APD791, 3-Methoxy-N-(3-(1-methyl-1H-pyrazol-5-yl)-4-(2-morpholinoethoxy)phenyl)benzamide, a Novel 5-Hydroxytryptamine 2A Receptor Antagonist: Pharmacological Profile, Pharmacokinetics, Platelet Activity and Vascular Biology

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ABSTRACT

We have evaluated the receptor pharmacology, antiplatelet activity, and vascular pharmacology of APD791 [3-methoxy-N-(3-(1-methyl-1H-pyrazol-5-yl)-4-(2-morpholinoethoxy)phenyl)benzamide] a novel 5-hydroxytryptamine 2A (5-HT2A) receptor antagonist. APD791 displayed high-affinity binding to membranes (Kᵢ = 4.9 nM) and functional inverse agonism of inositol phosphate accumulation (IC₅₀ = 5.2 nM) in human embryonic kidney cells stably expressing the human 5-HT2A receptor. In competition binding assays, APD791 was greater than 2000-fold selective for the 5-HT2A receptor versus 5-HT2C and 5-HT2B receptors, and was inactive when tested against a wide panel of other G-protein-coupled receptors. APD791 inhibited 5-HT-mediated amplification of ADP-stimulated human and dog platelet aggregation (IC₅₀ = 8.7 and 23.1 nM, respectively).

Similar potency was observed for inhibition of 5-HT-stimulated DNA synthesis in rabbit aortic smooth muscle cells (IC₅₀ = 13 nM) and 5-HT-mediated vasoconstriction in rabbit aortic rings. Oral administration of APD791 to dogs resulted in acute (1-h) and subchronic (10-day) inhibition of 5-HT-mediated amplification of collagen-stimulated platelet aggregation in whole blood. Two active metabolites, APD791-M1 and APD791-M2, were generated upon incubation of APD791 with human liver microsomes and were also indentified in dogs after oral administration of APD791. The affinity and selectivity profiles of both metabolites were similar to APD791. These results demonstrate that APD791 is an orally available, high-affinity 5-HT₂ₐ receptor antagonist with potent activity on platelets and vascular smooth muscle.

Serotonin (5-hydroxytryptamine, 5-HT) is a naturally occurring indoleamine found primarily in brain, enterochromaffin tissue, and platelets. 5-HT exerts a multitude of biological effects mediated through interaction with specific cell surface G-protein-coupled receptors (GPCRs). To date, at least 14 different human 5-HT GPCRs are known (Kaumann and Levy, 2006). Among them, 5-HT₂ₐ receptors on vascular smooth muscle cells and platelets play an important role in the regulation of cardiovascular function.

The uptake process and storage capacity for 5-HT by platelets are such that minimal amounts of the amine exist in normal plasma. However, upon platelet activation at sites of vessel injury, 5-HT is released from the dense granules in platelets (Ashton et al., 1986). Although 5-HT by itself is only a weak activator of platelet aggregation, it effectively amplifies aggregation induced by other agonists including collagen, ADP, epinephrine, and thrombin (De Clerck and Herman, 1983; De Clerck and Janssen, 1990).

5-HT, 5-hydroxytryptamine, serotonin; HEK293, human embryonic kidney 293; GPCR, G-protein-coupled receptor; DOI, (±)-1-(2,5-dimethoxy-4-iodomethyl)-2-aminopropane; PRP, platelet-rich plasma; ANOVA, analysis of variance; ketanserin, 3-[(4-(4-fluorobenzoyl)piperidin-1-yl)quinazoline-2,4(1H,3H)-dione; sarpogrelate, 4-[2-(dimethylamino)-1-{2-[2-(3-methoxyphenyl)phenoxo]methyl}ethoxy]-4-oxobutanoic acid; R-86544, (2R,4S)-5-[2-[2-(3-methoxyphenyl)ethoxy]ethyl]-1-methyl-3-pyridinol hydrochloride; SR 46349, trans-4-[323-(2-dimethylaminoethoxy)oxime]-3-fluorophenylpropen-1-yloxybenzene; AR295980, 3-(4-(2-hydroxyethoxy)phenyl)-3-(1-methyl-1H-pyrazol-5-yl)phenyl)-3-methoxybenzamide; AR295981, N-(4-(2-aminoethoxy)-3-(1-methyl-1H-pyrazol-5-yl)phenyl)-3-methoxybenzamide; AR246886, 1-(3-(4-bromo-1-methyl-1H-pyrazol-5-yl)-4-(2-pyrrolidin-1-y1)ethoxy)phenyl)-3-(4-chlorophenoxy)urea.

Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; HEK293, human embryonic kidney 293; GPCR, G-protein-coupled receptor; DOI, (±)-1-(2,5-dimethoxy-4-iodomethyl)-2-aminopropane; PRP, platelet-rich plasma; ANOVA, analysis of variance; ketanserin, 3-[2-[4-(4-fluorobenzoyl)piperidin-1-yl]quinazoline-2,4(1H,3H)-dione; sarpogrelate, 4-[2-(dimethylamino)-1-[2-[2-(3-methoxyphenyl)phenoxo]methyl]ethoxy]-4-oxobutanoic acid; R-86544, (2R,4S)-5-[2-(3-methoxyphenyl)ethoxy]ethyl]-1-methyl-3-pyridinol hydrochloride; SR 46349, trans-4-[323-(2-dimethylaminoethoxy)oxime]-3-fluorophenylpropen-1-yloxybenzene; AR295980, 3-(4-(2-hydroxyethoxy)phenyl)-3-(1-methyl-1H-pyrazol-5-yl)phenyl)-3-methoxybenzamide; AR295981, N-(4-(2-aminoethoxy)-3-(1-methyl-1H-pyrazol-5-yl)phenyl)-3-methoxybenzamide; AR246886, 1-(3-(4-bromo-1-methyl-1H-pyrazol-5-yl)-4-(2-pyrrolidin-1-y1)ethoxy)phenyl)-3-(4-chlorophenoxy)urea.

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released from activated platelets induces further platelet aggregation and enhances thrombosis. These phenomena may be causally related to the clinical observation associating elevated blood 5-HT with increased cardiac events (Vikenes et al., 1999). Moreover, hyperactive 5-HT_{2A} receptor activity has been implicated in the increased coronary events observed in patients with depressive disorders (Schins et al., 2003).

In addition to its effect on platelets, 5-HT has potent effects on vascular smooth muscle cells including vasoconstriction, migration, and proliferation (Frishman and Grewall, 2000; Kaumann and Levy, 2006). The platelet and smooth muscle responses to 5-HT have been shown to be mediated predominantly by the 5-HT_{2A} receptor (Yang et al., 1996; Pawlak et al., 1998; Ogawa et al., 2002; Nishihira et al., 2006). Thus, it is reasonable to explore the potential beneficial therapeutic effects of 5-HT_{2A} receptor antagonists in patients with cardiovascular disease.

The 5-HT_{2A} receptor antagonist ketanserin was shown in clinical studies to reduce the incidence of myocardial infarction in patients with coronary artery stenosis (Prevention of Atherosclerotic Complications with Ketanserin Trial Group, 1989). However, these results have been clouded by a deleterious side-effect profile associated with ketanserin, presumably resulting from a lack of 5-HT_{2A} receptor selectivity. Other 5-HT_{2A} antagonists with improved selectivity profiles have shown promise in human clinical studies (Israilova et al., 1973). For example, sarpogrelate was shown to inhibit restenosis after coronary stenting in humans (Fujita et al., 2003).

Thus, the role of serotonin in the setting of coronary artery disease is likely to involve stimulation of 5-HT_{2A} receptors on platelets and smooth muscle cells, resulting in increased platelet aggregation, vasoconstriction, and proliferation of vascular smooth muscle cells. Together, these 5-HT_{2A}-mediated effects could contribute significantly to arterial stenosis and thrombosis. The aim of this study was to examine the ability of APD791, a novel selective 5-HT_{2A} receptor antagonist, to inhibit the serotonin-mediated platelet and smooth muscle activities that contribute to thrombus formation.

**Materials and Methods**

**Animals.** Animal studies were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (1996). All study protocols were reviewed and approved by the Animal Care and Use Committee. Rats were housed in standard cages and were maintained at 25 ± 1°C under 12-h light and dark cycles. The animals were fed standard diet and water ad libitum except in indicated experiments.

**Chemicals.** APD791 and its major metabolites were synthesized as described previously (Teegarden et al., 2006). APD791 was dissolved in dimethyl sulfoxide for in vitro assays (receptor profiling, human and dog platelet aggregation, and rabbit proliferation studies), and was dissolved in water for rabbit vasoconstriction experiments.

**Transfection of Recombinant Human and Rat 5-HT_{2} Receptors.** For development of functional human 5-HT_{2A} inositol accumulation assays, the receptor was stably expressed in human embryonic kidney 293 (HEK293) cells as described previously (Adams et al., 2008). Stably transfected HEK293 cells were also used to develop radioligand-binding assays for human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Transiently expressed rat 5-HT_{2A} receptors in HEK293 cells were used to determine the affinity of APD791 as described previously (Adams et al., 2008). Similar methods were used to determine the affinity of APD791 for monkey, dog, and rabbit 5-HT_{2A} receptors transiently expressed in HEK293 cells.

125I-labeled [(-)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane Binding to Recombinant 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} Receptors. Membranes from HEK293 cells stably transfected with recombinant human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors or HEK293 cells transiently transfected with rat, rabbit, dog, or monkey 5-HT_{2A} receptor were collected as described previously (Adams et al., 2008). Radioligand binding assays were conducted by use of the 125I-labeled 5-HT_{2} agonist ((+/-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)) (PerkinElmer Life and Analytical Sciences, Waltham, MA) as the radioligand. Nonspecific binding was determined in the presence of 10 μM unlabeled DOI. Competition experiments were conducted as described previously (Adams et al., 2008). IC_{50} values for competition curves were calculated by use of the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

**Inositol Phosphate Accumulation Assays.** Inhibition of accumulation of total inositol phosphates (IP, IP_{1}, and IP_{2}) was used as a functional assay to determine the inverse agonist potency and efficacy of APD791 and its metabolites in cells stably expressing human recombinant 5-HT_{2A} receptors. IP accumulation assays were performed as described previously (Adams et al., 2008). For all IP accumulation experiments, IC_{50} values were obtained by fitting data to a nonlinear curve-fitting program (GraphPad Software, Inc., La Jolla CA).

**Human Platelet Aggregation Assay.** Platelet aggregation was measured turbidimetrically as described previously (Adams et al., 2008). In brief, platelet-rich plasma was prepared from anticoagulated whole blood obtained from male and female donors. Platelet concentrations in the platelet-rich plasma were adjusted to 250,000 platelets/μl with use of platelet-poor plasma. Platelet-rich plasma (500 μl) was preincubated at 37°C and stirred at 1200 rpm with APD791 or its metabolites for 1 min before induction of aggregation by the simultaneous addition of 5-HT (final concentration, 1 μM) and ADP (final concentration, 1 μM). The maximal amplitude of aggregation response within 3 min was measured in triplicate by use of the Chrono-log model 490 aggregometer (Chrono-log Corp., Havertown, PA). Percentage inhibition of aggregation was calculated from the maximum decrease in optical density of the controls and of the samples containing inhibitor.

**Dog Platelet Aggregation Assay.** Whole blood from male beagle dogs was provided for platelet studies by Perry Scientific, San Diego, CA. Approximately 15 ml of venous blood was collected into sodium citrate Vacutainer tubes by jugular venipuncture from three individual dogs. Anticoagulated blood was pooled and platelet-rich plasma (PRP) was obtained as described earlier for human PRP. Platelet aggregation in dog PRP was measured as described earlier except that 5 μM ADP was used to provide the baseline amplification response and 2 μM 5-HT was used to produce maximal amplification of the aggregation response. Data are presented as the mean of triplicate measurements ± standard error of the mean.

**Dog Pharmacokinetics.** The in-life portion of this study was conducted by MPI Research (Mattawan, MI). Adult male beagle dogs (n = 3) received a single oral gavage dose of APD791 (hydrochloride salt) at 10 mg/kg. APD791 was formulated in sterile water at 5 ml/kg. Animals were fasted before compound delivery. Serial sampling was used to obtain plasma concentration versus time profiles. Whole blood samples were collected via jugular vein venipuncture over a 24-h period. Plasma was prepared by centrifugation from sodium heparin-treated whole blood, frozen, and stored at approximately −20°C until bioanalytical analysis.

Heparinized male beagle dog plasma was analyzed for APD791 and its active metabolites, APD791-M1 and APD791-M2, by use of a selective liquid chromatography/tandem mass spectrometry method. Plasma proteins were removed from plasma with the addition of acetonitrile at 3-fold the plasma volume, followed by centrifugation.
The supernatant from the processed plasma samples was injected into an high-pressure liquid chromatography system equipped with an API 4000 mass spectrometer (Applied Biosystems, Foster City, CA) operated in positive ion multiple reaction monitoring mode. Quantitation was performed with regression analysis generated from calibration standards.

Noncompartmental pharmacokinetic analysis was performed with a commercial software package (WinNonlin Professional version 4.1b; Pharsight, Mountain View, CA).

**Vasoconstriction of Rabbit Aortic Rings.** Measurement of vasoconstriction was performed on aortic rings prepared from the thoracic aorta of New Zealand white rabbits as described previously (Adams et al., 2008). Quadruplicate measurements were made simultaneously from four individual rings treated identically in four separate water baths.

**Vascular Smooth Muscle Cell DNA Synthesis.** Rabbit aortic smooth muscle cells were obtained from Cell Applications, Inc. (San Diego, CA). Cells were used between passages 2 and 5. DNA synthesis measurements were performed as described previously (Adams et al., 2008).

**Ex Vivo Platelet Aggregation.** Seven male beagle dogs (Perry Scientific, Inc.) were used to test for ex vivo antiplatelet activity after oral administration of APD791.

**Time Course Study.** To determine the time course of inhibition of platelet aggregation, dogs were given a single oral bolus dose of either 10 mg/kg APD791 (n = 4) or vehicle (n = 3). Whole blood was collected into sodium heparin Vacutainer tubes by jugular venipuncture at 1, 6, 12, and 24 h after dosing for analysis of platelet aggregation. The effect of drug or vehicle treatment on ex vivo platelet aggregation activity in whole blood was evaluated by use of a whole-blood aggregometer (Chrono-log Corp.). In brief, heparinized whole blood (400 μl) was added to saline (600 μl) with constant stirring and aggregation was stimulated with 5 μg of collagen (Chrono-log Corp.). The serotonin amplification response was obtained by adding 5-HT (Sigma-Aldrich, St. Louis, MO) to a final concentration of 2.5 μM. Blood samples from each animal were measured in quadruplicate for platelet aggregation in response to either collagen alone (baseline) or collagen plus 5-HT (amplification). Plasma was prepared from each whole-blood sample to determine concentrations of APD791 and its active metabolites APD791-M1 and APD791-M2.

**Subchronic Study.** This crossover-designed study was used to evaluate dog platelet aggregation in whole blood after 10 days of repeated oral dosing with APD791. Seven adult male beagle dogs received once-daily oral doses of APD791 for 10 days (1 mg/kg in distilled H2O), or vehicle for 10 days, or vehicle for 9 days followed by a single dose of APD791 on day 10. An 11-day washout period was then followed by another round of dosing in which animals that had received compound were dosed with vehicle, and animals that had received vehicle were dosed with compound. Ex vivo whole-blood platelet aggregation assays were performed 1 h after the last dose on day 10. Quadruplicate platelet aggregation measurements from each blood sample were averaged to yield a single data point for each treatment condition. Data were subjected to one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison post test. Plasma was prepared from each whole-blood sample to determine concentrations of APD791, APD791-M1, and APD791-M2.

### Results

**APD791 Receptor Affinity and Functional Antagonism Assays.** Our effort to identify a novel, potent, selective, and orally bioavailable 5-HT2A receptor antagonist resulted in the discovery of APD791 (Fig. 1). Competition binding assays using $[^{125}\text{I}]$DOI were used to determine the affinity of APD791 for 5-HT2A receptors from several species. APD791 displayed high affinity for human, dog, rabbit, and monkey 5-HT2A receptors ($K_i = 4.9$ for human 5-HT2A) and lower affinity for rat 5-HT2A (Table 1). The affinity of APD791 for related 5-HT2B and 5-HT2C receptors was also measured with $[^{125}\text{I}]$DOI-binding displacement assays, resulting in $K_i$ values of $>10,000$ nM. The affinity of APD791 for other 5-HT receptor family members, including 5-HT1A, 5-HT1D, 5-HT3, 5-HT2A, 5-HT6, and 5-HT7, was considerably lower compared with 5-HT2A (see Supplemental APD791 Cerep Data). The selectivity of APD791 was further assessed in competitive binding assays by use of a panel of 70 human GPCRs, ion channels, and transporters (Cerep). At 1 μM APD791, less than 50% inhibition of reference ligand binding was observed (see Supplemental APD791 Cerep Data).

The 5-HT2A receptor has been shown to mediate intracellular signaling by coupling to Goq, by activation of phospholipase C, and by generation of inositol phosphates (Grotewiel and Sanders-Bush, 1999; Kroese et al., 2002). Thus, to determine whether APD791 behaves as a neutral antagonist or as a functional inverse agonist, we measured its ability to inhibit constitutive inositol phosphate accumulation in HEK cells (see Supplemental Data). Consistent with data obtained from binding experiments, APD791 effectively lowered inositol phosphates, demonstrating that it is a potent functional inverse agonist at the 5-HT2A receptor ($IC_{50} = 5.2 ± 1.6$ nM).

Two major metabolites of APD791 were detected in human liver microsomes (data not shown). The major metabolites, APD791-M1 and APD791-M2 (Fig. 1), were synthesized and quantitated using radioligand competition binding assays.

### Table 1

Affinity of APD791 and its major metabolites for human 5-HT2A, 5-HT2B, and 5-HT2C receptors using radioligand competition binding assays.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_i$ (nM ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human 5-HT2A</td>
<td>4.9 ± 0.42 (26)</td>
</tr>
<tr>
<td>Rat 5-HT2A</td>
<td>3435 ± 686 (7)</td>
</tr>
<tr>
<td>Rabbit 5-HT2A</td>
<td>3.6 (1)</td>
</tr>
<tr>
<td>Dog 5-HT2A</td>
<td>5.5 ± 1.3 (6)</td>
</tr>
<tr>
<td>Monkey 5-HT2A</td>
<td>1.2 ± 0.3 (2)</td>
</tr>
<tr>
<td>Human 5-HT2B</td>
<td>&gt;10,000 (20)</td>
</tr>
<tr>
<td>Human 5-HT2C</td>
<td>&gt;10,000 (24)</td>
</tr>
</tbody>
</table>

Fig. 1. The chemical structure of APD791 and its active metabolites.
evaluated for binding affinity toward the human, rat, dog, rabbit, and monkey 5-HT2A receptors, and for functional inverse agonism at the human 5-HT2A receptor. Both metabolites were similar to APD791 in binding affinity and inhibition of inositol phosphate accumulation (Tables 1 and 2). In addition, both metabolites were highly selective for the human 5-HT2A receptor compared with 5-HT2B and 5-HT2C receptors (Table 1 and Supplemental APD791-M1/M2 Cerep Data). Although both active metabolites showed modest activity at 1 μM at the 5-HT1B receptor in the Cerep panel analysis (Supplemental APD791-M1/M2 Cerep) further analysis confirmed >1000-fold binding affinity for 5-HT2A for each metabolite (Supplemental 5-HT1B Table). Furthermore, 5-HT1B cAMP assay data confirmed the functional selectivity of both metabolites for 5-HT2A (Supplemental 5-HT1B Table).

**Inhibition of In Vitro 5-HT Amplified Platelet Aggregation.** Standard turbidometric aggregometry assays were used to assess the ability of APD791 and its major metabolites to inhibit 5-HT-mediated amplification of ADP-induced human platelet aggregation. At a concentration of 1 μM, ADP alone partially and transiently aggregated human platelets in vitro (Fig. 2A), and 5-HT alone did not cause aggregation at any concentration tested. The combination of 5-HT (1 μM) with the submaximal concentration of ADP (1 μM) produced an amplified aggregation response. The 5-HT-mediated amplification of ADP-stimulated aggregation was completely blocked when platelets were preincubated with 1 μM APD791. An average IC50 of 8.7 ± 0.2 nM was determined for APD791 from three independent experiments by use of platelet-rich plasma from three different human donors. An example of one IC50 determination is shown in Fig. 2B. The APD791 metabolites had similar inhibitory activity in the human platelet aggregation assay, with average IC50 values of 5.8 ± 0.2 and 7.2 ± 0.1 nM for APD791-M1 and APD791-M2, respectively (data not shown). APD791 was also tested for inhibition of platelet aggregation in platelet-rich plasma prepared from male beagle dogs (Fig. 2C). The average IC50 for inhibition of the 5-HT-mediated amplification in PRP from three individual dogs was 23 ± 6 nM.

**Inhibition of 5-HT-Mediated Vasoconstriction by APD791.** To determine whether APD791 could block 5-HT-mediated vasoconstriction, we studied its effects on aortic rings isolated from rabbits. 5-HT produced reproducible vasoconstriction in aortic rings, with an approximate ED50 of 20 μM (data not shown). Pretreatment of aortic rings with APD791 prevented the vasoconstriction caused by 20 μM 5-HT in a concentration-dependent manner (Fig. 3). In contrast, APD791 had no inhibitory effect on KCl or norepinephrine-stimulated vasoconstriction demonstrating that the inhibitory effects of APD791 were selective for 5-HT.

**Inhibition of 5-HT-Stimulated Smooth Muscle Cell Proliferation by APD791.** The effect of APD791 on 5-HT-stimulated rabbit aortic smooth muscle cell proliferation was evaluated indirectly by measuring [3H]thymidine incorpora-

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**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 nM ± S.E.M. (n)</th>
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<tbody>
<tr>
<td>APD791</td>
<td>5.2 ± 1.6 (3)</td>
</tr>
<tr>
<td>APD791-M1</td>
<td>1.7 ± 0.42 (3)</td>
</tr>
<tr>
<td>APD791-M2</td>
<td>6.1 ± 0.27 (3)</td>
</tr>
</tbody>
</table>
APD791 significantly inhibited the 5-HT-stimulated DNA synthesis with an IC\textsubscript{50} of 13 ± 7 nM. The data presented in Fig. 4B are representative of three individual experiments. APD791 had no inhibitory effect on increased DNA synthesis observed in response to 500 nM angiotensin II (data not shown), demonstrating selectivity for the 5-HT response.

**Pharmacokinetics of APD791.** After a 10 mg/kg oral dose in dogs, APD791 absorption from the gastrointestinal tract into the systemic circulation was rapid, resulting in a maximum plasma concentration (C\textsubscript{max}) of 2.28 ± 0.32 µg/ml at 0.3 ± 0.1 h after dose (Fig. 5, Table 3). The terminal-phase half-life was 0.94 ± 0.04 h. APD791-M1 and APD791-M2 were present in plasma within 0.3 h, with resulting t\textsubscript{max} values at 0.5 and 2.0 h, respectively, after administration of APD791. Plasma exposures (AUC\textsubscript{0-inf}) of APD791-M1 and APD791-M2 were 62.3% and 7.66%, respectively, of the APD791 exposure. APD791-M1 and APD791-M2 C\textsubscript{max} values for were 0.336 and 0.0154 µg/ml, respectively; the corresponding terminal-phase half-lives were 5.1 and 12.5 h, respectively.

Because the affinity and selectivity profiles for APD791-M1 and APD791-M2 are similar to APD791, both metabolites are likely to contribute to the pharmacological response. The combined C\textsubscript{max} value for the active components (APD791 plus both active metabolites) after oral administration of APD791 was 2.45 µg/ml at 0.3 h. The combined plasma exposure for the active components (AUC\textsubscript{0-inf}) was 5.84 hµg/ml with a corresponding half-life of 5.7 h after oral administration of APD791.

The effects of serotonin and the role of 5-HT\textsubscript{2A} receptors in the brain have been widely studied. Minimal penetration of the blood-brain barrier is preferred to effectively inhibit the brain. We measured exposure of APD791 and its active metabolites in the central nervous system and plasma in rats after oral administration. Measurement of combined exposure of APD791 and its active metabolites resulted in a favorable brain/plasma ratio of 0.08 (Supplemental APD791 brain and plasma exposure data).

**Ex Vivo Inhibition of 5-HT-Amplified Platelet Aggregation by APD791.** The effect of APD791 on ex vivo platelet aggregation was determined by use of whole blood after oral

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**Fig. 3.** Inhibition of 5-HT-stimulated vasoconstriction by APD791. Rabbit aortic rings were incubated with 20 µM 5-HT, and vasoconstriction was measured in each ring relative to maximal vasoconstriction determined previously with 50 mM KCl. Pretreatment of aortic rings with indicated concentrations of APD791 for 10 min inhibited the vasoconstriction response to 5-HT. Selectivity of APD791 for the 5-HT vasoconstriction response is demonstrated by lack of effect of APD791 on vasoconstriction stimulated by 50 mM KCl or 30 nM norepinephrine (NE).

**Fig. 4.** Inhibition of vascular smooth muscle cell proliferation by APD791. A, 5-HT stimulates DNA synthesis in cultured rabbit aortic smooth muscle cells. DNA synthesis was analyzed by measuring the incorporation of [\textsuperscript{3}H]thymidine into cellular DNA. Labeling was done during the final 4 h of 24-h stimulation with the indicated doses of 5-HT. *, p < 0.001. B, effect of APD791 on 5-HT-induced aortic smooth muscle cell DNA synthesis. Rabbit aortic smooth muscle cells were incubated in the presence of APD791 at the concentrations shown for 20 min before stimulation of DNA synthesis with 10 µM 5-HT for 24 h. Labeling was done during the final 4 h of stimulation. A and B, values are means ± S.E.M. Data represent the results from three separate experiments.

**Fig. 5.** APD791, metabolite, and total (APD791 and active metabolites) plasma concentration versus time profiles after oral administration of APD791 at 10 mg/kg in male beagle dogs. Male beagles were dosed orally with APD791 at 10 mg/kg. Whole-blood samples were collected via jugular vein cannulation. Plasma samples were prepared and frozen at −70°C until assayed. Composite sampling was used to define the plasma concentration versus time profile (n = 2–3 animals per time point).
administration of the compound to dogs. After oral dosing of 10 mg/kg APD791 or vehicle, blood was removed at various intervals to evaluate the time course of inhibition of 5-HT-mediated platelet aggregation. Nonaggregating concentrations of 5-HT (2.5 μM) were used to amplify aggregation in combination with submaximal levels of collagen (5 μg/ml). Under these conditions, the baseline response to collagen in samples from vehicle-treated dogs was variable, but significant amplification by 5-HT was observed in all samples at all points (Fig. 6A). Complete inhibition of 5-HT-mediated aggregation was observed in samples obtained 1 h after dosing of APD791, and was maintained at the 6-, 12-, and 24-h time points with 86%, 57%, and 58% inhibition, respectively. Statistical significance was observed at all time points except at 24 h.

The corresponding plasma concentrations for APD791 and metabolites are shown in Table 4. The total plasma concentration of the three active components (sum of the plasma concentrations of APD791, APD791-M1, and APD791-M2) was well correlated with the extent of inhibition of ex vivo platelet aggregation. Platelet aggregation was effectively inhibited at the 1-, 6-, and 12-h time points where the total drug concentrations were substantially higher than the \( K_i \) values for the 5HT2A receptor, whereas inhibition became nonsignificant at the 24-h time point as the drug levels fell to approximately 6 nM.

To determine whether APD791 was effective after repeat dosing, dogs were treated daily with 1 mg/kg APD791 or vehicle (distilled water) for 10 consecutive days. The ex vivo platelet aggregation data shown in Fig. 6B indicate that the inhibitory effects were maintained after 10 days of dosing. The magnitude of the inhibition was similar to that seen in dogs to which vehicle had been administered for 9 days followed by APD791 on day 10. The plasma levels of APD791 and its metabolites in the subchronic group were similar to the drug levels observed after a single acute administration of drug (Table 5), indicating that repeat dosing did not alter the metabolism of APD791 or the associated pharmacokinetic parameters.

### Discussion

In this study, we evaluated the pharmacology, antiplatelet activity, vascular effects, and pharmacokinetics of APD791, a novel 5-HT2A receptor antagonist. APD791 and its active metabolites were shown to possess high affinity for human, monkey, dog, and rabbit 5-HT2A receptors in competitive binding assays. In addition, APD791 and its metabolites inhibited intracellular signaling (inositol phosphate accumulation) through the Gqq pathway at concentrations consistent with their binding affinities. APD791 was found to have substantial selectivity for the human 5-HT2A receptor relative to other GPCRs, including the closely related 5-HT2C and 5-HT2C receptors for which the affinity of APD791 was estimated to be at least 2000-fold lower. Selectivity of APD791

### Table 3

Pharmacokinetic parameters for APD791 and its metabolites, and the sum of APD791 and its metabolites after oral administration of APD791 at 10 mg/kg in male beagle dogs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>APD791 (^a)</th>
<th>APD791-M1 (^b)</th>
<th>APD791-M2 (^b)</th>
<th>Total (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_{1/2} ) (h)</td>
<td>0.94 ± 0.04</td>
<td>5.1</td>
<td>12.5</td>
<td>5.7</td>
</tr>
<tr>
<td>( t_{\text{max}} ) (h)</td>
<td>0.3 ± 0.1</td>
<td>0.5</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (μg/ml)</td>
<td>2.28 ± 0.32</td>
<td>0.336</td>
<td>0.0154</td>
<td>2.45</td>
</tr>
<tr>
<td>( \text{AUC}_{0-\text{inf}} ) (hrμg/ml)</td>
<td>3.55 ± 0.77</td>
<td>2.21</td>
<td>0.272</td>
<td>5.84</td>
</tr>
</tbody>
</table>

\(^a\) Values were determined from individual animal plasma concentration (nanogram equivalents of APD791·HCl) versus time profiles (mean ± S.D., \( n = 3 \)).

\(^b\) Values were determined from pooled plasma concentration (nanogram equivalents of APD791·HCl) versus time profiles.

\(^c\) Values were determined from the sum of the plasma concentrations of APD791, M1, and M2 (nanogram equivalents of APD791·HCl) versus time profiles.

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**Fig. 6.** Inhibition of ex vivo platelet aggregation by APD791. A, time course of platelet inhibition. APD791 was administered orally to dogs at 10 mg/kg. The effect of APD791 on the 5-HT amplification of collagen-induced platelet aggregation was evaluated in the whole blood impedance assay at the indicated time points after oral dosing. For each blood sample, collagen-stimulated aggregation (5 μg) served as a baseline and was compared directly with the collagen (5 μg) + 5-HT (2.5 μM) aggregation response to determine the 5-HT amplification response measured in Ohms (Ω). Values are means ± S.E.M., \( * \), \( p < 0.001 \), compared with vehicle by one-way ANOVA followed by Tukey’s multiple comparison test. B, effect of subchronic administration of APD791 on platelet inhibition. APD791 or vehicle was administered orally to dogs once daily for 10 days at 1 mg/kg. The effect of APD791 on the 5-HT amplification of collagen-induced platelet aggregation was evaluated in the whole-blood impedance assay 1 h after the final dose as described in A. The inhibitory effect of repeat dosing of APD791 was compared directly with the effect of an acute dose given on day 10 after 9 days of dosing with vehicle. Values are means ± S.E.M., \( * \), \( p < 0.001 \), compared with vehicle by one-way ANOVA followed by Newman-Keuls multiple comparison post-test. Col, Coll, collagen.
...and its active metabolites was also confirmed against a panel of 70 GPCRs, ion channels, and transporters.

Although platelets express functional 5-HT<sub>2A</sub> receptors (Drummond and Gordon, 1974), 5-HT alone does not effectively promote aggregation. Our data confirm that 5-HT acts to amplify platelet aggregation induced by strong agonists such as ADP and collagen. Under the conditions used in this study, we observed a highly reproducible and robust 5-HT-mediated amplification of ADP-induced platelet aggregation in human platelet-rich plasma from multiple donors. Whereas APD791 had no effect on ADP-induced aggregation per se, it was a potent inhibitor of 5-HT-mediated amplification. Similar results have been reported for other less selective 5-HT<sub>2A</sub> antagonists including ketanserin, sarpogrelate (M-1), R-96544, SR 46349, and AR246686 (Van Nueten et al., 2008). The IC<sub>50</sub> of APD791 inhibition of human platelet aggregation assay after either acute or repeated oral dosing, was closely correlated with the total concentration of the three components in blood, and was consistent with the K<sub>i</sub> values of APD791 and the metabolites for the 5-HT<sub>2A</sub> receptor.

The simultaneous blockade of 5-HT<sub>2A</sub> receptors on both platelets and vascular smooth muscle cells should contribute to the inhibition of thrombotic arterial occlusion, because, in addition to thrombosis, vasoconstriction and cell proliferation can contribute to narrowing of arterial vessels. Accordingly, we evaluated the effects of APD791 on smooth muscle cell contraction and proliferation. APD791 inhibited 5-HT-induced contraction of isolated rabbit aortic rings in a dose-dependent manner. The observed inhibition of 5-HT-induced contraction by APD791 was selective because there was no effect on vasoconstriction induced by norepinephrine or KCl. In addition, we confirmed the proliferative effects of 5-HT by measuring DNA synthesis in cultured rabbit aortic smooth muscle cells. APD791 inhibited the proliferative response with an IC<sub>50</sub> (13.4 nM), consistent with its K<sub>i</sub> for the rabbit 5-HT<sub>2A</sub> receptor (3.6 nM).

Previous studies in animal models have demonstrated that 5HT<sub>2A</sub> receptor antagonists, including ketanserin, sarpogrelate, and AR246686, can effectively block arterial thrombosis without increasing bleeding (Ashton et al., 1986; Kihara et al., 2001; Adams et al., 2008). However, lack of selectivity and suboptimal pharmacokinetics limit the usefulness of these compounds in the clinic. Thus, our goal was to improve the selectivity and pharmacokinetic profiles, which resulted in the discovery of APD791. Recently, APD791 was tested in the Folts dog model of coronary stenosis and was shown to effectively block coronary thrombosis without increasing bleeding time (Przyklenk et al., 2007). The separation of antithrombosis effects and bleeding is of potential clinical relevance. Current therapies for thrombotic diseases, including GPIIb/IIIa and P<sub>2</sub>Y<sub>12</sub> antagonists, are well known to

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**TABLE 4**

Plasma concentrations of APD791, AR295980, AR295981, and total (APD791 and active metabolites) in male beagle dogs after oral administration of APD791 at 1 mg/kg

<table>
<thead>
<tr>
<th>Time</th>
<th>APD791&lt;sup&gt;a&lt;/sup&gt;</th>
<th>APD791-M1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>APD791-M2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>1500 ± 291&lt;sup&gt;c&lt;/sup&gt;</td>
<td>400 ± 124</td>
<td>10.8 ± 4.7</td>
<td>1910 ± 292</td>
</tr>
<tr>
<td>6 h</td>
<td>29.5 ± 14.6</td>
<td>2.3 ± 0.74</td>
<td>3.5 ± 0.09</td>
<td>32.4 ± 9.2</td>
</tr>
<tr>
<td>12 h</td>
<td>31.9 ± 8.7</td>
<td>5.6 ± 1.07</td>
<td>1.52</td>
<td>42.0 ± 1.78</td>
</tr>
<tr>
<td>24 h</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
</tbody>
</table>

<sup>a</sup>APD791, M1 and M2 were converted to APD791-HCl equivalents.  
<sup>b</sup>Total: sum of the plasma concentrations of APD791, M1 and M2 (ng equivalents of APD791-HCl).  
<sup>c</sup>Mean ± S.D.; n = 4.

**TABLE 5**

Mean plasma concentrations of APD791, AR295980, AR295981, and total (APD791 and active metabolites) in male beagle dogs after oral dosing of APD791 at 1 mg/kg after acute or subchronic administration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APD791&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vehicle</td>
<td>BLQ</td>
</tr>
<tr>
<td>Acute</td>
<td>34.7 ± 5.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Subchronic</td>
<td>56.4 ± 9.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>APD791, M1, and M2 were converted to APD791-HCl equivalents.  
<sup>b</sup>Total: sum of the plasma concentrations of APD791, M1, and M2 (nanogram equivalents of APD791-HCl).  
<sup>c</sup>Mean ± S.D.; n = 1–7.
increase serious bleeding events (Cooke and Goldschmidt-Clermont, 2006). We recently demonstrated a pharmacological separation of the effects of a novel 5-HT2A antagonist on thrombosis and bleeding (Adams et al., 2008). In the same study no separation of thrombosis and bleeding was observed for the P2Y12 antagonist clopidogrel.

In conclusion, the present study demonstrates that APD791 is a potent, selective, and orally available 5-HT2A receptor antagonist, and suggests that clinical evaluation of this drug is warranted because this profile offers potential improvements over previously described 5-HT2A receptor antagonists (Van Nueten et al., 1981; Herbert et al., 1993; Ogawa et al., 2002). The combined inhibition of 5-HT-mediated thrombosis, vasoconstriction, and smooth muscle cell proliferation by APD791 could provide unique benefits for the treatment of thrombotic disease.

References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22:3099–3108.


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