the detection of G-protein subunit rearrangements, HEK293 cells were transiently cotransfected with cDNA for hGPR17, Go₁q–91Rluc, GPR₁–G₂y, and the complementary subunit Gβ, using FuGENE HD transfection reagent (Promega, Mannheim, Germany). Forty-eight hours after transfection, BRET² between Go₁q and G₂y was detected 2 minutes after agonist exposure as described in detail previously (Gales et al., 2006; Bock et al., 2012).

Label-Free Assays (Dynamic Mass Redistribution, Bioimpedance). Dynamic mass redistribution (DMR) was recorded using the Corning Epic (Corning, Corning, NY) biosensor or the PerkinElmer Ensite (PerkinElmer, Waltham, MA) in conjunction with the Cybi-SELMA semiautomated electronic pipetting system (Analytik Jena AG, Jena, Germany) as described previously (Schröder et al., 2010, 2011, Simon et al., 2016). For bioimpedance measurements using the CellKey system (Molecular Devices, Biberach an der Riss, Germany), cells were seeded at a density of 12,000 cells (CHO and 1321N1) and 18,000 cells (HEK293) per well into 384-well microtitr plates and grown to confluence for 16 hours (37°C, 5% CO₂). Before the assay, cells were washed twice with Hanks’ balanced salt solution buffer containing 20 mM Hepes and incubated for at least 1 hour in the DMR reader for temperature equilibration. The sensor plate was scanned for 5 minutes to record a baseline optical signature and then compound solutions were transferred into the biosensor plate to monitor bioimpedance changes for at least 1200 seconds. The effects of antagonists were observed for 1 hour before agonist addition.

Data Analysis. Data points were fitted to both three-parameter (fixed Hill slope) and four-parameter nonlinear regression isotherms using Prism 5.00 or 6.05 (GraphPad Software, San Diego, CA). Quantification of DMR and bioimpedance signals was performed by calculation of the maximum response or the area under the curve between at least 0 and 1200 seconds. All data presented are mean values ± S.E.M. of n (number) independent experiments, unless stated otherwise. Comparison between two experimental groups was performed with a two-way analysis of variance with Bonferroni multiple comparison test. P value significance thresholds were *P < 0.05, **P < 0.001, and ***P < 0.0001. For statistical reasons, the n (number) of each individual compound in the different figure panels is listed in Supplemental Tables 1 and 2.

Results

Uracil Nucleotides and Cysteinyl Leukotrienes Do Not Activate Orphan GPR17 in Three Recombinant Cell Hosts Stably Expressing hGPR17. To study possible activation by uracil nucleotides and cysteinyl leukotrienes of GPR17, we initially used two techniques: loading of hydrolysis-resistant [³²S]GTPyS onto the α subunit of hetero-

trimeric GTP binding proteins and mobilization of Ca²⁺ from intracellular stores. Both methods were used successfully in the original deorphaning report (Ciana et al., 2006); however, incubation of membranes collected from 1321N1 astrocytoma cells that stably express the short isoform of human GPR17 (hGPR17) with the putative endogenous ligands did not unveil detectable [³²S]GTPyS incorporation. Lack of activity in response to these ligands was not due to deficient GPR17 expression or function because MDL29,951, a synthetic GPR17 agonist (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016), promoted robust and concentration-dependent increase of [³²S]GTPyS binding over basal levels (Fig. 1A). Because GTP exchange is an event proximal to receptor activation, we wondered whether a low degree of signal amplification might have precluded detection of low-efficiency agonists in this assay. Akin to the results obtained from [³²S]GTPyS binding, effects of MDL29,951 were not mimicked by the endogenous ligands in fluorescence-based Ca²⁺ measurements despite the large degree of signal amplification in this assay (Fig. 1B). Because no evidence of GPR17 coupling to Go₁q or Gqₙ upon stimulation with the putative endogenous ligands was obtained, we took advantage of label-free whole-cell recordings, which are powerful biosensor methods to report cell activation in real-time by stimulated GPCRs regardless of their primary signaling pathway (Verdonk et al., 2006; Schröder et al., 2010, 2011). Yet, no indication of cell activity was observed in optical, DMR-based biosensor
recordings for all five endogenous ligands contrasting with the robust and concentration-dependent DMR traces of cells exposed to MDL29,951 (Fig. 1, C–I). Analogous results were obtained in label-free bioimpedance measurements where hGPR17-1321N1 cells were brought in contact with gold electrodes embedded in microtiter plates whereby the cell layer’s change in electrical impedance was followed over time after application of the activating stimuli (Fig. 1, J–P). MDL29,951 and all five endogenous ligands were completely inactive in label-free optical and impedance-based recordings on native 1321N1 cells, confirming GPR17 specificity of MDL29,951 action and ruling out that inactivity of endogenous

---

**Fig. 1.** Functional analysis of activated GPR17 in response to the small-molecule surrogate agonist MDL29,951 and a panel of putative endogenous activators in hGPR17-1321N1 cells. (A) GPR17-mediated G protein activation is captured by [35S]GTPγS binding to membranes collected from hGPR17-1321N1 cells (n = 3–5). (B) Concentration-effect curves of intracellular Ca2+ flux triggered by MDL29,951, UDP, UDP-gal, UDP-glc, LTC4, and LTD4 (n = 3–5). (C–H) Label-free real-time traces of MDL29,951 (C), LTC4 (D), LTD4 (E), UDP (F), UDP-gal (G), and UDP-glc (H) recorded with an optical biosensor based on detection of DMR. (I) Concentration-effect curves of compound-induced DMR responses (C–H) (n = 4–6). (J–O) Label-free bioimpedance sensing of MDL29,951 (J) and proposed endogenous ligands (K–O) in living hGPR17-1321N1 cells. (P) Concentration-effect relationships calculated from impedance recordings (J–O) (n = 4–6). Label-free recordings are shown as representative traces (mean ± S.E.M.); each trace is an average of three replicates. Concentration-effect curves are mean values ± S.E.M.
ligands in hGPR17-1321N1 cells is due to compensation of activity profiles with opposing deflection in this cellular background (Fig. 2). We also examined the possibility that GPR17 function might be attenuated by uracil nucleotides and cysteinyl leukotrienes. To this end, hGPR17-1321N1 cells were preincubated with increasing concentrations of each endogenous ligand before stimulation with MDL29,951 at its EC80. Supplemental Fig. 1 shows that the integrated cellular response observed with MDL29,951 was essentially unaffected by the presence of each compound. Thus, canonical second-messenger and innovative label-free biosensor recordings do not indicate any type of functional interaction between UDP, UDP-glucose, UDP-galactose, leukotriene C4 (LTC4), as well as leukotriene D4 (LTD4) and orphan GPR17.

The same set of experiments was recapitulated in an independent cell line, hGPR17-CHO, to ensure that the negative results were not a consequence of the expression system. Akin to the results obtained with the hGPR17-1321N1 cells, a combination of membrane-based [35S]GTPγS binding (Fig. 3A), intact-cell Ca2+ mobilization (Fig. 3B), real-time label-free DMR (Fig. 3, C–I) and impedance-based assays (Fig. 3, J–P) permitted only the detection of cell activation by MDL29,951. Although we noted cellular activity in response to some high concentrations of UDP in both DMR and impedance recordings (Fig. 3, F and M), these traces were not GPR17-specific because they also occurred in cells deficient in GPR17 expression (Supplemental Fig. 2, D and J). Contrary to the lack of activity of all five endogenous ligands in hGPR17-1321N1 and -CHO cells, uracil nucleotides and cysteinyl leukotrienes markedly activated their cognate receptors P2Y14 and CysLT1, respectively, confirming both the chemical integrity of the applied ligands and the validity of assay design and performance (Fig. 4).

To exclude the possibility that the commonly used mammalian 1321N1 and CHO cell hosts lack cellular components that might be required for detection of agonist activity of the purported endogenous activators, we also investigated potential stimulation of GPR17 stably expressed in HEK293 cells. We initially examined cellular localization of GPR17 because we are aware of orphan receptors (e.g., GPR18) that show predominant intracellular expression in stably transfected but surface expression in transiently transfected HEK293 cells (Finlay et al., 2016). Unlike GPR18, however, GPR17 displayed a considerable surface-resident population (Fig. 5A). In spite of significant surface expression, label-free DMR and impedance assays failed to report GPR17 activation in response to all five endogenous ligands (Fig. 5, C–H and J–O). By contrast, prominent real-time activation profiles were evoked with MDL29,951, confirming functional expression of GPR17 in this cell line (Fig. 5, B, I, H, and O). Control experiments in which hGPR17 was not transfected showed no significant cell activation in both DMR and impedance recordings (Supplemental Fig. 3). These data ascertain MDL29,951 as a GPR17-specific probe in the HEK293 cell background and both label-free platforms as suitable for unraveling potential biologic effects set in motion via
activation of this orphan receptor. After our multitiered approach, in this expression system also, we failed to detect agonist activity in Ca\textsuperscript{2+} and a BRET–based live-cell G\textsubscript{a}i assay conceived with G\textsubscript{a}i-R\textsubscript{Luc} as energy donor, GFP\textsubscript{10}-G\textsubscript{g}\textsubscript{2} as energy acceptor, along with unlabeled G\textsubscript{b}1 (Fig. 6, A and B) (Galés et al., 2006; Bock et al., 2012). Activation of numerous GPCRs converges on the level of ERK1/2, a key signaling molecule that is consistently activated in cells by G\textsubscript{aq}, G\textsubscript{ai}, and some G\textsubscript{as} GPCRs, but also in an arrestin-dependent, G protein–independent manner (Goldsmith and Dhanasekaran, 2007; Rajagopal et al., 2010; Rakesh et al., 2010; Reiter et al., 2012). Therefore, the detection of ERK1/2 phosphorylation might provide an advantage to capture signaling of the purported endogenous ligands via pathways that were not
Uracil nucleotides and cysteinyl leukotrienes are robust activators of their cognate receptors P2Y14 and CysLT1, respectively. (A–C) DMR recordings of UDP (A), UDP-gal (B), and UDP-glc (C) in HEK293 cells stably expressing the human ortholog of the P2Y14 receptor. (D) Concentration-effect curves of the DMR traces (A–C) are depicted \((n = 3)\). (E) Concentration-effect relationships of Ca^{2+} flux mediated by LTC4 and LTD4 on HEK293 cells stably expressing the hCysLT1 receptor \((n = 3 \pm 5)\). (F and G) Label-free real-time traces of LTC4 (F) and LTD4 (G) in hCysLT1 receptor expressing HEK293 cells. (H) Concentration-effect curves from the DMR assays depicted in (F and G) \((n = 3 \pm 4)\).

**Fig. 4.** Uracil nucleotides and cysteinyl leukotrienes are robust activators of their cognate receptors P2Y14 and CysLT1, respectively. (A–C) DMR recordings of UDP (A), UDP-gal (B), and UDP-glc (C) in HEK293 cells stably expressing the human ortholog of the P2Y14 receptor. (D) Concentration-effect curves of the DMR traces (A–C) are depicted \((n = 3)\). (E) Concentration-effect relationships of Ca^{2+} flux mediated by LTC4 and LTD4 on HEK293 cells stably expressing the hCysLT1 receptor \((n = 3 \pm 5)\). (F and G) Label-free real-time traces of LTC4 (F) and LTD4 (G) in hCysLT1 receptor expressing HEK293 cells. (H) Concentration-effect curves from the DMR assays depicted in (F and G) \((n = 3 \pm 4)\).

yet addressed experimentally. Robust phospho-ERK1/2 production was evoked by treating cells with MDL29,951 but not with endogenous stimuli, which were completely inactive (Fig. 6C). These results contrast sharply with the prominent ERK1/2 phosphorylation we observed when all endogenous ligands were acting via their cognate receptors P2Y14 and CysLT1, respectively (Fig. 6D and E). In agreement with these findings, BRET-based detection of induced interaction between GPR17 and β-arrestin2 indicated strong activity of MDL29,951 but inactivity of all five endogenous ligands (Fig. 6F). As β-arrestin recruitment is perceived as a distinct intracellular signaling route (Walters et al., 2009; Rajagopal et al., 2010; Violin et al., 2010; Reiter et al., 2012), our results exclude the possibility that uracil nucleotides and cysteinyl leukotrienes are biased toward β-arrestin over classic G protein pathways.

**Uracil Nucleotides and Cysteinyl Leukotrienes Do Not Activate Mouse and Rat GPR17.** Since marked differences in ligand pharmacology have been encountered with species orthologs of GPCRs (Milligan, 2011; Hudson et al., 2013; Strasser et al., 2013), we investigated the possibility that uracil nucleotides and cysteinyl leukotrienes might eventually serve to stimulate rodent GPR17 orthologs. To this end, we compared label-free whole-cell activity profiles evoked by MDL29,951 and the endogenous ligands in HEK293 transfectants stably expressing rat and mouse GPR17 (rGPR17, mGPR17) (Fig. 7, A and B). Marked and concentration-dependent activation of rGPR17 and mGPR17 was triggered by MDL29,951, ensuring accurate function of both orthologs in this expression system (Fig. 7; C, I, J, and P). Endogenous ligands were inactive at all applied concentrations (Fig. 7; D–I and K–P). In agreement with the inactivity of uracil nucleotides and cysteinyl leukotrienes on recombinant rodent GPR17 orthologs, the same activity pattern was replicated in the immortalized murine oligodendrocyte cell line Oli-neu (Fig. 8, A–G) and in primary rat oligodendrocytes using whole-cell DMR recordings (Fig. 8, H–N). Both cell lines endogenously express GPR17 during differentiation and were functionally responsive to MDL29,951 in a concentration-dependent and GPR17-specific manner (Fig. 8, A, G, H, and N) (Hennen et al., 2013; Simon et al., 2016). We conclude that uracil nucleotides and cysteinyl leukotrienes are completely inactive on both native and recombinant rodent GPR17 orthologs and are, therefore, inappropriate for exploring the function of this receptor in rodent cells or tissues.

**Antiplatelet Drugs Ticagrelor and Cangrelor Do Not Inhibit Human, Mouse, and Rat GPR17.** Because of the great discrepancies between the original deorphaning and essentially all subsequent studies, including our own, concerning the responses of human, rat, and mouse GPR17 to uracil nucleotides and cysteinyl leukotrienes, we also reassessed the antagonist activity of ligands previously reported to inhibit GPR17 (Ciana et al., 2006; Gelosa et al., 2014). Among these are cangrelor and ticagrelor, two drugs marketed for the inhibition of platelet aggregation (Ingall et al., 1999; Nicholas, 2001; Qamar and Bhatt, 2016) and used in extensive in vivo investigations by virtue of their purported capacity to inhibit GPR17 (Lecca et al., 2008; Ren et al., 2012). Yet, we noted that akin to literature on GPR17 agonists, literature on antagonists is both relatively limited and inconsistent [compare Ciana et al. (2006) with Hennen et al. (2013)]. Interestingly, using three distinct functional assay platforms we found that cangrelor and ticagrelor were completely inactive as inhibitors of MDL29,951-stimulated human, mouse, and rat GPR17 (Fig. 9). Pranlukast, in contrast, an antiasthma medicine originally described as CysLT1 receptor antagonist, fully reversed the agonist action of an EC_{50} concentration of MDL29,951 in Ca^{2+} mobilization assays at either ortholog (Fig. 9, A–C) and did so—albeit less efficiently—in the other assay platforms (Fig. 9, D–I). As control, cangrelor and ticagrelor were examined for inhibition of their cognate P2Y12 receptor, the function of which was effectively attenuated (Supplemental Fig. 4). Thus, our data suggest that the CysLT1 inhibitor pranlukast, but not the antiplatelet drugs cangrelor and ticagrelor, serves to attenuate function of MDL-activated GPR17.
Signaling Complexes Composed of GPR17 and a Partner Receptor Do Not Explain its Enigmatic Pharmacology. As we failed to confirm inhibition of GPR17 by cangrelor and ticagrelor, we questioned whether the inhibition observed previously might have been due to coexpression of GPR17 and P2Y12 in the cell system under investigation. Following this line of thought, a GPR17-P2Y12 receptor signaling unit might also be the pharmacological entity responding to uracil nucleotides and cysteinyl leukotrienes. We chose whole-cell phenotypic label-free DMR biosensor assays to test this assumption because coexpressed receptors may modulate each other’s signaling profile via heteromer formation or cross-talk (Milligan and Bouvier, 2005; Ellis et al., 2006; Sedej et al., 2012; Vischer et al., 2015), but holistic

![Signaling Complexes Composed of GPR17 and a Partner Receptor Do Not Explain its Enigmatic Pharmacology](image_url)

**Fig. 5.** Membrane-localized GPR17 is functionally responsive to MDL29,951 but not uracil nucleotides and cysteinyl leukotrienes in stable hGPR17-HEK293 transfectants. (A) Immunocytochemical localization of human GPR17 in hGPR17-HEK293 cells. Permeabilized cells were treated with an antibody directed against the C terminus of GPR17 and then incubated with a Cy3-conjugated goat anti-rabbit antibody. Scale bar = 20 μm. (B–G) Label-free real-time traces of MDL29,951 (B), LTC4 (C), LTD4 (D), UDP (E), UDP-gal (F), and UDP-glc (G) recorded with an optical DMR biosensor. (H) Quantification of DMR responses as concentration-effect curves (n = 4 or 5). (I–N) Activity of MDL29,951 (I) and putative endogenous activators (J–N) as determined in whole-cell label-free bioimpedance sensing. (O) Concentration-effect relationships from impedance assays depicted in (I–N) (n = 4–8). Label-free signatures are shown as representative traces (mean + S.E.M.), measured in triplicate. Concentration-effect curves are mean values ± S.E.M.
Label-free assays are pathway-unbiased and should, therefore, be ideally suited to visualize impacts of coexpression on quantitative or qualitative aspects of receptor signaling (Supplemental Fig. 5). In cells coexpressing both receptors, we evoked distinct activity patterns with the GPR17 agonist MDL29,951, the P2Y12 agonist ADP, but not cysteinyl leukotrienes or UDP-glucose, with the latter inducing only a hint of agonism at the very high concentration of 100 μM (Supplemental Fig. 5, A–C). From these data, we conclude that coexpression of GPR17 with P2Y12 in the same cell does not form the elusive molecular entity that helps to reconcile the inconsistent data surrounding GPR17 and its putative endogenous ligands. We then performed analogous experiments using HEK293 cells transfected to stably coexpress GPR17 and CysLT1 (Supplemental Fig. 5, D–F). Functional expression of each receptor was verified by robust whole-cell activity profiles evoked upon application of individual receptor agonists (Supplemental Fig. 5, D and E). Therefore, inability to trigger whole-cell responses with uracil nucleotides indicates that formation of a GPR17-CysLT1 signaling unit does not account for the elusive GPR17 pharmacology: the agonist specificity of coexpressed receptors does not differ from that observed in the absence of a partner receptor.

**Activation of GPR17 Does not Depend on the Simultaneous Presence of Uracil Nucleotides and Cysteinyl Leukotrienes.** Finally, we also explored the possibility that cysteinyl leukotrienes and uracil nucleotides, functionally inert on GPR17 when applied alone, might evoke a cellular response upon coadministration. LTD4 and UDP-glucose, in particular, have already been coadministered to specifically target GPR17 in vivo with a novel paradigm on satiety control by GPR17 emerging from these experiments (Ren et al., 2012). Because this conclusion was centered in part on promotion of food intake by pharmacologic ligands that do not activate GPR17 in their own right, we tested the hypothesis that orphan GPR17 requires a cocktail of endogenous ligands to boost full functional activation. To this end, we expressed GPR17 and CysLT1, alone and in combination, and exposed each transfectant to LTD4 in conjunction with each uracil nucleotide (Fig. 10, A–D), but also to GPR17 agonist MDL29,951 (Fig. 1, E–H), CysLT1 agonist LTD4 (Fig. 10, I–L), or carbachol as viability control (Fig. 10, M–P). Robust responses to the ligand mix were obtained only in cells expressing CysLT1 individually and in combination with GPR17 (Fig. 10, C and D) but were not seen in cells expressing GPR17 or empty vector control (Fig. 10, A and B). Whole-cell activity patterns therefore indicated substantial contribution of the CysLT1 receptor to the signal generated by the ligand mix (Fig. 10, C and D) but are incongruent with the notion that coadministration of cysteinyl leukotrienes and uracil nucleotides suffices to trigger functional activation of GPR17. Because treatment of GPR17 cells with ligand mix did not evoke any functional response, we conclude that cellular GPR17 activity does not depend on coordinated activation of a single receptor by two classes of endogenous ligands.

**Discussion**

Erroneous designation of orphan receptors and their cognate endogenous ligands may cause considerable confusion within the scientific community (Lauwers et al., 2006; Civelli et al., 2013; Rueda et al., 2016). GPR17 is such an orphan...
GPCR, the pharmacology of which should be of great interest owing to several recent studies that have implicated this receptor in demyelinating central nervous system diseases such as multiple sclerosis, but also in the regulation of food intake and aging of the brain (Lecca et al., 2008; Ren et al., 2012; Marschallinger et al., 2015). All these studies have in common that major conclusions were drawn based on utilization of ligands, activity of which at GPR17 is still ill-defined. Thus, there is pressing need to revisit the issues surrounding GPR17 pharmacology and authenticity of both activators, as

Fig. 7. Membrane-localized rodent GPR17 orthologs are unresponsive to uracil nucleotides and cysteinyl leukotrienes in label-free DMR studies. (A, B) Immunocytochemical localization of mouse GPR17 (A) and rat GPR17 (B) in HEK293 cells stably expressing these receptors. Permeabilized cells were treated with an antibody directed against the C terminus of GPR17 and then incubated with a Cy3-conjugated goat anti-rabbit antibody. Scale bars = 20 μm. (C–H) Label-free real-time DMR responses of MDL29,951 (C), LTC4 (D), LTD4 (E), UDP (F), UDP-gal (G), and UDP-glc (H) in HEK293 cells stably expressing mGPR17. (I) Concentration-effect curves calculated from the peak DMR responses in C–H (n = 3 or 4). (J–O) Label-free DMR sensing of MDL29,951 (J) and putative endogenous stimuli (K–O) in rGPR17-HEK293 cells. (P) Concentration-effect relationships from DMR assays in J–O (n = 3–5). Label-free signatures are means ± S.E.M. of representative traces, each representing the mean of three replicates. Concentration-effect curves are mean values ± S.E.M.
well as inhibitors, proposed to target this receptor. If pharmacologic tools, inert on GPR17, are being used in extensive ex vivo/in vivo investigations to uncover the physiologic functions of this receptor, conclusions drawn from such studies may mislead an entire field (Lecca et al., 2008; Ren et al., 2012; Marschallinger et al., 2015).

Originally, GPR17 was proposed as a dualistic receptor responding to two distinct classes of signaling molecules: uracil nucleotides and cysteinyl leukotrienes (Ciana et al., 2006). Whereas this receptor ligand assignment agrees well with the phylogenetic position of GPR17, located just intermediate between P2Y nucleotide and CysLT receptors (Bläsius et al., 1998; Ciana et al., 2006), the variability of GPR17 responses in a ligand- and system-dependent manner is striking (Bläsius et al., 1998; Heise et al., 2000; Ciana et al., 2006; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Davenport et al., 2013; Harden, 2013; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014). In spite of apparently identical experimental conditions, only a single independent laboratory succeeded to recapitulate activity of uracil nucleotides but not cysteinyl leukotrienes on GPR17 (Benned-Jensen and Rosenkilde, 2010). Instead, constitutive Gαi activity was proposed for GPR17, a receptor feature that was not observed in the original deorphaning report and subsequent publications from this laboratory (Ciana et al., 2006; Lecca et al., 2008; Pugliese et al., 2009).

The scenario seems even more ambiguous, taking into account another independent study showing neither constitutive nor ligand-mediated activity of GPR17 (Maekawa et al., 2009). In fact, the latter revealed an intriguing, ligand-independent regulatory role of GPR17 in suppressing the function of the leukotriene CysLT1 receptor as a dominant negative inhibitor.

Fig. 8. Label-free whole-cell DMR recordings of activated GPR17 in immortalized and primary rodent oligodendrocytes natively expressing the receptor. Label-free, real-time DMR traces of GPR17 agonist MDL29,951 and the panel of putative stimuli in the immortalized oligodendrocyte line Oli-neu (A–F) and in primary rat oligodendrocytes (H–M). Oli-neu cells were pretreated with 1 μM EGFR tyrosine kinase inhibitor PD174265 for 24 hours to induce GPR17 expression (Simon et al., 2016). (G and N) Concentration-effect curves of ligand-induced DMR responses calculated from peak maxima in Oli-neu (G) (n = 3) and primary rat oligodendrocytes (N) (n = 3–7). Shown are individual DMR recordings (mean ± S.E.M.) of triplicate determinations and concentration-effect curves (mean values ± S.E.M.) calculated for data as given in (A–F) and (H–M), respectively.
within the context of a heterodimer. In this study, inactivity of both uracil nucleotides and cysteinyl leukotrienes on GPR17 in different cellular backgrounds was communicated, but experimental data were not provided (Maekawa et al., 2009). In 2013, Qi and coworkers (2013) confirmed suppression of CysLT1 receptor function by coexpressed GPR17 but failed to recapitulate activation by all purported endogenous ligands when applied in a single high concentration (Qi et al., 2013). Apparently, researchers do not all observe activation of GPR17-mediated signaling events by the proposed endogenous signaling molecules and there is no consensus among these reports as to the ability of the individual ligand classes to activate this orphan receptor (Bläsius et al., 1998; Heise et al., 2000; Maekawa et al., 2009; Benned-Jensen and Rosenkilde, 2010; Wunder et al., 2010; Hennen et al., 2013; Qi et al., 2013; Köse et al., 2014).

From our perspective, it is highly unlikely that fundamental differences in molecular function are the bottleneck in GPR17 deorphanization; rather, GPR17 utilizes the same basic molecular mechanisms as other family A GPCRs in terms of signal transduction (Hennen et al., 2013). Consistent functional activity of MDL29,951 across all tested cell lines, species, and assay platforms attests to this (Hennen et al., 2013; Simon et al., 2016). Thus, GPR17 is either poorly behaved when it comes to functional assays with uracil nucleotides and cysteinyl leukotrienes, or the purported endogenous ligands are simply inactive. Regardless, our data clearly rule out the possibility that orphan GPR17 accounts for the direct effects reported for uracil nucleotides and cysteinyl leukotrienes. Consequently, these ligands should not be used to probe the physiology and function of GPR17 in complex systems.

Fig. 9. CysLT1 receptor antagonist pranlukast, but not P2Y12, inhibitors cangrelor and ticagrelor interdict activation of MDL29,951-stimulated GPR17. The ability of cangrelor and ticagrelor, effective as antiplatelet drugs, to dampen signaling of GPR17 orthologs from humans, rats, and mice was compared with the reported GPR17 inhibitor pranlukast in assays measuring intracellular Ca2+ flux ($n = 4–8$), IP1 accumulation ($n = 3–5$) and label-free DMR ($n = 3–7$). MDL29,951 was added to the cells at its EC80 in all cases. Data shown are means ± S.E.M.
biologic settings. The same applies to ticagrelor and cangrelor: both P2Y12 inhibitors do not blunt function of GPR17. One might argue that lack of inhibition is a consequence of agonist use in our study, but one may just as well challenge their purported GPR17 inhibition because both “antagonists” were identified when GPR17 was stimulated with inactive ligands (Ciana et al., 2006; Gelosa et al., 2014). MDL29,951, in contrast, is the first small-molecule agonist reliably activating GPR17 irrespective of the cellular background and expression system (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016). Therefore, we argue that MDL29,951 currently represents the only pharmacologic agonist probe likely to accelerate research surrounding pharmacology and function of this exciting orphan GPCR and its implication in central nervous system physiology.

It is well known that matching of GPCRs with their cognate ligands may be error-prone because GPCRs cannot be expressed and analyzed in isolation but instead require rather complex cell-based assay systems harboring an array of endogenous receptors in addition to the overexpressed receptor of interest (Atwood et al., 2011). It follows that the readout of GPCR activation is not necessarily specific to the overexpressed receptor, but rather it represents the sum signal produced by all GPCRs in the assay tube. Nevertheless, differences between the receptor overexpressing and the native cell system is interpreted as receptor-specific response.

---

**Fig. 10.** Concerted application of uracil nucleotides and cysteinyl leukotrienes does not result in functional activation of GPR17. HEK293 cells expressing GPR17 and CysLT1 alone and in combination were stimulated with LTD4 in conjunction with each uracil nucleotide (A–D), GPR17 agonist MDL29,951 (E–H), CysLT1 agonist LTD4 (I–L), or carbachol as viability control (M–P), and cellular activity was monitored with a label-free DMR biosensor. Shown are traces (mean + S.E.M.) of a single experiment representative of three such experiments. Each trace is an average of three replicates.
to form the elusive GPR17 along with its phylogenetic neighbors is not sufficient per se, they clearly indicate that coexpression of the purported GPR17 pharmacology. Whereas our data do not the presence of neither receptor was sufficient to re-establish onto GPR17 upon coexpression with these receptors; however, cells with specific cDNAs coding for the nucleotide P2Y12 but tory effects of the lipid mediator leukotriene E4 (LTE4) lated previously as complex transmitting the proinflamma-
involving P2Y12 and an unknown receptor has been postu-
lation range head-to-head with the known GPR17 agonist MDL29,951 on human and rodent orthologs using eight different functional assays platforms and five distinct cellular backgrounds. We took advantage of the known small-molecule agonist MDL29,951 (Hennen et al., 2013; Ou et al., 2016; Simon et al., 2016) to ascertain functional GPR17 expression in all cell systems and included positive controls, P2Y14 and CysLT1R, to verify ligand integrity as well as assay design and performance. In spite of this broad effort, evidence in favor of ligand-mediated responses of uracil nucleotides and cysteinyl leukotrienes remained elusive across all assays, cell lines, and receptor species. As we failed to recapitulate both activation of GPR17 by all proposed endogenous ligands and inhibition by the antiplatelet drugs ticagrelor and cangrelor, which target GPR17 by all proposed endogenous ligands and inhibition by
receptor species. As we failed to recapitulate both activation of GPR17 by all proposed endogenous ligands and inhibition by the antiplatelet drugs ticagrelor and cangrelor, which target the nucleotide P2Y12 receptor (Ingall et al., 1999; Nicholas, 2001; Qamar and Bhatt, 2016), we speculated that the elusive GPR17 pharmacology may be explained by the absence of partner receptors lacking in our assays. Indeed, a heteromer involving P2Y12 and an unknown receptor has been postulated previously as complex transmitting the proinflammato-
tory effects of the lipid mediator leukotriene E4 (LTE4) (Paruchuri et al., 2009). To test our assumption, we enriched cells with specific cDNAs coding for the nucleotide P2Y12 but also the cysteinyl leukotriene CysLT1 receptor and examined whether novel pharmacologic properties may be conferred onto GPR17 upon coexpression with these receptors; however, the presence of neither receptor was sufficient to re-establish the purported GPR17 pharmacology. Whereas our data do not rule out dimerization between GPR17 and P2Y12 as well as CysLT1R per se, they clearly indicate that coexpression of GPR17 along with its phylogenetic neighbors is not sufficient to form the elusive “receptor pair” accounting for the enigmatic pharmacology of GPR17.

We are aware that our results have not provided an explanation for the contradictory data regarding GPR17 and the functional consequences ensued by purported endogenous and surrogate ligands. Although it is conceivable that inconsistencies may be explained in part by the use of different cell expression systems, cell growth conditions, receptor expres-
sion levels, and assay methods used to assess receptor func-
tion, our comprehensive study based on numerous assay platforms that capture proximal and distal signaling events convincingly demonstrates that GPR17 is functionally expressed, yet completely inert, toward uracil nucleotides and cysteinyl leukotrienes. In view of these results, we strongly recommend that GPR17 be considered as orphan and anticipate our findings to stimulate future efforts into deorphanization of this receptor that is still in search of its true endogenous ligand(s).

Acknowledgments

The authors thank Marianne Vasmer-Ehses for expert technical assistance with conductance of the DMR experiments and Corning Inc. as well as PerkinElmer for their support on the Epic- and the Ensitks-DMR readers.

Authorship Contributions

Participated in research design: Simon, Merten, Schröder, Hennen, Preis, Schmitt, Peters, Schrage, Vermeiren.


Wrote or contributed to the writing of the manuscript: Simon, Merten, Kostenis.

References


Finlay DB, Joseph WR, Grimsey NL, and Glass M (2016) GPR18 undergoes a high degrees of constitutive trafficking but is unresponsive to N-arachidonyl glycerin. Peep 4:1835.


Gelosa P, Lecca D, Fumagalli M, Wypych D, Pignieri A, Cimino M, Verderio C, Enerback M, Nikooshahes E, Tremoli E, et al. (2014) Microglia is a key player in the redution of stroke damage promoted by the new antiinflammatory agent tica-

Goldsmith ZG and Dhaneanaskaran DN (2007) G protein regulation of MAPK net-


Supplemental Figures

THE ORPHAN RECEPTOR GPR17 IS UNRESPONSIVE TO URACIL-NUCLEOTIDES AND CYSTEINYL-LEUKOTRIENES

Katharina Simon, Nicole Merten, Ralf Schröder, Stephanie Hennen, Philip Preis, Nina-Katharina Schmitt, Lucas Peters, Ramona Schrage, Celine Vermeiren, Michel Gillard, Klaus Mohr, Jesus Gomeza, and Evi Kostenis

Journal: Molecular Pharmacology.

Supplemental Figure 1

Suppl. Fig. 1. Pharmacologically inert GPR17 agonists do not inhibit GPR17. hGPR17-1321N1 cells were pre-treated with the indicated concentrations of UDP (A), UDP-gal (B), UDP-glc (C), LTC4 (D) and LTD4 (E) prior to stimulation with MDL29,951 at its EC_{80} concentration and DMR responses were recorded as a measure of receptor activity (upper panels). Quantification of real-time DMR traces based on the peak maxima of three independent experiments shown as mean values ± S.E.M. (lower panels).
Suppl. Fig. 2. Real-time label-free activity profiles of GPR17 agonist MDL29,951 and purported endogenous GPR17 ligands in native CHO-K1 cells. MDL29,951- (A, G), LTC4- (B, H), LTD4- (C, I), UDP- (D, J), UDP-gal- (E, K) and UDP-glc-mediated (F, L) whole-cell responses recorded with an optical DMR-based biosensor (A-F) and by bio-impedance (G-L). ATP and PGE1 were applied as indicators for cell vitality. Signatures are shown as representative traces (mean + S.E.M.), each trace is an average of three replicates.
Suppl. Fig. 3. Head-to-head comparison of a set of putative endogenous GPR17 ligands and the synthetic GPR17 agonist MDL29,951 applying label-free assays in native HEK293 cells. Label-free real-time traces of MDL29,951 (A, G), LTC4 (B, H), LTD4 (C, I), UDP (D, J), UDP-gal (E, K) and UDP-glc (F, L) recorded with an optical DMR-based biosensor (A-F) and a label-free bio-impedance detection (G-L). Carbachol and ATP were applied as viability controls. Signatures are shown as representative traces (mean + S.E.M.), each trace is an average of three replicates.
Suppl. Fig. 4. Cangrelor and ticagrelor inhibit cellular DMR responses of the activated P2Y12 receptor.

Cangrelor and ticagrelor counteract ADP-induced activation of the nucleotide P2Y12 receptor upon stable expression in 1321N1 cells. Shown are means ± S.E.M. of five independent experiments each performed in triplicate.
Suppl. Fig. 5. Co-expression of nucleotide P2Y12 and CysLT1R does not confer novel pharmacology onto GPR17. HEK293 cells co-expressing GPR17 and P2Y12 (A-C) or GPR17 and CysLT1 (D-F) were stimulated with the synthetic GPR17 agonist MDL29,951 (A, D), P2Y12 agonist ADP (B), CysLT1 agonist LTD4 (E) to verify receptor expression in the co-transfection paradigm, and the indicated endogenous stimuli suggested to activate GPR17 (C, F) in label-free DMR recordings. Shown are representative traces (mean + S.E.M.) indicative of three such experiments each performed in triplicate.
Supplemental Table 1
Listing of precise sample sizes for individual figure panels to allow for interpretation of S.E.M. values. Slash denotes that a ligand was not applied.

<table>
<thead>
<tr>
<th>Fig</th>
<th>MDL29,951</th>
<th>LTC4</th>
<th>LTD4</th>
<th>UDP</th>
<th>UDP-gal</th>
<th>UDP-glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1B</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1H</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1P</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>3A</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3B</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>3I</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3P</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4D</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4E</td>
<td>/</td>
<td>5</td>
<td>3</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>4H</td>
<td>/</td>
<td>4</td>
<td>3</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>5H</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>5O</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>6A</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>6B</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>6C</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>6D</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6E</td>
<td>/</td>
<td>4</td>
<td>4</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>6F</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>7I</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>7P</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>8G</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>8N</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>
Supplemental Table 2
Listing of precise sample sizes for individual figure panels to allow for interpretation of S.E.M. values.

<table>
<thead>
<tr>
<th>Fig</th>
<th>pranlukast</th>
<th>cangrelor</th>
<th>ticagrelor</th>
</tr>
</thead>
<tbody>
<tr>
<td>9A</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>9B</td>
<td>8</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>9C</td>
<td>8</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>9D</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>9E</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>9F</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>9G</td>
<td>7</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>9H</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>9I</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>