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REVIEW ARTICLE

EP_3 RECEPTOR ANTAGONISTS: POTENTIAL FOR IMPROVING SAFETY IN THE ANTIPLATELET THERAPY FIELD

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SUMMARY

Current antiplatelet therapies offer significant benefits for the treatment of patients at risk for recurrent cardiovascular events. However, clinical data suggest that many of these agents lead to an increased risk of severe or fatal hemorrhage by affecting general platelet function. It has been suggested that targeting the inflammatory component of the disease instead may address this issue. Human genetic data from peripheral arterial occlusive disease (PAOD), heart attack and stroke converged on the identification of the EP_3 receptor as a target, linking variations in the gene encoding EP_3 to an increased risk for the disease. Several small-molecule EP_3 antagonists employed either as monotherapy or in combination with clopidogrel and aspirin have been shown to inhibit platelet aggregation at the site of lesions in the vasculature, without increasing bleeding time. This suggests that targeting the inflammatory mechanism of arterial thrombosis mediated by the prostaglandin E_2 (PGE_2)–EP_3 receptor system may lead to a new generation of antiplatelet drugs with an enhanced safety and efficacy profile.

INTRODUCTION

Peripheral arterial occlusive disease (PAOD) affects over 10% of the adult population in the industrialized world and one in five people over the age of 70. The condition is caused by the narrowing of major arteries in the legs due to atherosclerotic plaques. The reduction of blood flow leads to insufficient oxygenation of muscle tissue. The initial symptoms include intermittent pain in the legs (claudication). As the disease progresses, atherosclerotic plaques occlude distal arteries, causing ischemic ulcers and in severe cases gangrene. Surgical intervention including scission of the affected lesion(s) and their replacement with grafts has been reported to be an effective treatment for this condition (1, 2).

Current pharmacological treatments for PAOD affect platelet function in order to either address the cardiovascular component of the disease or to provide symptomatic relief from claudication. Examples of the former approach include treatment with low doses of aspirin (acetylsalicylic acid, ASA) or inhibition of the platelet adenosine diphosphate (ADP) receptor. The platelet phosphodiesterase type 3 (PDE3) inhibitor cilostazol provides symptomatic relief from claudication. This agent also increases intracellular levels of cAMP. Population genetics research conducted by deCODE in Iceland identified common versions of the *PTGER3* gene encoding EP_3 leading to a significantly increased risk of PAOD via elevated expression of EP_3.

CURRENT ANTIPLATELET AGENTS

The benefits of ASA for the prevention of heart attack are well established (3). In patients with an acute heart attack, treatment with ASA lowered the occurrence of subsequent cardiac events (4). Antagonists of the platelet purinergic $P2Y_{12}$ receptor for ADP showed additional benefits for the treatment of recurrent heart attack (5); namely, the marketed agent clopidogrel and a second-generation $P2Y_{12}$ antagonist, prasugrel, lowered the risk of myocardial infarction further when compared to ASA (6, 7). The two currently FDA-approved $P2Y_{12}$ antagonists ticlopidine and clopidogrel are thienopyridines which are metabolized through cytochrome P450 in the liver (Fig. 1). These products irreversibly antagonize the $P2Y_{12}$ receptor. Ticlopidine was the first FDA-approved $P2Y_{12}$ antagonist, but it has been replaced in clinical practice by clopidogrel. This agent displays fewer side effects (8) and is well established as an antiplatelet therapy in clinical practice (9, 10). However, clopidogrel therapy is associated with certain challenges (11), including: 1) a significant incidence of stent thrombosis in patients treated with clopidogrel and aspirin (12); 2) the phenomenon of hyporesponsiveness or "resistance" (13); and 3) the relatively slow onset of action. A number of novel $P2Y_{12}$ antagonists are therefore under investigation to determine whether they can result in better or more rapid antithrombotic effects than

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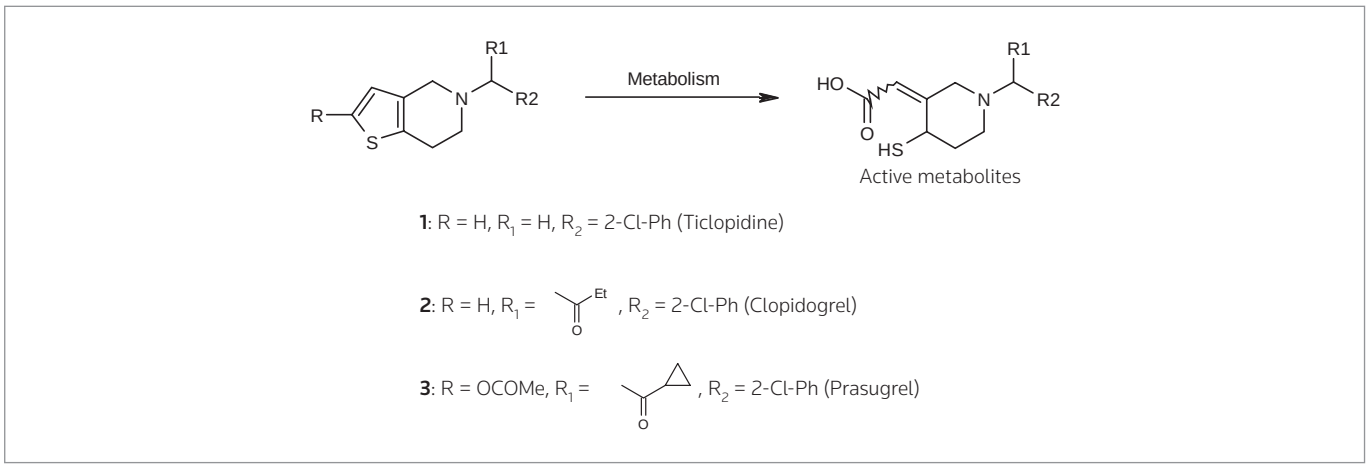

Figure 1. Thienopyridines and their active metabolites as P2Y$_{12}$ receptor antagonists.

clopidogrel, without an unacceptable increase in hemorrhagic (or other) side effects.

Prasugrel (Lilly/Daiichi Sankyo; Fig. 1) is a recently approved thienopyrimidine. This molecule is also metabolized by cytochrome P450 in the liver to irreversibly bind the P2Y$_{12}$ receptor (14-16). Prasugrel was designed to enhance in vivo generation of the active metabolite to result in a much more rapid, potent and consistent inhibition of platelet function at a dose of 60 mg compared to the standard clopidogrel loading dose of 300 mg (17). However, major bleeding was observed in 2.4% of patients receiving prasugrel (18).

Ticagrelor (AZD-6140; AstraZeneca) is an investigational competitive P2Y$_{12}$ receptor antagonist (19). This molecule is a derivative of an earlier candidate, AR-C109318XX, with enhanced bioavailability (Fig. 2) (20). Ticagrelor featured greater and more potent inhibition of platelet aggregation than clopidogrel, and it did not increase bleeding compared with clopidogrel. The molecule is given orally

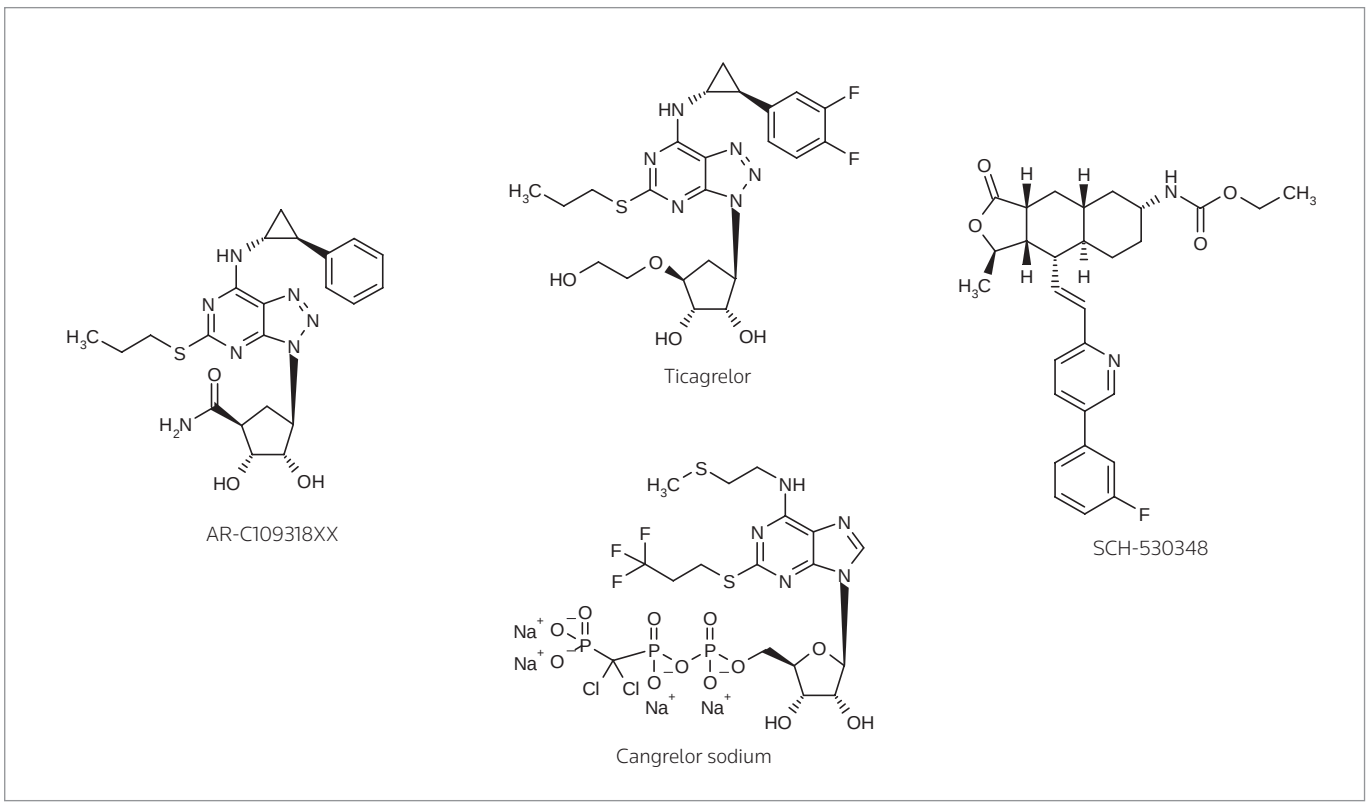

Figure 2. Additional P2Y$_{12}$ and PAR1 antagonists in clinical trials.

twice a day and is currently undergoing a phase III trial (21). Similar to ticagrelor, cangrelor (The Medicines Company) is a reversible P2Y$_{12}$ antagonist administered intravenously. The agent provided a rapid onset of action and greater degree of platelet inhibition compared to prasugrel and ticagrelor. It did not increase bleeding compared with clopidogrel in phase II studies (22).

The PAR1 receptor is activated by thrombin and signals via multiple regulatory proteins, including G_{qa}, to mobilize Ca^{2+}, triggering actin skeleton rearrangement, and G_i, inhibiting adenylate cyclase, suggesting that an antagonist would selectively inhibit thrombus formation without affecting bleeding. Indeed, the PAR1 receptor antagonist SCH-530348 (Bayer Schering Pharma) has recently been a focus of clinical studies (23, 24). The compound was generally well tolerated, without affecting bleeding; however, the studies were not powered to detect differences in clinical event rates.

As mentioned above, the development of novel antiplatelet therapies that specifically target thrombosis at the diseased vascular wall versus general amplification of the thrombosis cascade is of critical importance. It is expected that these mechanistically distinct agents would not affect normal hemostatic mechanisms. One of the strategies launched recently involves augmentation of inflammatory signaling mediated by prostanoids.

INFLAMMATION AND PLATELET ACTIVATION

Prostanoids have been described to play an essential role in vascular homeostasis, including the regulation of platelet function. In support of the physiological link between prostanoid signaling and POAD, several clinical studies suggested that treatment with prostaglandins improves symptoms of the disease (25-27). A diverse prostanoid family of molecules is formed via metabolism of arachidonic acid. There are five physiologically important prostanoids, i.e., PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. Prostanoids elicit their effect by direct modulation of respective prostanoid receptors. Eight of these have been cloned and characterized to be members of the G protein-coupled receptor (GPCR) rhodopsin-like family. The PGD_2 molecule binds preferentially to DP and FP receptors, PGE_2 to the $EP_{1\text{-}4}$ receptor subfamily, $PGF_{2\alpha}$ to the FP and EP_3 receptors, PGI_2 to the IP receptor and TXA_2 to the TP receptor. Of these, PGE_2 is of particular interest due to its distribution and confirmed effect on platelet function.

PGE_2 mediates signaling in diverse systems, including the immune, endocrine, cardiovascular, renal and reproductive systems, as well as the contraction and relaxation of smooth muscle (28). It is one of the most abundant prostanoids in the brain (27, 29), being critical to many cerebral functions, especially during development (30, 31). PGE_2 influences mitogenesis, promotes the growth and metastasis of tumors (32-34) and modulates the transcription of many genes (35-39). The biological actions of PGE_2 have been attributed to its interaction with cell-surface $EP_{1\text{-}4}$ receptors (40). Several research teams have suggested that prostaglandins may also act intracellularly to promote a nuclear action (41-45). There are currently eight known subtypes of the human EP_3 receptor, designated EP_{3A}, EP_{3B}, EP_{3C}, EP_{3D}, $EP_{3\text{-}V}$, $EP_{3\text{-}VI}$, EP_{3E} and EP_{3F} (46). Mouse (47), rat (48) and rabbit (49) homologues have also been identified for some of these EP_3 subtypes. They differ in the primary sequence of the cytoplasmic carboxyl terminus, as well as in their coupling to respective GPCRs (50).

The EP_3 receptor subtypes inhibit adenylate cyclase via the $G_{i\alpha}$ family of proteins. Four subtypes of bovine EP_3 have been cloned (designated A, B, C and D) (49). Notably, the EP_{3A} homologue signals via $G_{i\alpha}$ to inhibit adenylate cyclase, EP_{3B} and EP_{3C} receptors signal through $G_{s\alpha}$ to activate adenylate cyclase, and EP_{3D} is coupled to $G_{i\alpha}$, $G_{s\alpha}$ and $G_{q\alpha}$ to afford both inhibition and activation of adenylate cyclase, as well as the activation of phospholipase C (Table I). It was also found that the EP_3 receptor subtypes may differ in their levels of constitutive activity, as suggested by studies of the mouse isoforms of the EP_3 receptor (designated α, β and γ) (51).

Knockout of the *Ptger3* gene ($EP_3^{-/-}$) has been reported. Based on phenotypic evidence, the receptor is involved in PGE_2-induced pyrexia (52) and urinary concentrating function (53). The $EP_3^{-/-}$ mice did not secrete duodenal bicarbonate on luminal perfusion with PGE_2 relative to wild-type animals (54). A gender-specific effect of EP_3 on vasopressor responses in a murine model has been described (55). Namely, male but not female $EP_3^{-/-}$ mice exhibited more PGE_2-induced hypotension relative to wild-type mice. Mice lacking EP_3 developed pronounced allergic inflammation that was suppressed by a receptor agonist, suggesting that the PGE_2/EP_3 pathway is an important negative modulator of allergic reactions (56).

It has been suggested that regions in the third intracellular loop and in the carboxyl termini of the prostanoid receptors contribute to the specificity of GPCR–G protein interactions (57, 58). EP_3 receptors inhibit adenylate cyclase (59) and can also couple to voltage-sensitive and -insensitive Ca^{2+} and Cl^- channels (60, 61).

Analysis of RNA prepared from human platelets revealed that platelets express three of the eight human EP_3 isoforms, along with other GPCRs (62). In addition to the EP_3 family, platelets feature multiple GPCRs, including receptors for ADP and PGE_2 (Fig. 3) (63). ADP can vicariously mobilize Ca^{2+}, and through the P2Y$_{12}$ receptor can inhibit cAMP production, causing platelet activation and aggregation. Thomboxane A_2 (TXA_2) is a stimulator of platelet function, whereas prostaglandin I_2 (PGI_2) inhibits its activation. Prostacyclin and TXA_2 are of particular importance in the control of hemodynamics and hemostasis. Prostacyclin is a physiological antagonist of platelet function. It also features selectivity towards TXA_2-mediated platelet activation. The chronic inflammatory condition of atherosclerotic plaque leads to increased content of COX-2 and microsomal PGE_2 synthase (64), with consequently increased production of PGE_2 (65). Both an increase in intracellular Ca^{2+} and a decrease in cAMP are needed to induce platelet activation and aggregation. ADP signals via two GPCRs expressed on platelets, namely the P2Y$_1$ and P2Y$_{12}$ receptors (66, 67). P2Y$_1$ signals via the G_q protein to mobilize platelet Ca^{2+}, whereas P2Y$_{12}$ couples through G_i to inhibit adenylate cyclase and consequently decrease intracellular cAMP (Fig. 3) (68).

Table I. *Classification of bovine prostanoid EP_3 receptors and their signal transduction.*

Ligand	Isoform	G protein	Signal transduction
PGE_2	EP_{3A}	G_i	cAMP ↓
	EP_{3B}	G_s	cAMP ↑
	EP_{3C}	G_s	cAMP ↑
	EP_{3D}	$G_{i/s/q}$	cAMP ↓↑, PLC activation

EP_3 RECEPTOR AS ANTIPLATELET TARGET

It has been reported that PGE_2 induces opposite effects on platelet aggregation. Specifically, low levels of PGE_2 promote platelet aggregation via the EP_3 receptor. At high concentrations it activates the receptor for PGI_2, inhibiting aggregation (69, 70). To study the role of the platelet EP_3 receptor, Gross et al. provoked atherothrombosis in the carotid arteries of $ApoE^{-/-}$ mice, without interrupting blood flow, by scratching plaques with a needle (71). It has also been reported that arterial walls subjected to inflammatory stimuli produce PGE_2, which in turn facilitates arterial thrombosis. Notably, study of mouse atherosclerotic plaques suggested that PGE_2 can activate EP_3 on blood platelets. The authors noted that in this model of plaque rupture the effect was significantly reduced in the absence of EP_3. It was further suggested that EP_3 antagonism may specifically affect thrombosis resulting from small local injuries occurring at inflammatory site(s) (72). In contrast, the platelet response to traumatic vascular breach does not result in PGE_2 induction and therefore should not be affected by an EP_3 antagonist. It has been reported that arterial walls subjected to inflammatory stimuli produce PGE_2, which in turn facilitates arterial thrombosis.

To further support the antiplatelet effect of EP_3 receptor blockade, several research teams studied small-molecule agonists. For example, analogues of PGE_2 were reported to enhance platelet aggregation and inhibit adenylate cyclase (73). Administration of the selective EP_3 receptor agonist ONO-AE-248 to rats increased infarct size in the middle cerebral artery occlusion model in a dose-dependent manner (74).

On platelets, EP_3 acts synergistically with $P2Y_{12}$ through G_i to ultimately inhibit adenylate cyclase activity and thereby lower platelet cAMP (62, 75). However, facilitation of platelet aggregation via the PGE_2/EP_3 pathway is dependent on co-agonists (e.g., collagen, TXA_2, ADP) that can cause Ca^{2+} mobilization (69, 70). At low concentrations PGE_2 can synergize with TXA_2 by acting on EP_3 receptors to abrogate the protective function of prostacyclin. Both the IP_1 receptor and the PGD_2 receptor are modulated at high levels of PGE_2 to turn off platelet activation.

In three different models –arachidonic acid superfusion, $FeCl_3$-mediated endothelial damage and mechanical atherosclerotic plaque rupture– thrombosis was impaired if platelets lacked EP_3. Circulating levels of prostanoids are extremely low in healthy individuals (76), whereas the local concentration of PGE_2 can markedly increase in inflammatory states (77). Both COX-1 and COX-2 are present in arteriosclerotic plaque, but only COX-1 is present in the healthy arterial wall (63, 78). PGE_2 binding to EP_3 decreases cAMP in platelets and opposes the effect of PGI_2 by enhancing the effects of primary aggregating agents. Since the healthy arterial wall produces negligible PGE_2, the platelet EP_3 system should minimally affect bleeding time, although contradictory results have been reported for bleeding times in EP_3 gene-deleted mice (69, 70). An antagonist is expected to elicit both symptomatic and disease-modifying benefit(s) in PAOD.

DESIGN OF POTENT AND SELECTIVE EP_3 RECEPTOR ANTAGONISTS

Several research teams have reported success in developing specific EP_3 receptor antagonists. Analogues **5** and **6** introduced by Merck

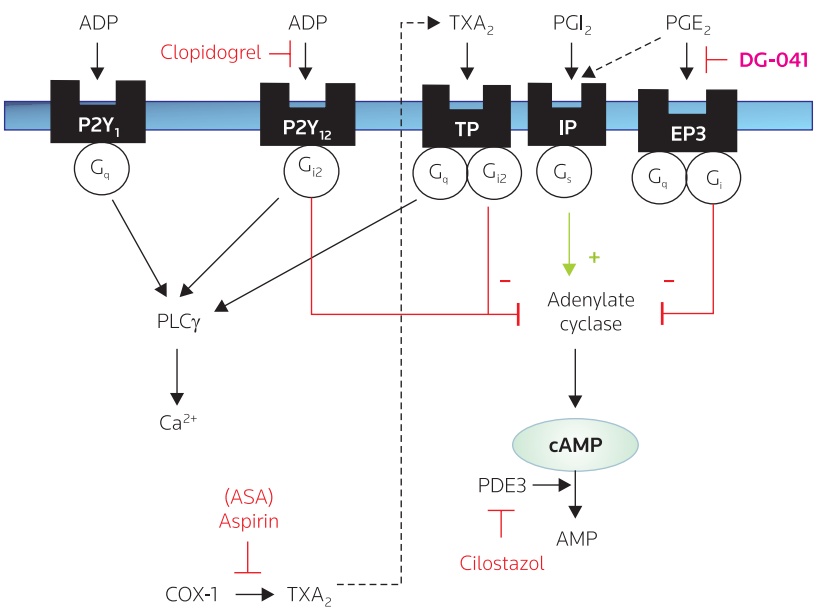

Figure 3. Amplification of a platelet response via GPCRs. ADP signals via both $P2Y_1$ and $P2Y_{12}$ receptors to mobilize Ca^{2+} and activate the G_q pathway, respectively, to inhibit adenylate cyclase and ultimately the formation of cAMP. TXA_2 signals through the TP receptor to mobilize Ca^{2+}. PGE_2 and PGI_2 have opposing effects on the formation of cAMP. EP_3 receptor activation does not trigger Ca^{2+} mobilization, and therefore PGE_2 alone does not promote platelet aggregation. PGI_2 blocks platelet responses to most agonists by elevating cAMP. Several inhibitors block this platelet activation cascade at mechanistically distinct points.

show good potency against the human receptor, although their affinity towards the mouse receptor is reduced by > 30- and 290-fold, respectively (79-81). On the other hand, the GlaxoSmithKline lead compound **8** shows fairly similar affinity for the rat and dog EP$_3$ receptors, 6- and 12-fold lower, as reported (82). This analogue shows very good selectivity versus other prostanoid receptors. Good oral pharmacokinetic data have been reported following single doses in rats. Both Merck and GlaxoSmithKline analogues have been reported to behave as full antagonists (Fig. 4).

Our group at deCODE focused on the synthesis of 1,3-disubstituted five-membered heterocycles (Fig. 4). The rationale for these analogues was based on a pharmacophore model generated from limited structure–activity relationship (SAR) studies for the cinnamic acid-based EP$_3$ antagonists (**9**) reported earlier. After limited SAR studies, however, we failed to discover compounds with potency better than 1-10 μM (83).

We then turned to the analysis of derivatives that would represent combined features of the endogenous ligand PGE$_2$ (**10**) (84), the potent and selective EP$_3$ agonist sulprostone (**11**) and cinnamic acid-based EP$_3$ antagonists (**9**) (Fig. 5). Based on this analysis, we derived a pharmacophore hypothesis featured in Figure 6. We had considered two main avenues of ligand assembly, both of which would potentially provide preorganization and a hairpin motif for the putative small-molecule ligands. The respective U-shaped analogues are represented by peridisubstituted bicyclic analogues (**A**) (83, 85) and the V-shaped series (**B**) is represented by 1,3-disubstituted mono- and bicyclic heterocyclic cores (82).

We hypothesized that if our pharmacophore model (Fig. 6) for EP$_3$ receptor binding is correct, then as long as the preorganization of the key binding elements, namely "the acid and lipophilic tail", maintain optimal interaction with the key receptor sites, a significant structural variation in the bicyclic core portion of the molecule should be tolerated. This notion has since been supported by several distinct bicyclic series. The diversity of these cores is exemplified in Figure 7. Selected analogues and the receptor affinity data are shown in Figure 8. We have recently reported the SAR for series of analogues represented by compounds **20** (86), **21** (87) and **22** (88).

In parallel to generating SAR for in vitro affinities for various series, we evaluated their metabolic stability via incubation of molecules with rodent and nonrodent liver microsomal preparations. For 1,7-

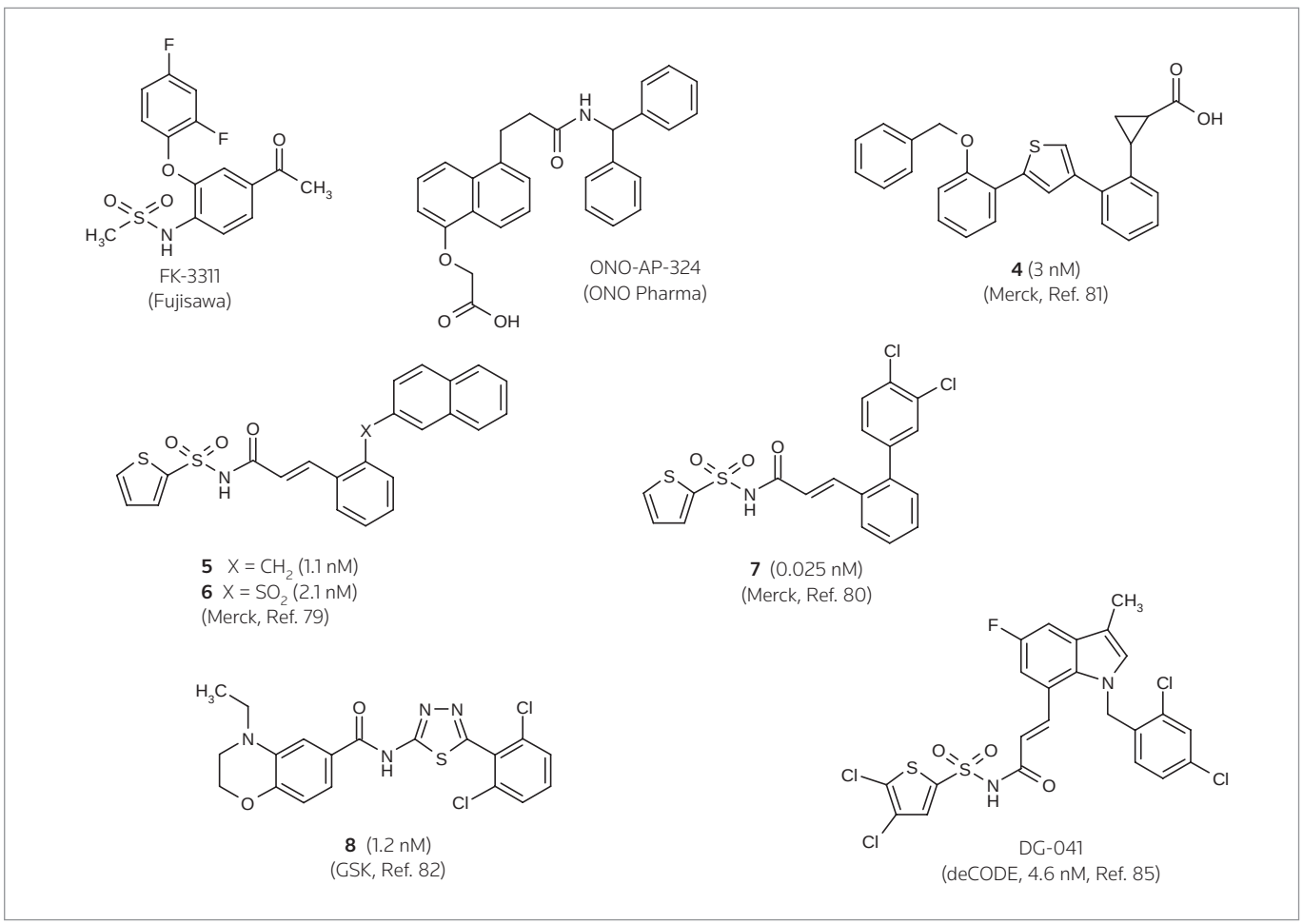

Figure 4. Examples of EP$_3$ receptor antagonists reported in the literature. Data shown are the IC$_{50}$ for the human EP$_3$ receptor.

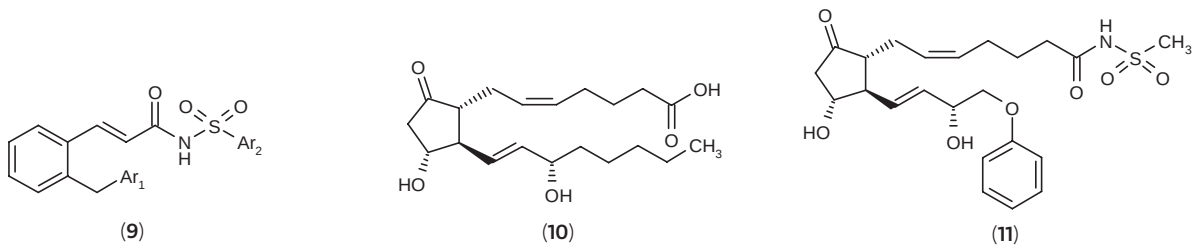

Figure 5. Selected templates for the early design of EP$_3$ receptor antagonists.

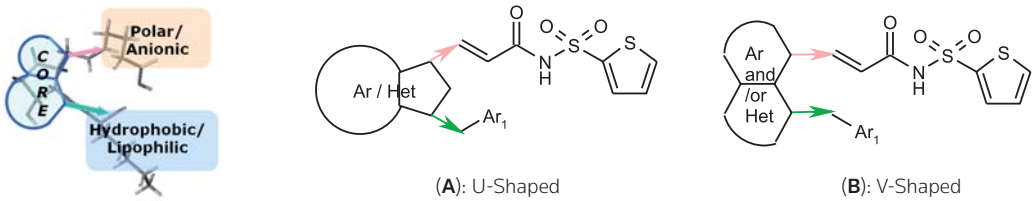

Figure 6. EP$_3$ receptor antagonist pharmacophore hypothesis and initial analogue series.

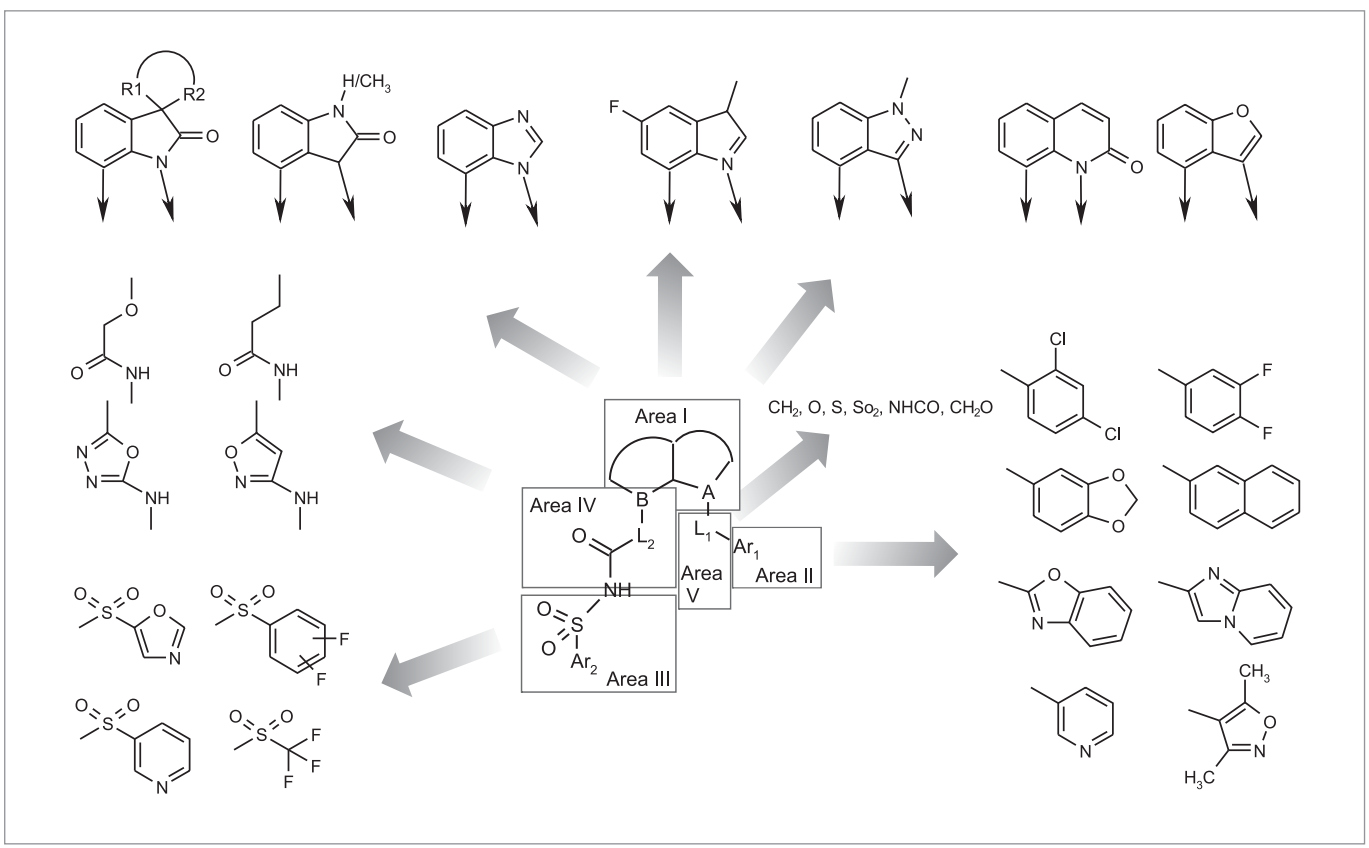

Figure 7. An overview of potent hEP$_3$ antagonists representing diverse *peri*-disubstituted bicyclic cores.

disubstituted indole derivatives, metabolism at C3 and C5 sites was addressed by incorporation of Me and F atoms, respectively, to yield **23** (Fig. 8). Further screening for selectivity provided several analogues with > 100-1,000-fold selectivity against a panel of targets, including prostanoid receptors EP$_1$, EP$_2$, EP$_4$, DP, IP$_1$ and FP (83).

In comparison to the reported EP$_3$ receptor antagonists (*vide infra*), DG-041 shows essentially identical affinity for the human and mouse receptors (4.6 nM vs. 5.3 nM; Table II). This further translated into functional in vitro results for DG-041 showing very similar IC$_{50}$ values for rat and human platelet aggregation (85).

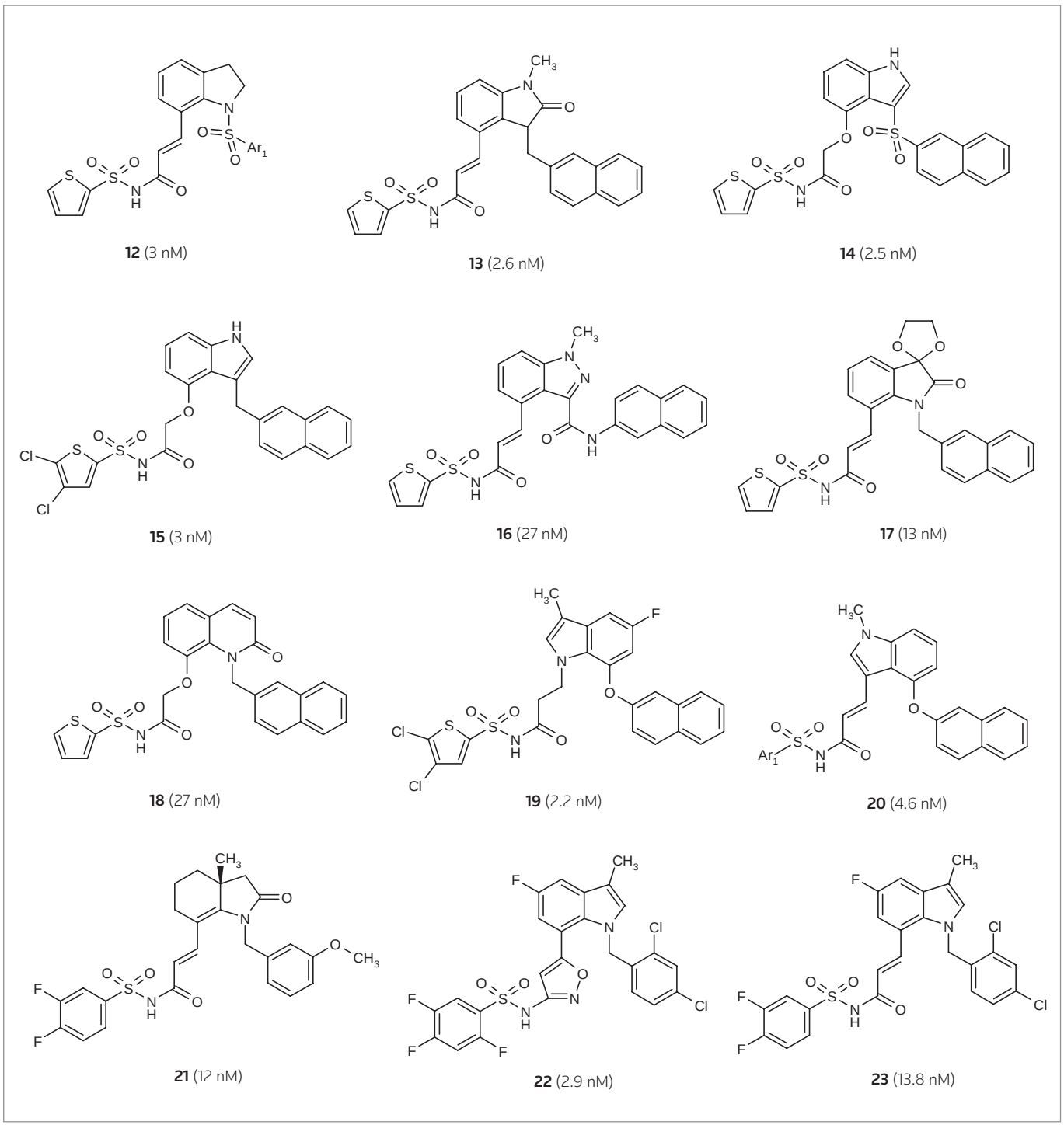

Figure 8. Selected examples of potent *peri*-disubstituted bicyclic cores (human hEP$_3$ IC$_{50}$ values in nM) (83, 85-88).

Table II. *Comparison of selected EP_3 receptor antagonists.*

Activities		Merck series (79-81)			GSK (82)	DG-041
		4	**6**	**7**	**8**	
In vitro	hEP_3 IC_{50} (nM)	3	2.1	25	1.25	4.6
	mEP_3 IC_{50} (nM)	NR	NR	NR	6.31[a]	5.3
	Plasma protein binding (fold shift)	NR	20x (+0.05% HSA)	NR	NR	1.3x (+ 10% HS)
	Selectivity profile vs. EP_1, EP_2, EP_4, DP, FP, IP_1, TP	DP40x; all others > 100x	EP_1, EP_2, EP_4 > 1,000x; others NR	DP 33x, TP 30x; all others > 100x	> 1,000x; NR for IP	EP_2, EP_4, IP_1, FP and DP_2 > 100x; EP_1 60x; TP 90x; DP 16x
	Platelet aggregation (using PGE_2 in the presence of collagen as coaggregant) (51)	NR				130 nM (human); 298 nM (rat)
Pharmacokinetics (rat) – t½, %F		NR			5.6 h, 100%	4 h, 27 ± 5.6%
Indication		Not disclosed			Bladder function	Antiplatelet agent
Overall status		Lead	Lead	Lead	Lead	Phase IIb

NR, not reported; HAS, human serum albumin; HS, human serum. [a]Data reported for rat EP_3 receptor.

In subsequent studies of the agent, we attempted to replicate the physiologically relevant pharmacology of the inflamed plaque by employing both PGE_2 and collagen to facilitate platelet responses. Under these conditions, PGE_2 consistently restored the response of platelets inhibited with clopidogrel at 10 mg/kg to collagen and ADP. Although the increased doses of clopidogrel blocked this PGE_2 effect, bleeding time was dramatically increased. On the contrary, DG-041 inhibited PGE_2-induced platelet aggregation in the presence of agonists (collagen, ADP) while having no effect on bleeding (Fig. 9) (85).

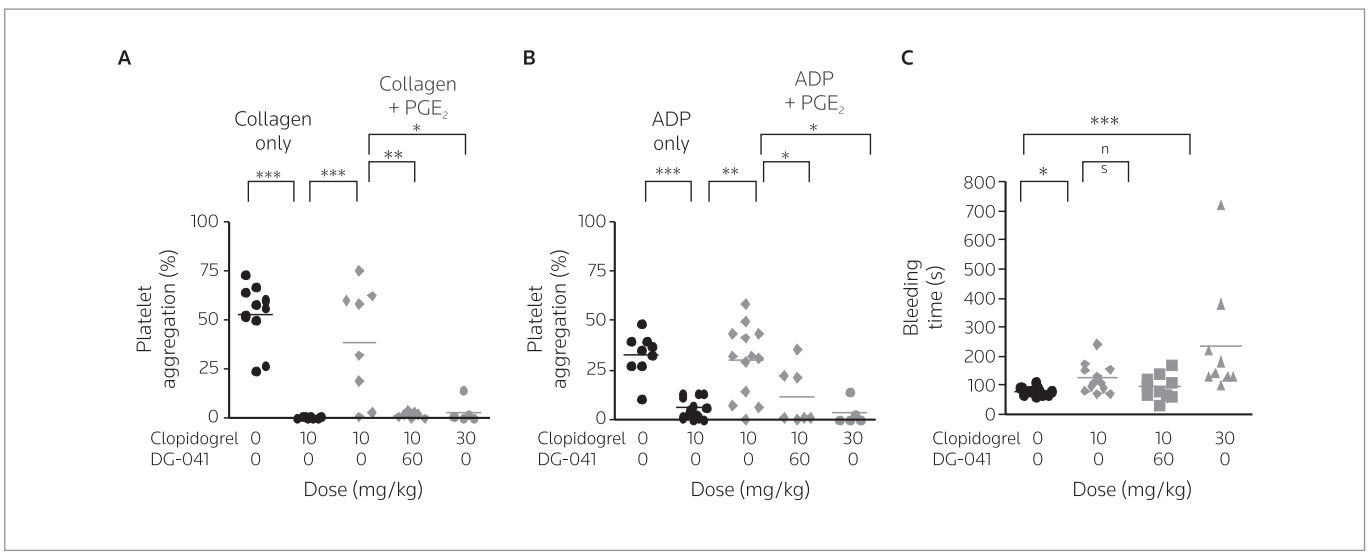

Figure 9. Effects of clopidogrel as a single agent versus combination with DG-041 on rat ex vivo platelet aggregation and bleeding times. **A**) Rat platelet aggregation tested with collagen (5 μg/mL) ± PGE_2 (1 μM); clopidogrel as a single agent (10 mg/kg) fully inhibited collagen-induced platelet aggregation, but the effect was reversed by the addition of PGE_2. Coadministration of DG-041 (60 mg/kg) or an increased dose of clopidogrel as a single agent (30 mg/kg) completely blocked PGE_2-facilitated platelet aggregation. **B**) Rat platelet aggregation tested with ADP (0.5 μM) ± PGE_2 (1 μM). Clopidogrel as a single agent provided partial protection, yielding an 83% reduction in platelet aggregation; PGE_2 reversed this inhibitory effect. A combination of clopidogrel and DG-041 provided a further decrease in platelet aggregation compared to clopidogrel alone (61% reduction); a higher dose of clopidogrel (30 mg/kg) fully blocked aggregation. **C**) Clopidogrel at 10 mg/kg increased bleeding times compared to control (from 80 ± 4 s to 129 ± 16 s); the addition of DG-041 (60 mg/kg) did not further affect bleeding times (95 ± 13 s). Increasing the dose of clopidogrel to 30 mg/kg caused a 4-fold increase in bleeding compared to controls (237 ± 67 s). *P < 0.05, **P < 0.01, ***P < 0.001. Adapted in part with permission from ACS ChemBio. Copyright 2009 American Chemical Society.

Some of the key characteristics and highlights of the DG-041 profile are summarized below. DG-041 inhibits the platelet aggregation response to PGE_2 but does not affect bleeding in rats as a single agent or in combination with clopidogrel. Several successful phase II clinical studies of DG-041 as a first-in-class antiplatelet agent have been completed. The molecule exhibited a favorable safety profile in terms of bleeding as compared to the reported data for $P2Y_{12}$ antagonists such as clopidogrel and prasugrel in a similar clinical setting. These findings underscore the potential of DG-041 as a next-generation oral antiplatelet therapy, as a compound that can reduce the risk of thrombus formation without increasing overall bleeding risk. DG-041 is currently undergoing phase II clinical studies as an antithrombotic agent.

CONCLUSIONS

Clinical data suggest that many of the current antiplatelet agents are associated with an increased risk of severe or fatal hemorrhage by affecting general platelet function. Targeting inflammatory components of the disease, specifically the PGE_2/EP_3 pathway, opens up the possibility to differentiate thrombosis from hemostasis. Several small-molecule EP_3 antagonists employed both as monotherapy or in combination with clopidogrel and aspirin have been shown to inhibit platelet aggregation at the site of lesions in the vasculature, without increasing bleeding time. This suggests that antagonists of the EP_3 receptor may become the next generation of antiplatelet drugs with an enhanced safety and efficacy profile.

DISCLOSURES

The authors are employees of deCODE Chemistry.

NOTE

Subsequent to the completion of this work that led to the identification of DG-041 and initiation of its evaluation in clinical settings, a recent publication from GlaxoSmithKline reported a thiadiazole core-derived analogue as a human EP_3 receptor antagonist. The structure of the GSK lead compound as reported is shown in Figure 4 as compound 8. See reference 82 for GSK publication.

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