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Antithrombotic $P2Y_{12}$ receptor antagonists: recent developments in drug discovery

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The $P2Y_{12}$ receptor is one of eight known P2Y receptor subtypes, and belongs to the G-protein-coupled receptor (GPCR) family. The $P2Y_{12}$ receptor is highly expressed on blood platelets and in the brain. Potent, selective, peripherally acting antagonists for the $P2Y_{12}$ receptor are used clinically as antithrombotic drugs. Several different scaffolds have been identified as $P2Y_{12}$ receptor antagonists, including irreversibly acting thienotetrahydropyridines (prodrugs), and reversible competitive antagonists, including adenine nucleotide analogs, piperazinyl-glutamate-quinolines, -pyridines, and -pyrimidines, and anthraquinone derivatives. Here, we provide an overview of the different scaffolds that have been developed as $P2Y_{12}$ receptor antagonists, some of which have become important therapeutics.

Introduction

Cardiovascular diseases have been the leading cause of death and disability globally for the past 15 years, accounting for ~30% of all deaths [1,2]. In industrialized countries in particular, this increase is thought to be the result mainly of unhealthy lifestyles [1–3].

Platelets have a fundamental role in the formation and stabilization of cardiovascular thrombosis [4]. They adhere to the subendothelial matrix following endothelial damage as a result of a ruptured atherosclerotic plaque, and then aggregate to form a prothrombotic surface that promotes clot formation and subsequently vascular occlusion [5]. The activation step involves a series of reactions, including interaction with, and release of, several platelet-activating agonists, such as ADP, thrombin, and thromboxane A2 [5,6]. As a result, therapies that influence platelet functions are considered useful for the prevention and treatment of heart diseases associated with thrombus formation [e.g. acute coronary syndrome (ACS)]. Currently, the standard therapeutics for ACS are antiplatelet agents, such as clopidogrel (**1**, $P2Y_{12}$ antagonist) or prasugrel (**2**, $P2Y_{12}$ antagonist), often in combination with acetylsalicylic acid (**3**, aspirin, COX-1 inhibitor). These drugs have been found to be useful in reducing the risk of heart attack and stroke [7,8].

Ticlopidine (**4**), a predecessor of clopidogrel (**1**), was discovered in 1972 to have antiplatelet aggregation properties *in vivo* and, a few years later, it was marketed in France for those at high risk for ischemic heart disease and stroke [9], without its target being known. In 1991, it was also approved for the US market [10]. A decade later, in 2001, the $P2Y_{12}$ receptor was cloned and identified as the target of ticlopidine and clopidogrel [11]. ADP is the endogenous agonist of the $P2Y_{12}$ receptor, whereas ATP blocks the receptor [12] (for structures see Figs 1 and 2).

$P2Y_{12}$ receptors show a restricted distribution, they are expressed in platelets [13] and in the central nervous system (CNS), mainly in microglial cells [14], making it an ideal drug target. The $P2Y_{12}$ receptor belongs to the family of purine/pyrimidine nucleotide-activated P2Y receptors, which are class A, rhodopsin-like GPCRs [15]. They belong to the δ-branch of class A GPCRs, which additionally comprises receptors activated by lipids or peptides, as well as many orphan receptors, the cognate agonists of which are still unknown or unconfirmed [16].

The $P2Y_{12}$ receptor represents a successful drug target, with several clinical drugs on the market, including clopidogrel (**1**), prasugrel (**2**), ticlopidine (**4**), cangrelor (**5**), and ticagrelor (**6**), and more drugs in clinical trials [17,18]; for structures, see Fig. 1.

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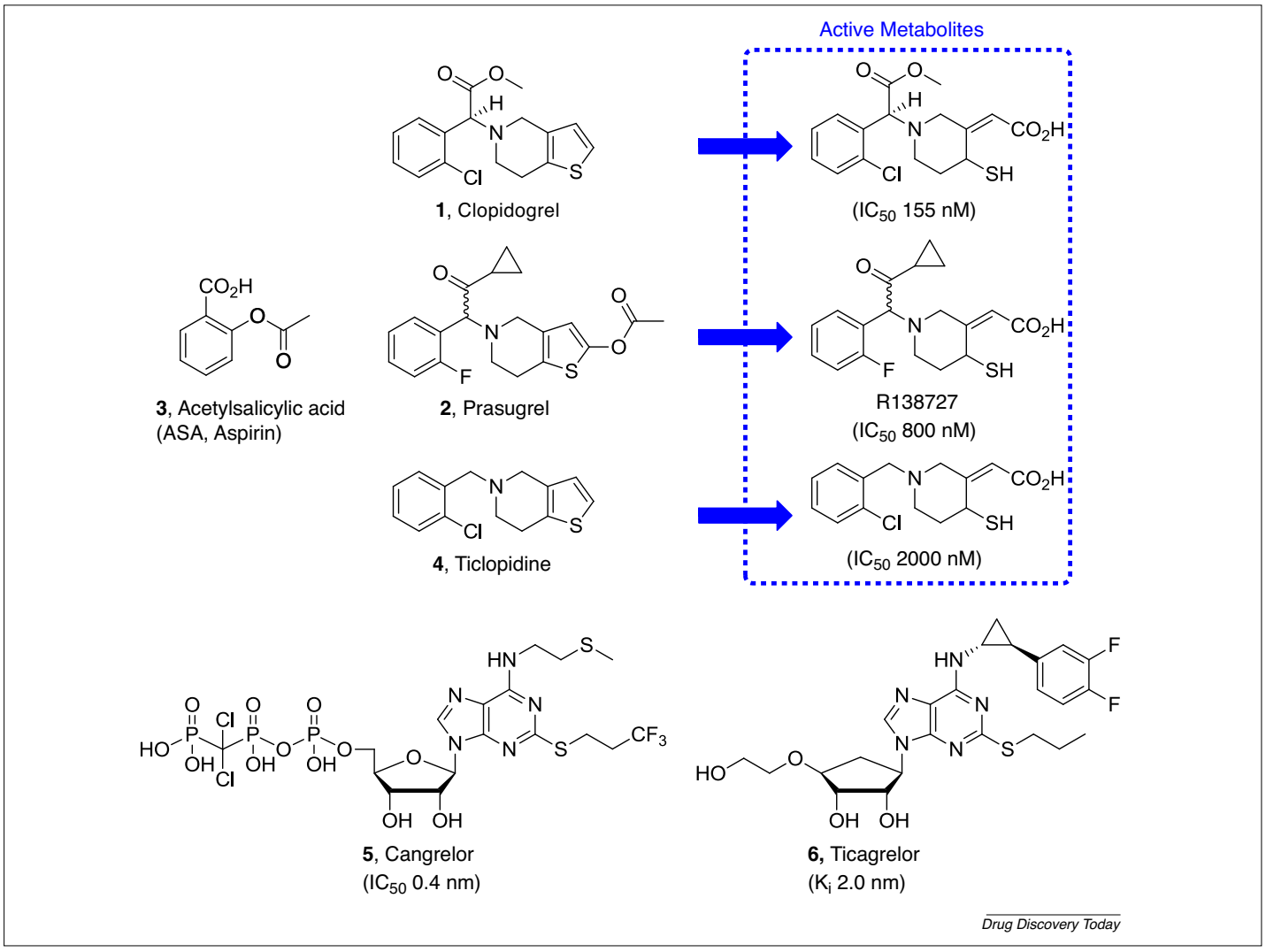

FIGURE 1

Chemical structures of marketed anticoagulant agents and their active metabolites with $P2Y_{12}$ receptor-antagonistic activity, and of the cyclooxygenase (COX) inhibitor aspirin.

Therefore, the development of $P2Y_{12}$ antagonists has been an active area of drug development, and efforts have been based on various chemical scaffolds, including (i) thienotetrahydropyridines; (ii) adenine nucleotides and analogs; (iii) piperazinyl-glutamate-pyridines, -pyrimidines, and -quinolones; (iv) anthraquinones; and (v) various other scaffolds, with the aim to obtain new anticoagulant (antiplatelet) drugs.

Platelet aggregation

Platelet aggregation involves three different purinergic P2 receptors, namely the ATP-gated ion channel receptor P2X1 and two purinergic GPCRs, $P2Y_1$ and $P2Y_{12}$, which are both activated by ADP [19]. Thus, the extracellular concentration of ADP has a crucial role in the process of platelet aggregation [20]. The P2X1 receptor, a cation channel (for Ca^{2+}, Na^+ and K^+), mediates changes in platelet shape, the G_q-coupled $P2Y_1$ receptor induces a transient rise in intracellular calcium ion concentration, platelet shape change, and the initial, easily reversible platelet aggregation, whereas the G_i-coupled $P2Y_{12}$ receptor mediates progressive and persistent platelet aggregation (Fig. 2) [21–23].

X-ray structures of the $P2Y_{12}$ receptor

Crystal structures of the human $P2Y_{12}$ receptor have recently been resolved (Fig. 3). Structures of the receptor in a complex with the agonists 2-methylthio-adenosine diphosphate (2MeS-ADP, **7**, Fig. 3a) and 2-methylthio-adenosine triphosphate (2MeS-ATP, **8**, Fig. 3b) [24], and with the non-nucleotide antagonist ethyl 6-(4-[(benzylsulfonyl)carbamoyl]piperidin-1-yl)-5-cyano-2-methylnicotinate (AZD1283, **9**, Fig. 3c) [25] were obtained. These represent the first crystallographic determinations of a receptor of the δ-branch of class A GPCRs in both agonist- and antagonist-bound states. The crystal structures showed that the agonists and the non-nucleotide competitive antagonist adopt different orientations in the $P2Y_{12}$ receptor, with a partial overlap in the orthosteric binding pockets (Fig. 2a–c) [24,25]. Whereas the agonists lead to a contraction of the binding site, the antagonist has the opposite effect, and its binding pocket appears to be open and flexible. A crystal structure of the $P2Y_{12}$ receptor bound to an irreversible allosteric antagonist would be of interest, but has not yet been published. Mutagenesis studies and the fact that the reaction of the purified

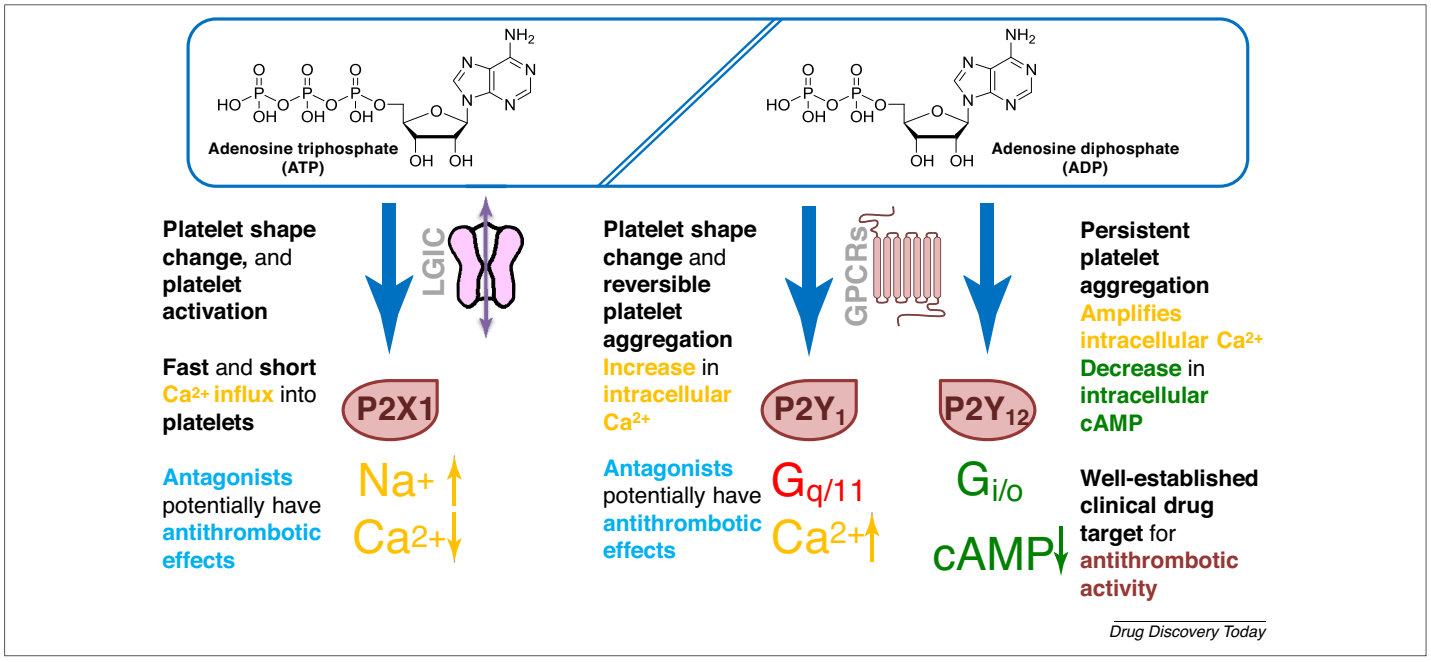

FIGURE 2

Prothrombotic activity of ATP-gated ion channel P2X1 and ADP-activated $P2Y_1$ and $P2Y_{12}$ receptors.

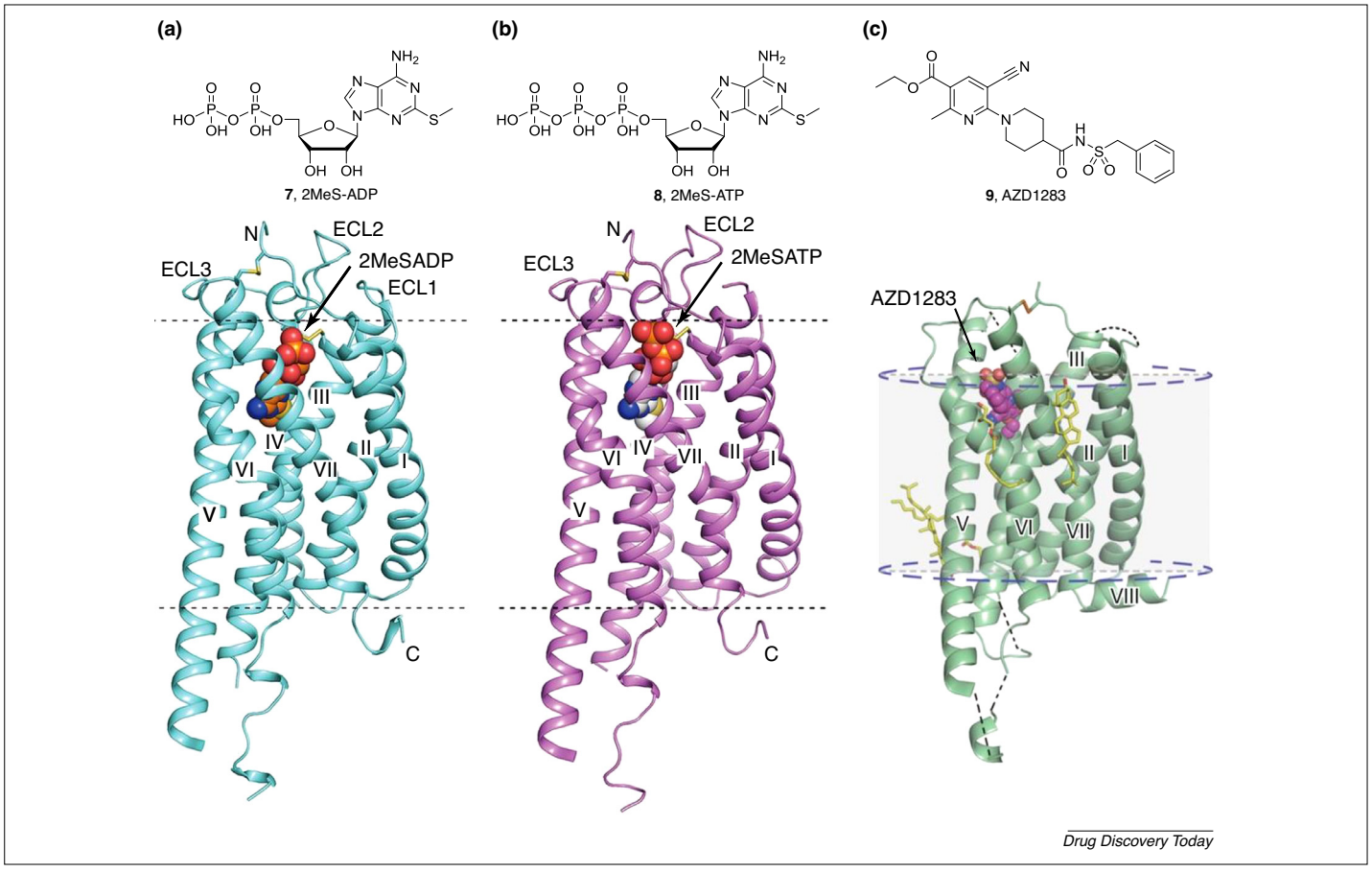

FIGURE 3

Cartoon representation of the crystal structures of the human $P2Y_{12}$ receptor in complex with: (a) the full agonist 2MeS-ADP (**7**); (b) the partial agonist 2MeS-ATP (**8**), and (c) the competitive antagonist AZD1283 (**9**) [24,25].

$P2Y_{12}$ receptor with the prasugrel metabolite led to stabilization of the C175A mutant, but not of the C97A mutant, led to the conclusion that cysteine 97 is the most likely reaction partner for the active metabolites of the thienotetrahydropyridine derivatives forming a disulfide bond. This cysteine residue does not form a disulfide bond in the X-ray structure; and is located in the upper part of transmembrane helix 3, not far from the orthosteric ligand-binding site [20].

Thienotetrahydropyridines: prodrugs of irreversible allosteric $P2Y_{12}$ receptor antagonists

The basic structure of thienotetrahydropyridines [International Union of Pure and Applied Chemistry (IUPAC) name: 4,5,6,7-tetrahydrothieno[3,2-c]pyridines, **10**, Fig. 4] comprises two rings; thiophene, a five-membered aromatic ring, which is fused with a partially saturated tetrahydropyridine ring. The fusion occurs between the 2,3-position of the thiophene ring and the 3,4-position (*c*-bond) of the tetrahydropyridine moiety. This scaffold is highlighted in dark blue in Fig. 4. The thienotetrahydropyridine drugs are further substituted by a benzyl residue at the nitrogen function, as highlighted in red in Fig. 4.

Thienotetrahydropyridine drugs, such as clopidogrel, prasugrel, and ticlopidine (see compounds **1**, **2**, and **4**, Fig. 4), represent well-established perorally bioavailable clinically used antithrombotic drugs. Vicagrel (**11**), the structure of which represents a combination of clopidogrel and prasugrel, is currently in Phase 2 clinical trials in China [26].

These compounds are prodrugs that produce their therapeutic effects only after enzymatic activation. Their active metabolites react with cysteine-97 of the $P2Y_{12}$ receptor (see earlier) forming a disulfide bond, which results in its irreversible inhibition [27]. Therefore, the antithrombotic effects of the compounds last for the lifetime of an affected thrombocyte, which is 8–12 days on average.

The first generation of thienotetrahydropyridine drugs, ticlopidine and the more potent, less toxic clopidogrel, require hepatic bioactivation by cytochrome P450 enzymes (see Fig. 5a for clopidogrel). Oxidation is catalyzed by CYP1A2, CYP2B6, and CYP2C19, respectively, to 2-oxo-clopidogrel (**13**) and its sulfoxide derivative **14** followed by ring-opening to the corresponding highly unstable sulfenic acid (**16**) or the thiol (**18**), which are believed to covalently bind to the receptor protein, forming a disulfide bond (**19**); thus, they act as covalent, allosteric antagonists at $P2Y_{12}$ receptors (Fig. 5) [28,29].

Hepatic esterases hydrolyze the ester function in clopidogrel, generating the inactive metabolites **12** and **14**. As a result, <10% of the absorbed clopidogrel is converted into the active metabolites **16** and **18** in the liver (Fig. 5) [30–34].

Despite the success of the first generation of thienotetrahydropyridines in reducing an array of thrombotic events, their slow onset of action (up to several days) because of the required metabolism, long duration of action because of irreversible inhibition, drug resistance in a high percentage of patients (up to 30%) resulting from polymorphisms in CYP2C19 preventing the production of the active metabolites, and in other proteins (e.g., the ABCB1 transporter),moderate potency, extensive enzymatic degradation by ester hydrolysis, and difficulties in controlling the antithrombotic effects represent major disadvantages associated in particular with the most widely used drug, clopidogrel [28–30]. Clopidogrel also shows interactions with other drugs, such as with omeprazole, which inhibits CYP enzymes, leading to reduced effects of clopidogrel [35].

Therefore, there has been a need to develop $P2Y_{12}$ receptor antagonists without the drawbacks associated with ticlopidine and clopidogrel. In 2009, prasugrel was approved as a second-generation $P2Y_{12}$ receptor antagonist. The chemical structure of prasugrel (**2**, Fig. 5b) lacks the methyl ester function. The introduction of a cyclopropylcarbonyl residue has been reported to have several benefits, including enhancement of potency, reducing off-target effects, and increasing metabolic stability [36]. In prasugrel, oxidation of the thiophene ring by CYP enzymes is not required; therefore, its activation is CYP2C19 independent. It is activated by ester hydrolysis instead, mediated by esterases in the intestine and in blood serum to intermediate **20**, which is then further converted to its active metabolite R-138727 by CYP enzymes (**21**) (Fig. 5b). Therefore, less resistance in patients and fewer drug interactions are observed for prasugrel.

Shan *et al.* developed vicagrel (**11**, Fig. 4) as another orally bioavailable drug to overcome some of the drawbacks of clopidogrel. It was reported to show a faster onset of action, and lower

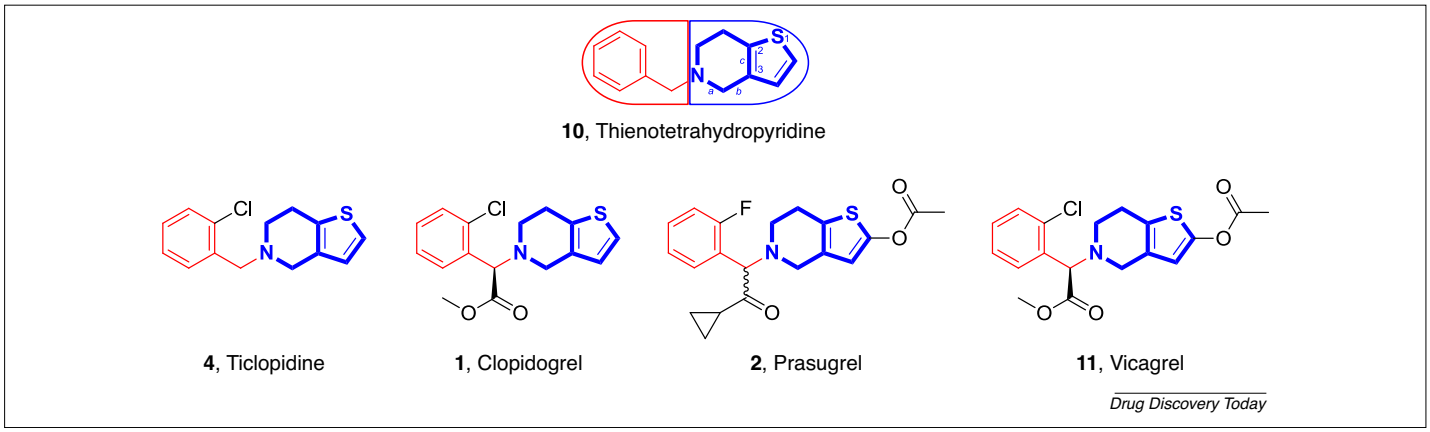

FIGURE 4

Thienotetrahydropyridine scaffold and derived antithrombotic drugs: the thienotetrahydropyridine ring is in blue, whereas the benzyl residue is in red.

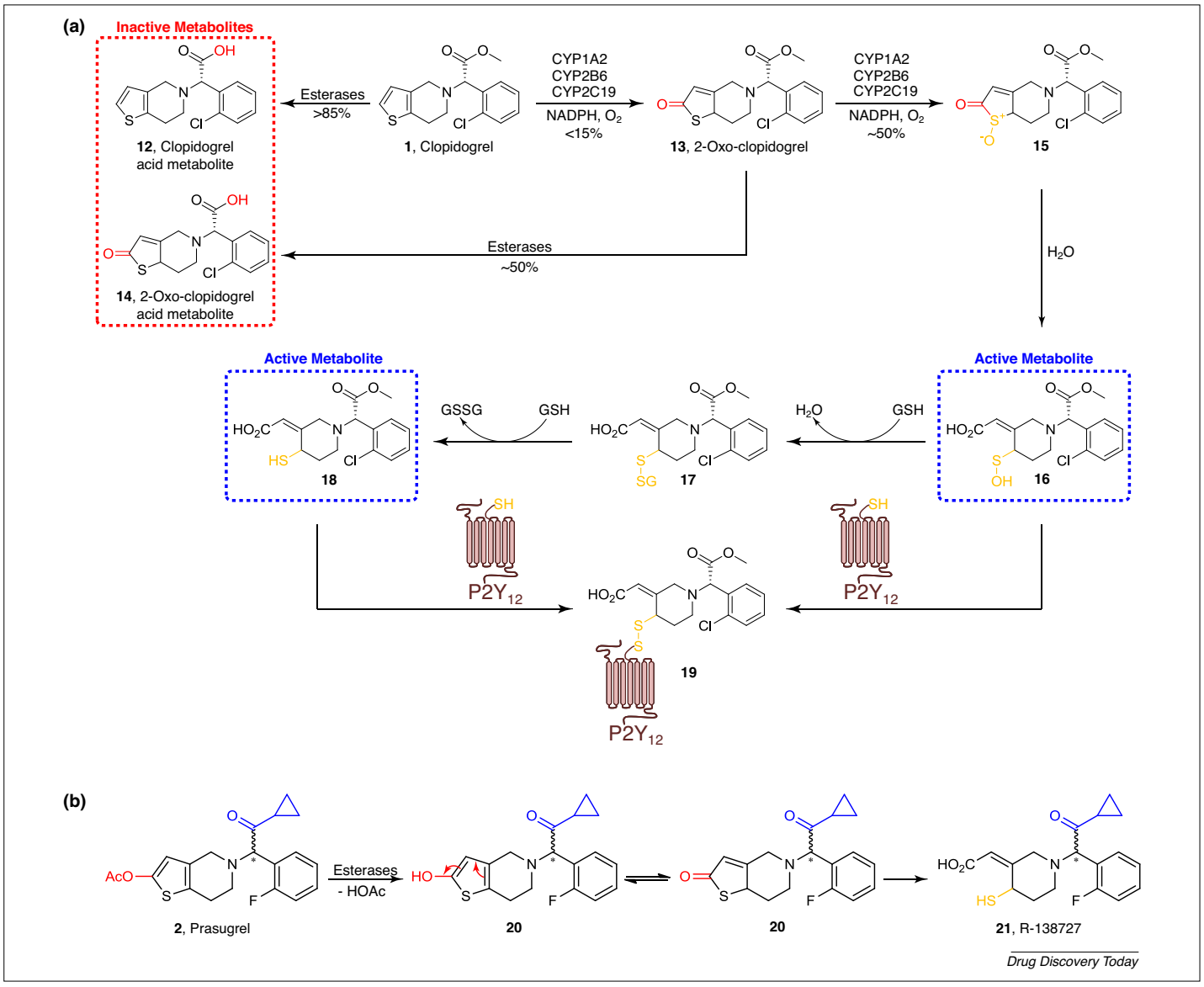

FIGURE 5

Proposed mechanisms of thienotetrahydropyridine activation. (a) Clopidogrel activation and action on platelet $P2Y_{12}$ receptors [27–30]. (b) Prasugrel activation by esterases.

dose-related toxicity and drug resistance [37]. Vicagrel shares its activation mechanism with prasugrel, but still bears the ester group of clopidogrel, which is metabolically unstable [26].

Adenine nucleotides and analogs

Several groups have developed competitive, reversible $P2Y_{12}$ receptor antagonists that might be superior to allosteric irreversible thienotetrahydropyridine prodrugs. Most approaches used adenine nucleotides as the lead structure, either ADP, the physiological agonist, or ATP, which is an antagonist at the $P2Y_{12}$ receptor. AstraZeneca developed a series of ATP analogs, including cangrelor (AR-C69931MX, **5**, Fig. 1) and AR-C67085MX (**22**, Fig. 6a) [38–40]. These compounds are polar because they are negatively charged at a physiological pH of 7.4, and, therefore, cannot be perorally administered. In addition, they are metabolically unstable because of hydrolysis by nucleotide pyrophosphatases [41]; thus, cangrelor has a short half-life (3–6 min), which results in a rapid offset of action [42]. Cangrelor (**5**, Fig. 1) was developed as an intravenously administered antiplatelet agent [43]. It is proposed for patients with coronary artery disease (CAD) undergoing percutaneous coronary intervention (PCI), and to maintain $P2Y_{12}$ inhibition when oral therapy is interrupted because of surgery. The drug was approved by the US Food and Drug Administration (FDA) in 2015 [44]. Cangrelor is particularly useful when a fast onset and termination of the anticoagulatory effect is desirable. Platelet function is recovered within 30–60 min of stopping the infusion of cangrelor. The infusion can be given for several hours, and is well tolerated. Given that it is not excreted via the kidneys, it does not require dose adjustment in patients with kidney disease. Following treatment, the patients are typically transitioned to peroral $P2Y_{12}$ receptor antagonists (e.g., ticagrelor or clopidogrel) [45,46]. Further applications for cangrelor are currently being investigated [47].

Ticagrelor (**6**, Fig. 1) is another nucleotide-derived reversible, and likely competitive [48] $P2Y_{12}$ receptor antagonist, but without

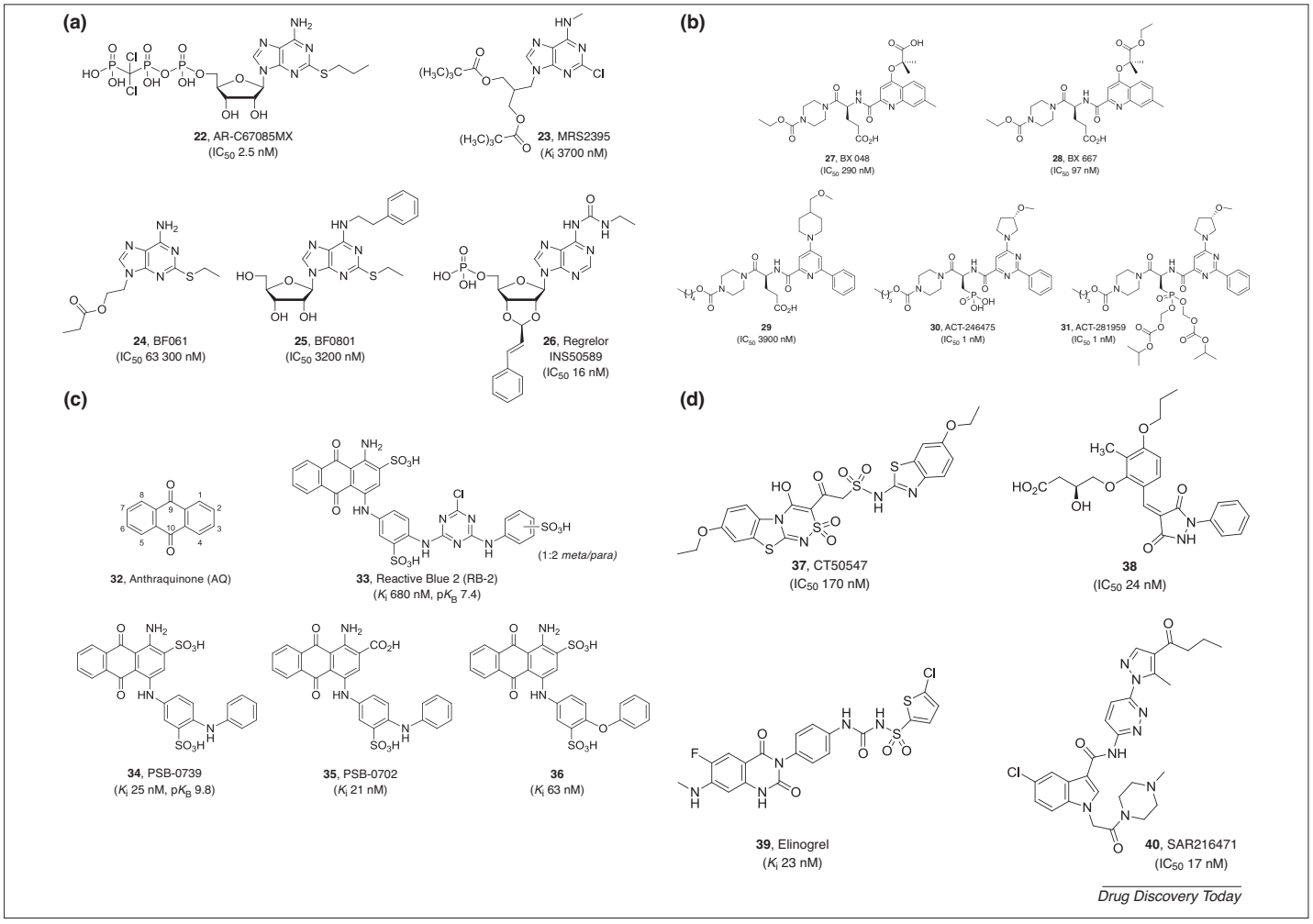

FIGURE 6

Structures of $P2Y_{12}$ receptor antagonists: (a) Selected adenine and associated nucleotide scaffolds. (b) Piperazinyl-glutamate-substituted heterocyclic quinolines, pyridines, and pyrimidines. (c) Anthraquinone derivatives. (d) Various scaffolds reported as potent and selective reversible $P2Y_{12}$ receptor antagonists.

the phosphate/phosphonate groups of cangrelor. Therefore, it is an orally available antiplatelet drug, which lacks many of the disadvantages associated with clopidogrel [49], although it can induce shortness of breathing, which is one of its characteristic adverse effects. The drug was approved by the FDA in 2011 for patients with cardiovascular diseases to prevent heart attacks in patients with ACS [50]. In 2015, ticagrelor received further FDA approval to be used as a crushed tablet for oral administration to expand its indications to include long-term use in patients with a history of heart attack [51].

Several structurally simple adenine derivatives were also reported to antagonize the $P2Y_{12}$ receptor. Jacobson and colleagues described a series of adenine derivatives [e.g., MRS2395 (**23**, Fig. 6a) [52]], while Ding and colleagues reported on BF061(**24**, Fig. 6a) [53], although these compounds exhibit moderate potency. In addition, the ester groups in the molecules may be metabolically unstable. In an additional study by Ding and colleagues, the adenosine derivative BF0801 was reported to block $P2Y_{12}$ receptors, but only with a moderate activity at micromolar concentrations (**25**, Fig. 6A) [54]. Inspire Pharmaceuticals Inc. developed nucleoside 5′-monophosphates, including regrelor (INS50589, **26**, Fig. 6a) as short-acting, competitive, reversible $P2Y_{12}$ receptor antagonists for intravenous application [55]. However, development of compound **26**, which was evaluated in Phase 2 clinical trials, has been discontinued because of an increased risk of bleeding complications [56].

Given the limitations of currently available drugs, and several drawbacks that have been encountered, discovery of new scaffolds to block the $P2Y_{12}$ receptor remains a hot area of research for the development of novel antiplatelet agents. Several classes of non-nucleotide-derived heterocyclic compounds, which act as competitive inhibitors, have been identified.

Piperazinyl-glutamate-quinolines, -pyridines and -pyrimidines

The piperazinyl-glutamate-quinoline derivative BX 048 (**27**, Fig. 6b) was described as a competitive $P2Y_{12}$ receptor antagonist, and its ethyl ester prodrug (BX 667, **28** Fig. 6b) was shown to be orally bioavailable [57,58]. Compound **27** was reported to block ADP-induced platelet aggregation, whereas compound **28** had low effects on collagen-induced aggregation and weakly inhibited arachidonic acid-induced aggregation. *In vitro* administration of

compound **28** resulted in a rapid and sustained inhibition of platelet aggregation in human blood, where the extent and duration of platelet inhibition was directly proportional to circulating drug plasma levels [57,58].

As a results of these findings, Pfizer Global Research developed a series of piperazinyl-glutamate-pyridines as $P2Y_{12}$ receptor antagonists. Among these, compound **29** (Fig. 6b) showed good potency, selectivity, oral bioavailability, and *in vivo* antithrombotic efficacy in preclinical species [59].

More recently, the related pyrimidine derivative ACT-246475 (**30**, Fig. 6b) was described as a potent, selective, and reversible $P2Y_{12}$ receptor antagonist. The compound is currently in Phase 2 clinical trials and has so far been found to be well tolerated [60]. As a result of its phosphonate group, **30** is very polar and not perorally bioavailable, but is instead applied subcutaneously (s.c.). In addition, to increase its oral bioavailability, a bis[(isopropoxycarbonyl) oxy]methyl ester prodrug (ACT-281959, **31**, Fig. 6b) has been developed [61].

Anthraquinones

Anthraquinone (AQ, IUPAC: 9,10-dioxoanthracene) is a condensed symmetrical aromatic hydrocarbon comprising 14 carbon atoms consisting of two benzene rings linked together with two carbonyl groups (**32**, Fig. 6c). AQs have been isolated naturally from medicinal plants and found to have pharmacological properties; moreover, they have also found applications as dyes [62,63]. Given the widespread applications of AQs, they have been intensively explored. Plant extracts containing AQs are increasingly being used in cosmetics, foods, and pharmaceuticals [e.g., AQs extracted from madder (*Rubia tinctorum*) are used as hair dyes [64], whereas natural extracts of senna (*Cassia senna*) have been used as laxatives [65]]. Moreover, rhubarb (*Rheum rhabarbarum*) extracts were found to have anticancer properties [66] and, recently, free total anthraquinones isolated and extracted from rhubarb were found to have several health-promoting effects, including antiviral, antibacterial, and anti-inflammatory activity, and they have been proposed for the treatment of severe acute pancreatitis [67].

AQ derivatives have been developed as antagonists of purine/pyrimidine P2 receptor subtypes (P2X and P2Y receptors) and as inhibitors of ectonucleotidases, including ecto-nucleoside triphosphate diphosphohydrolases (NTPDases, CD39) and ecto-5′-ectonucleotidase (ecto-5′-NT, CD73) [68,69]. The anthraquinone dye Reactive Blue 2 (RB-2, **33**, Fig. 6c) was found to interact with a variety of nucleotide-binding proteins in the human body, including several different P2 receptor subtypes and ectonucleotidases [49,70,71]. RB-2 was described to antagonize $P2Y_{12}$ receptor activation and to act as a direct $P2Y_{12}$ receptor antagonist [27,72]. Novel synthetic methodologies have been developed [73–75] to access novel anthraquinone derivatives, and these have been pharmacologically investigated and optimized as P2 receptors antagonists and ectonucleotidase inhibitors [76–81].

A new class of highly potent, competitive and reversible non-nucleotide $P2Y_{12}$ receptor antagonists has also been developed. The most potent compounds of this series were PSB-0739 (**34**), PSB-0702 (**35**), and compound **36** (Fig. 6c), which showed affinities in the lower nanomolar range and selectivity versus other P2 receptor subtypes in radioligand-binding studies [27,73]. In another study by Hoffman *et al.* using functional assays, PSB-0739 was found to be the most potent competitive non-nucleotide, non-nucleoside antagonist at the human $P2Y_{12}$ receptor described so far, exhibiting subnanomolar potency [72].

Various heterocyclic scaffolds

In recent years, several studies have aimed to develop potent, reversible, and selective non-nucleotide, non-nucleoside heterocyclic derivatives with $P2Y_{12}$ receptor antagonistic activity, especially to overcome the drawbacks of current clinical antithrombotic drugs.

Benzothiazolo[2,3-c]thiadiazines

Tricyclic derivatives based on a benzothiazolo[2,3-*c*]thiadiazine moiety derived from a library screening hit were reported to inhibit the $P2Y_{12}$ receptor reversibly with high affinity. Among these tricyclic derivatives, compound **37** (Fig. 6d) was found to be the most potent and selective $P2Y_{12}$ antagonist [82].

Pyrazolidinediones

Pyrazolidinedione derivatives were also reported to block the $P2Y_{12}$ receptor competitively, **38** being the most potent compound of this series [83].

Elinogrel

This potent and selective $P2Y_{12}$ receptor antagonist has a quinazoline-2,4-dione scaffold (see compound **39**, Fig. 6d). It is a direct-acting, reversible $P2Y_{12}$ receptor antagonist found to be suitable for both oral and intravenous administration [84]. The drug was evaluated in Phase 2 clinical trials [85], followed by Phase 3 trials, but was discontinued because of an increased incidence of dyspnea and elevated liver transaminases [86].

AZD1283

Among a series of ethyl 6-aminonicotinate acyl sulfonamides, the $P2Y_{12}$ receptor antagonist AZD1283 (**9**, Fig. 3) showed the strongest antithrombotic effect and was progressed to human clinical trials [87]; however, results have not yet been published, and the drug appears to have been discontinued. More recently, AZD1283 was successfully co-crystalized with the human $P2Y_{12}$ receptor, revealing the binding site of the receptor in a high-resolution (2.6 Å) crystal structure [18] (see earlier). This provides essential insights for the development of new antithrombotic drugs targeting the $P2Y_{12}$ receptor.

SAR216471

This indole derivative, derived from a screening hit, was reported by Boldron and colleagues [88]. SAR216471 (**40**) (Fig. 6d) was found to be a potent, highly selective, reversible inhibitor for the human P2Y12 receptor, exhibiting potent *in vivo* antithrombotic activities [88,89].

Concluding remarks and prospects

The development of drugs that modulate purinergic receptors is one of the most active areas of current research for the treatment of a variety of diseases [90]. Among these, the development of antagonists for the platelet $P2Y_{12}$ receptor for the treatment of thrombosis and stroke is the most advanced area. Several different drugs have been successfully introduced onto the market, includ-

ing the irreversibly acting allosteric antagonist prodrugs ticlopidine, clopidogrel, and prasugrel [91]. However, clopidogrel and, to some extent, the related thienotetrahydropyridine drugs are associated with several major drawbacks, including delayed onset of action (up to several days) because of the two-step drug activation required, a significant number of patients who are resistant to the drug, long duration of action because of irreversible inhibition, and difficulties in steering and controlling the effects [92]. For prasugrel, a novel type of thienotetrahydropyridine prodrug was developed that does not require initial oxidative activation by Cytochrome P450 (CYP450) enzymes in the liver, but is hydrolyzed by esterases in the intestine and in serum.

Another step forward was the development of cangrelor as a reversible $P2Y_{12}$ antagonist; however, this is an adenine nucleotide derivative, which is negatively charged at physiological pH, rendering it very polar; thus, it cannot be perorally administered. Cangrelor is a short-acting antithrombotic drug for intravenous application. Ticagrelor was the first reversible, competitive $P2Y_{12}$ antagonist for peroral application available on the market, although it can induce breathing problems. A variety of other heterocyclic compounds have been developed as potent and selective $P2Y_{12}$ receptor antagonists, including PSB-0739, SAR216471, AZD1283, and elinogrel. $P2Y_{12}$ antagonists currently in clinical trials include vicagrel (derived from clopidogrel and prasugrel) for peroral application, and ACT-246475, a phosphonic acid derivative intended for s.c. application. There is still room for improvement of current antithrombotic $P2Y_{12}$ receptor antagonists, given that each drugs has its drawbacks. However, it appears to be increasingly difficult to develop novel $P2Y_{12}$ antagonists for the market, because they have to display clear advantages over already approved drugs. Moreover, the development of $P2Y_{12}$ receptor antagonists that are brain-permeable would enable researchers to explore their potential applications in inflammatory brain diseases associated with microglial activation (e.g., neuropathic pain and neurodegenerative disorders). This could open a new field of indications for $P2Y_{12}$ receptor antagonists.

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