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Anti-thrombotic and vascular effects of AR246686, a novel 5-HT_{2A} receptor antagonist

John W. Adams *, Juan Ramirez, Danny Ortuno, Yunqing Shi, William Thomsen, Jeremy G. Richman, Michael Morgan, Peter Dosa, Bradley R. Teegarden, Hussien Al-Shamma, Dominic P. Behan, Daniel T. Connolly

Arena Pharmaceuticals, Inc. San Diego, CA 92121 USA

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Abstract

We have evaluated the anti-platelet and vascular pharmacology of AR246686, a novel 5-hydroxytryptamine2A (5-HT_{2A}) receptor antagonist. AR246686 displayed high affinity binding to membranes of HEK cells stably expressing recombinant human and rat 5-HT_{2A} receptors (K_i=0.2 nM and 0.4 nM, respectively). Functional antagonism (IC_{50}=1.9 nM) with AR246686 was determined by inhibition of ligand-independent inositol phosphate accumulation in the 5-HT_{2A} stable cell line. We observed 8.7-fold and 1360-fold higher affinity of AR246686 for the 5-HT_{2A} receptor vs. 5-HT_{2C} and 5-HT_{2B} receptors, respectively. AR246686 inhibited 5-HT-induced amplification of ADP-stimulated human platelet aggregation (IC_{50}=21 nM). Similar potency was observed for inhibition of 5-HT stimulated DNA synthesis in rat aortic smooth muscle cells (IC_{50}=10 nM) and 5-HT-mediated contraction in rat aortic rings. Effects of AR246686 on arterial thrombosis and bleeding time were studied in a rat model of femoral artery occlusion. Oral dosing of AR246686 to rats resulted in prolongation of time to occlusion at 1 mg/kg, whereas increased bleeding time was observed at a dose of 20 mg/kg. In contrast, both bleeding time and time to occlusion were increased at the same dose (10 mg/kg) of clopidogrel. These results demonstrate that AR246686 is a high affinity 5-HT_{2A} receptor antagonist with potent activity on platelets and vascular smooth muscle. Further, oral administration results in anti-thrombotic effects at doses that are free of significant effects on traumatic bleeding time.

Keywords: 5-hydroxytryptamine; 5-HT_{2A} receptor; Platelet; Thrombosis; Vasoconstriction; Serotonin; Platelet aggregation; Bleeding time

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a naturally occurring indoleamine found primarily in the brain, enterochromaffin tissue and platelets. 5-HT exerts a multitude of biological effects achieved through interaction with specific cell surface G-protein coupled receptors. To date, at least 14 different human 5-HT receptors are known (Kaumann and Levy, 2006). Amongst them, 5-HT_{2A} receptors on vascular smooth muscle cells and platelets play an important role in regulation of cardiovascular function.

The uptake process and storage capacity of 5-HT in platelets is such that minimal amounts of the amine exists in normal plasma. However, upon platelet activation at the site of vessel injury, 5-HT is released from the dense granules in platelets (Ashton et al., 1986). 5-HT by itself is a weak activator of platelet aggregation but amplifies aggregation induced by other agonists including collagen, ADP, epinephrine and thrombin (De Clerck and Herman, 1983; De Clerck and Janssen, 1990). Thus, subsequent to platelet adhesion and activation at the site of vascular injury, 5-HT released from platelets induces further platelet aggregation and enhances thrombogenesis. This phenomenon is supported by clinical data demonstrating that increased blood serotonin levels correlate with cardiac events (Vikenes et al., 1999). Moreover, hyperactive 5-HT_{2A} receptor activity has been implicated in the increased coronary events

* Corresponding author. Cardiovascular Biology, Arena Pharmaceuticals, Inc. San Diego, CA 92121 USA. Tel.: +1 858 453 7200.
E-mail address: jadams@arenapharm.com (J.W. Adams).

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demonstrated 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 (Ogawa et al., 2002; Nishihira et al., 2006; Pawlak et al., 1998; Yang et al., 1996). Thus, it is reasonable to explore the potential beneficial effects of 5-HT_{2A} receptor antagonists as therapy in patients with cardiovascular disease.

The 5-HT_{2A} receptor antagonist ketanserin was shown in clinical studies to reduce the incidence of myocardial infarctions 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 use, 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. For example, sarpogrelate was shown to be protective against angina pectoris and inhibited restenosis following coronary stenting in humans (Tanaka et al., 1998; 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 a selective 5-HT_{2A} receptor antagonist to inhibit thrombus formation and the serotonin mediated platelet and smooth muscle activities that contribute to thrombus formation.

2. Materials and methods

2.1. 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 Arena Pharmaceuticals Institutional Animal Care and Use Committee. Rats were housed in standard cages and they were maintained at 25 ± 1 °C under 12 h light and dark cycles. The animals were fed standard diet, except in indicated experiments, and water ad libitum.

2.2. Chemicals

AR246686 was synthesized at Arena Pharmaceuticals (San Diego, CA, USA) (Teegarden et al., 2006). Clopidogrel, and ferric chloride were obtained from Sigma (St. Louis, MO, USA). For in vitro human platelet aggregation studies, AR246686 was dissolved in dimethyl sulfoxide (DMSO). For rat ex-vivo platelet aggregation studies, AR246686 was dissolved in 80% polyethylene glycol 400 (PEG400). For the thrombosis and bleeding study, it was dissolved in 40% hydroxypropyl beta-cyclodextrin (HPBCD). Clopidogrel was dissolved in PEG400.

2.3. Cell growth and transfection of recombinant human and rat 5-HT_2 receptors

For development of functional human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} inositol phosphate accumulation assays, receptors were stably expressed in human embryonic kidney 293 (HEK293) cells. For generation of stable cell lines, cDNA expression plasmids encoding respective 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} genes and the neo^r gene were separately transfected into HEK293 cells (ATCC# CRL-1573) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Stable receptor expressing pools were then generated over 3 weeks by standard techniques in the presence of 500 μg/ml Geneticin (Gibco). Individual receptor stable pools were dilution cloned using standard techniques and clones were comparatively evaluated in radioligand binding assays as well as inositol phosphate accumulation assays. Preferred clones were banked and cultured as needed. The same 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 establish the affinity of AR246686 for rat receptor. Briefly, HEK293 cells were incubated in 15 cm plates with Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Inc.) and grown to 60–80% confluency, rinsed with Opti-MEM (Invitrogen) media, followed by addition of 16 μg cDNA and 60 μl of lipofectamine. After incubation of cells for 5 h at 37 °C in a 5% CO_2 incubator, plates were aspirated and 30 ml of DMEM was replaced.

2.4. Preparation of plasma membranes from cells stably expressing recombinant 5-HT_2 receptors

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 5-HT_{2A} receptor, were collected 24 h post-transfection, washed with ice-cold phosphate buffered saline (PBS), pH 7.4, and then centrifuged at 48,000 ×g for 20 min at 4 °C. The cell pellet was then resuspended in wash buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 and 0.1 mM EDTA, homogenized on ice using a Brinkman Polytron, and re-centrifuged at 48,000 ×g for 20 min at 4 °C. The resultant pellet was then resuspended in 20 mM HEPES, pH 7.4, homogenized on ice, and centrifuged (48,000 ×g for 20 min at 4 °C). Crude membrane pellets were stored at −80 °C until used for radioligand binding assays.

2.5. [^{125}I]DOI binding to recombinant 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors

Radioligand binding assays for human and rat 5-HT_{2A}, human 5-HT_{2B}, and human 5-HT_{2C} receptors were conducted

using the [^{125}I] labeled 5-HT$_2$ agonist (+/−)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (PerkinElmer, Inc.) as the radioligand and nonspecific binding was determined in the presence of 10 μM unlabeled DOI. Similar experiments were also performed using [3H]ketanserin as the radioligand. Competition experiments consisted of addition of 95 μl of assay buffer (20 mM Hepes, pH 7.4 and 10 mM $MgCl_2$), 50 μl of membranes (5–25 μg protein), 50 μl of [^{125}I]DOI (0.33–2 nM final assay concentration), and 5 μl of test compound diluted in assay buffer (final concentrations ranging from 1 pM to 10 μM) to 96-well Perkin Elmer GF/C microtiter plates. Incubations were performed for 1 h at room temperature. Each competition study consisted of testing compounds at eight concentrations in which triplicate determinations were performed at each test compound concentration. Assay incubations were terminated by rapid filtration of microtiter plates under vacuum pressure using a Brandell Cell Harvestor, followed by washing filter plates several times with ice-cold wash buffer (50 mM Tris–HCl, pH 7.4). Plates were then dried at 45 °C in an oven for a minimum of 2 h, 25 μl of scintillation cocktail was added to each well, and microtiter plates were counted in a Packard TopCount scintillation counter. K_i values for competition curves were calculated using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

2.6. Evaluation of AR246686 in 5-HT inositol phosphate accumulation assays

Inhibition of ligand-independent accumulation of total inositol phosphates (IP, IP_1, and IP_3) was used as a functional assay to determine the inverse agonist potency and efficacy of AR246686 for cells stably expressing human recombinant 5-HT$_{2A}$ receptors. For assay development, wild type human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ (INI; unedited version) receptors were stably expressed in HEK293 cells. For all three 5-HT$_2$ receptors, constitutive IP accumulation was sufficient to allow evaluation of AR246686 inverse agonist activity. Cells expressing receptor of interest were removed from cell culture plates by treatment with 2 ml of trypsin, additional growth medium was added to dilute the trypsin, and then cells were diluted to a final concentration of 550,000 cells/ml using growth medium. Subsequently, a total of 55,000 cells were added to 96-well microtiter plates pretreated with poly-D-lysine and incubated for 5 h at 37 °C with 5% CO_2, 100 μl of inositol-free Dulbecco's Modified Eagle's Medium (brought to pH 7.4 with sodium bicarbonate) containing 40 μCi [^{3}H]inositol was then added to each well, and plates were incubated overnight at 37 °C with 5% CO_2. The next day unincorporated [^{3}H]inositol was carefully removed from wells by aspiration and replaced with 90 μl of inositol-free Dulbecco's Modified Eagle's Medium (pH 7.4) also containing 10 mM LiCl and 10 μM pargyline followed by addition of 10 μl of test compound (1 pM to 10 μM, triplicate determinations at each concentration) containing a final concentration of 0.5% DMSO. Assay plates were then incubated for 2 h at 37 °C with 5% CO_2 and then the reaction mixture was then removed by gentle aspiration. Stop solution (160 μL) containing 0.1 M formic acid was added to each well and the plates were frozen at −80 °C overnight to promote cell lysis. Assay plates were thawed and [^{3}H]inositol was resolved from [^{3}H]inositol phosphates by Dowex resin chromatography and counted in a Packard TopCount scintillation counter. For radioligand binding experiments, IC_{50} values were obtained by fitting competition data to a nonlinear curve-fitting program (Prism, San Diego). In all cases, data were better fit to a single-site model (data not shown). For all IP accumulation experiments, IC_{50} values were obtained by fitting data to a nonlinear curve-fitting program (Prism, San Diego).

2.7. Human platelet aggregation assay

Aggregation was measured turbidometrically at 37 °C. Platelet rich plasma was prepared from anti-coagulated (0.32% Na-citrate) whole blood obtained from male and female donors by centrifugation for 15 min at 100 ×g. Platelet poor plasma was obtained by further centrifugation of donor blood for 10 min at 3000 ×g. Platelet concentration in the platelet rich plasma was determined using the Z series Beckman Coulter particle counter (Beckman, Fullerton, CA) and adjusted to 250,000 platelets/μL using platelet poor plasma. 500 μL of platelet rich plasma was pre-incubated at 37 °C and stirred at 1200 rpm with inhibitor for 1 min before induction of aggregation by the simultaneous addition of 5-HT (final concentration 1 μM) and ADP (final concentration 1 μM). Under these conditions, 1 μM ADP alone causes approximately 10–20% of maximal aggregation, whereas the combination of 1 μM 5-HT with 1 μM ADP produces maximal aggregation. The maximal amplitude of aggregation response within 3 min was determined and measured in triplicate using the Chronolog model 490 aggregometer (Chronolog Corp., Havertown, PA). Percent inhibition of aggregation was calculated from the maximum decrease in optical density of the controls and of the samples containing inhibitors.

2.8. Rat pharmacokinetics

Male Sprague–Dawley rats were dosed orally with AR246686 at 10 mg/kg. AR246686 was formulated in 80% PEG400 in PBS (pH 7.4) and administered using a dosing volume of 5 ml/kg. Animals were fasted overnight prior to dose administration. Whole blood samples were collected via cardiac puncture under light isoflurane anesthesia from .25 to 24 h post-dose. Plasma was prepared from sodium heparin-treated whole blood and separated by centrifugation. Plasma samples were frozen and stored at −70 °C until assayed. Composite sampling was used to define the plasma concentration vs. time profile (n=2–3 animals/time point). Noncompartmental pharmacokinetic analysis was performed with a commercial software package (WinNonlin Professional version 4.1.b., Pharsight, Mountain View, CA).

2.9. Vasoconstriction of rat aortic rings

Sprague–Dawley rats (average body weight 250–300 g) were euthanized using a CO_2 chamber. The thoracic aorta was

quickly harvested and placed in aerated (95% O_2 and 5% CO_2) Krebs–Henseleit buffer (Sigma K3753) (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, Glucose 11, $NaHCO_3$ 25, pH 7.4. The excess adventitial tissue was carefully removed and the endothelium was sloughed off by rinsing with H_2O via syringe. The vessel was then cut into 2–3 mm rings and mounted on wire stirrups connected to force transducers (World Precision Instruments, Myobath 4 channel system, FORT-10) to record changes in isometric force. The aortic rings were then placed in 10 ml organ baths filled with buffer at 37 °C with continuous aeration (95% O_2 and 5% CO_2). The absence of endothelium was confirmed by the lack of carbachol-induced relaxation of rings that had been pre-contracted with norepinephrine. The rings were left to equilibrate in buffer for 60 min, then tension was applied (2 g) and the rings allowed to equilibrate for an additional hour. During the equilibration period, the buffer was changed every 15 min. After equilibration was complete, 50 mM KCl was added to test for viability and to normalize individual aortic isometric force response to agonist. After removal of KCl, vehicle or doses of AR246686 were added to the organ baths 15 min prior to the addition of 20 μM 5-HT or 30 nM norepinephrine. A 30 min washout period was applied after each test dose of inhibitor. Quadruplicate measurements were made simultaneously from 4 individual rings treated identically in 4 separate water baths.

2.10. Vascular smooth muscle cell DNA synthesis

Rat aortic smooth muscle cells were obtained from Cell Applications, Inc. (San Diego, CA) and maintained in growth media at 37 °C in a humidified atmosphere containing 95% air, 5% CO_2. Cells were used from passage 2 through passage 5. DNA synthesis measurements were performed as previously described (Nebigil et al., 2000), with minor modifications. Briefly, cells were seeded onto 24-well plates to confluency and then serum-starved for 48 h. The quiescent cells were treated with 5-HT at different concentrations in the presence of 100 μM pargyline (a monoamine oxidase inhibitor) for 20 h. [^{3}H] thymidine (1 μCi) was added to the cultures for the last 4 h of incubation to measure DNA synthesis induced by 5-HT stimulation. For inhibitor studies, cells were treated with AR246686 30 min prior to addition of 5-HT (10 μM). The free [^{3}H]thymidine was washed away in 10% trichloroacetic acid and the incorporated [^{3}H]thymidine was quantified by scintillation counting.

2.11. Thrombosis and bleeding time measurements

This model was developed in an effort to concomitantly measure thrombus formation and bleeding time in the same animal. Following delivery of drug by oral gavage, rats were anesthetized using sodium pentobarbital (50 mg/kg). The right femoral artery was isolated in 2 different sections approximately 4–6 mm in length, one area for probe placement and one for ferric chloride patch positioning. The artery was then allowed to stabilize for a period of 20 min to allow recovery from the surgery. During stabilization a midline cervical incision was made to expose the trachea. A lateral incision was then made in the trachea, which was cannulated with a 18G blunt needle and tied with 4.0 silk suture. A Harvard rodent respirator (model 681) was used to ventilate the rats mechanically with room air set at 75 strokes/min with a stroke volume of 2.5 cm^3. A micro-arterial probe (Transonic Systems, Inc) was placed on the distal isolated femoral artery and femoral artery blood flow was monitored using a Powerlab recording system (AD Instruments). A small piece of filter paper (2 mm × 4 mm) soaked in 30% ferric chloride ($FeCl_3$) was placed on the area of the artery upstream of the probe for 10 min. To measure thrombosis, after removal of the $FeCl_3$ patch, the femoral artery blood flow was recorded until the artery was fully occluded and time to occlusion was recorded. To measure bleeding, 5 min after $FeCl_3$ patch placement, the last 3 mm of the tail was transected with a razor blade. The transected tail was immediately placed in saline at 37 °C, and the time at which bleeding stopped was recorded.

2.12. Ex-vivo platelet aggregation

2 h following P.O. dosing of male Sprague–Dawley rats with vehicle or AR246686, whole blood was collected in a 5 ml vacutainer with exogenous heparin (5 U/ml) added to the vacutainer. Aggregation studies were evaluated using a whole blood Aggregometer (Chronolog Corp.) Briefly, whole blood (400 μL) was added to saline (600 μL) with constant stirring and activated with 5 μg of collagen (Chronolog Corp.) The 5-HT response was obtained by also adding 5-HT to final concentration of 2.5 μM.

3. Results

3.1. AR246686 receptor affinity and functional antagonism assays

Our medicinal chemistry effort to identify a novel, potent and selective 5-HT_{2A} receptor antagonist that was orally bioavailable resulted in discovery of AR246686 (Fig. 1). Using competition displacement assays with [^{125}I]DOI, binding affinity estimates for AR246686 were 0.2 and 0.4 nM for the human and rat 5-HT_{2A} receptor respectively (Table 1). Competition studies using the 5-HT_{2A} antagonist [^{3}H]ketanserin rather than the agonist [^{125}I]DOI gave similar 5-HT_{2A} K_i values for AR246686. Published K_i values for several well

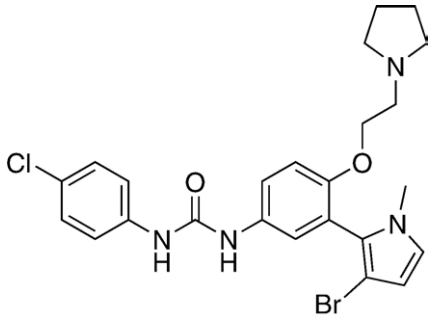

Fig. 1. The chemical structure of AR246686.

Table 1
Pharmacological properties of AR246686

Drug	Receptor	Binding assay	K_i±S.E.M. nM (*n*)	Reference
AR246686	Human 5-HT_{2A}	[^{125}I]DOI	0.2+/−0.02 (6)	
	Human 5-HT_{2A}	[^{3}H]ketanserin	0.4+/−0.03 (4)	
	Rat 5-HT_{2A}	[^{125}I]DOI	0.4+/−0.06 (2)	
	Rat 5-HT_{2A}	[^{3}H]ketanserin	0.3+/−0.06 (7)	
	Human 5-HT_{2B}	[^{125}I]DOI	300+/−151 (3)	
	Human 5-HT_{2C}	[^{125}I]DOI	2+/−0.3 (4)	
Ketanserin	Human 5-HT_{2A}	[^{3}H]ketanserin	1.1+/−0.1 (5)	Muntasir et al. (2006)
Ritanserin	Human 5-HT_{2A}	[^{3}H]ketanserin	1.7+/−0.2 (4)	Muntasir et al. (2006)
Sarpogrelate	Human 5-HT_{2A}	[^{3}H]ketanserin	23.0+/−2.0 (5)	Muntasir et al. (2006)
M-1 (sarpogrelate active metabolite)	Human 5-HT_{2A}	[^{3}H]ketanserin	10.0+/−1.8 (4)	Muntasir et al. (2006)

Affinity of AR246686 for human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors and rat 5-HT_{2A} receptors.

known 5-HT_{2A} antagonists are included in Table 1 for comparison (Muntasir et al., 2006). Affinity of AR246686 to related 5-HT_{2B} and 5-HT_{2C} receptors was also measured with [^{125}I]DOI binding displacement resulting in K_i values of 300 and 2 nM respectively. The affinity of AR246686 for other 5-HT receptor family members including 5-HT_{1A}, 5-HT_{1B}, 5-HT_3, 5-HT_{5A}, 5-HT_6, and 5-HT_7 was considerably lower (see Online Supplemental data). Competitive binding of 1 μM AR246686 against a panel of 70 human G-protein coupled receptors, ion channels and transporters (CEREP) demonstrated less than 50% inhibition of reference ligand binding with the exception of dopamine D1 (83%), dopamine D5 (79%), histamine H1 (74%), muscarinic M1 (93%), μ opiate (68%), L-type calcium channel (55%), and Na channel, site 2 (77%) (see Online supplemental data).

The 5-HT_{2A} receptor has been shown to mediate intracellular signaling by coupling to the GTP binding protein Gq, by activation of phospholipase C, and by generation of inositol phosphates (Grotewiel and Sanders-Bush, 1999; Kroeze et al., 2002). Thus, in order to determine whether AR246686 behaves as a neutral antagonist or as a functional inverse agonist of the 5-HT receptors, we measured its ability to inhibit constitutive inositol phosphate accumulation in HEK cells overexpressing either the 5-HT_{2A}, 5-HT_{2B}, or the 5-HT_{2C} receptors (Table 2). Consistent with the relative affinities obtained from binding experiments, AR246686 was a more potent functional inverse agonist at the 5-HT_{2A} receptor (IC_{50}=2 nM) than at the 5-HT_{2B} receptor (IC_{50}=572 nM). Inhibition of inositol phosphate accumulation was not observed in cells overexpressing the 5-HT_{2C} receptor, suggesting that AR246686 is a neutral antagonist at this receptor rather than an inverse agonist.

Table 2
Potency and Efficacy of AR246686 obtained in human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} IP accumulation assays

Drug	Receptor	IC_{50}+/−S.E.M. nM (*n*)	Reference
AR246686	Human 5-HT_{2A}	2+/−0.3 (4)	
	Human 5-HT_{2B}	572+/−149 (4)	
	Human 5-HT_{2C}	>10,000 (8)	
Ketanserin	Human 5-HT_{2A}	14+/−2 (4)	Muntasir et al. (2006)
Ritanserin	Human 5-HT_{2A}	26+/−4 (6)	Muntasir et al. (2006)
Sarpogrelate	Human 5-HT_{2A}	317+/−7 (5)	Muntasir et al. (2006)
M-1	Human 5-HT_{2A}	97+/−7 (5)	Muntasir et al. (2006)

Published IC_{50} values for several well known 5-HT_{2A} antagonists are included in Table 1 for comparison.

3.2. Pharmacokinetics in rats

AR246686 was rapidly absorbed from the gastrointestinal tract upon oral gavage (Fig. 2). Compound was present in the plasma at the first time point taken (0.25 h). The maximum absorption occurred between 2 and 4 h at plasma concentrations of 100 and 113 ng/ml, respectively. After 24 h, the plasma concentration declined to 1 ng/ml. The terminal phase half-life was 5.1 h.

3.3. In vitro 5-HT-induced platelet aggregation

Standard aggregometry assays were used to assess the ability of AR246686 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. 3A), whereas 5-HT alone did not cause aggregation at any concentration tested. Combining 5-HT (1 μM) with the sub-maximal concentration of ADP (1 μM), produced a maximal aggregation response. The 5-HT-mediated amplification of ADP-stimulated aggregation was completely blocked when platelets were pre-incubated with 1 μM

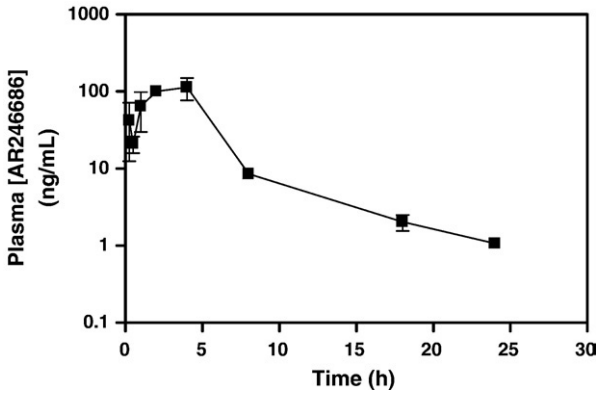

Fig. 2. Male Sprague–Dawley rats were dosed orally with AR246686 at 10 mg/kg. Whole blood samples were collected via cardiac puncture. Plasma samples were prepared and frozen at −70 °C until assayed. Composite sampling was used to define the plasma concentration vs. time profile (*n*=2–3 animals/time point).

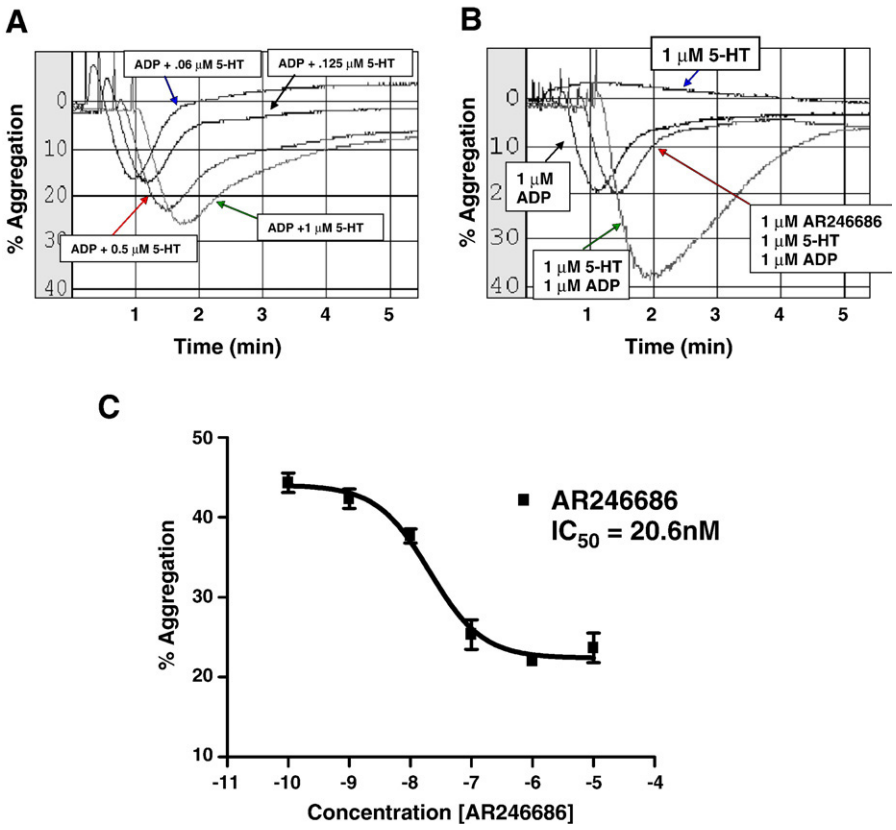

Fig. 3. A) Platelet aggregation in human platelet rich plasma was measured in response to 1 μM ADP in the presence of increasing concentrations of 5-HT as indicated in figure. No further increase in aggregation was seen with higher concentrations of 5-HT (not shown). B) 5-HT-mediated amplification of ADP-stimulated platelet aggregation is demonstrated in human platelet rich plasma. 1 μM AR246686 abolishes 5-HT-mediated amplification of aggregation. C) Dose–response of AR246686 inhibition of platelet aggregation stimulated by 1 μM 5-HT + 1 μM ADP. Values are means ± S.E.M. Data is representative of results from five separate experiments.

AR246686. (Fig. 3B). An average IC_{50} of 21 nM was determined from 5 independent experiments using platelet rich plasma from 5 different human donors. An example of one IC_{50} determination is shown in Fig. 3C.

3.4. Ex-vivo 5-HT-mediated vascular contraction

In order to determine if AR246686 could block 5-HT-mediated vasoconstriction, we studied its effects ex-vivo using aortic rings isolated from rats. 5-HT produced reproducible vasoconstriction in aortic rings, with an approximate ED_{80} of 20 μM (data not shown). The magnitude of vasoconstriction by 5-HT was similar to that produced maximally by 50 mM KCl. Pre-treatment of aortic rings with AR246686 prevented the vasoconstriction caused by 20 μM 5-HT in a concentration dependent manner, with a half maximal effect at 10 nM. (Fig. 4) In contrast, AR246686 had no inhibitory effect on norepinephrine stimulated vasoconstriction demonstrating that the effects of AR246686 are selective.

3.5. 5-HT stimulated smooth muscle cell proliferation

The effect of AR246686 on 5-HT stimulated rat aortic smooth muscle cell (RASMC) proliferation was evaluated indirectly by measuring [^{3}H]thymidine incorporation into newly synthesized DNA. Initial experiments demonstrated that 5-HT stimulated an increase in DNA synthesis in a concentration dependent manner resulting in approximately 4-fold increase at 10 μM 5-HT (Fig. 5A). Pre-incubation with AR246686 significantly inhibited 10 μM 5-HT stimulated DNA synthesis with an IC_{50} of 10 nM (Fig. 5B).

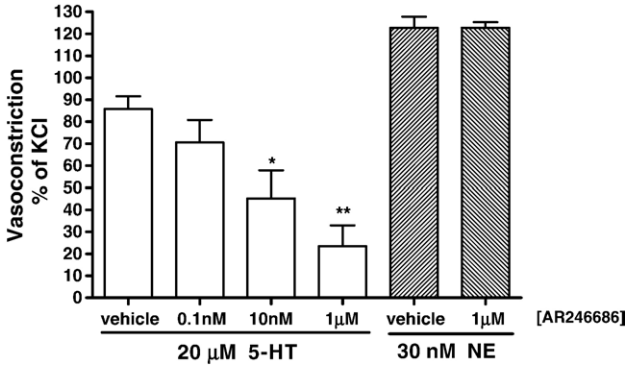

Fig. 4. AR246686 inhibits 5-HT stimulated vasoconstriction in rat aortic rings. Rat aortic rings were incubated with 20 μM 5-HT and vasoconstriction was measured in each ring relative to maximal vasoconstriction previously determined with 50 mM KCl. Pre-treatment of aortic rings with indicated concentrations of AR246686 for 10 min inhibited the vasoconstriction response to 5-HT. *$p<0.05$, **$p<0.001$ vs. 20 μM 5-HT alone. Selectivity of AR246686 for the 5-HT vasoconstriction response is demonstrated by lack of effect of 1 μM AR246686 on vasoconstriction stimulated by 30 nM norepinephrine (NE).

3.6. Ex-vivo 5-HT-induced platelet aggregation

The effect of AR246686 on ex-vivo platelet aggregation was determined using whole blood following oral administration of the compound to rats. Non-aggregating levels of 5-HT (1 μM) were used to amplify the effects of sub-threshold levels of collagen (1 mg/ml). Under these conditions, 5-HT amplified the effects of collagen alone approximately 5-fold in blood from vehicle treated animals. In blood from animals treated with AR246686, inhibition of 5-HT-mediated aggregation was demonstrated at 5 mg/kg and maximal inhibition at 10 mg/kg with an ED_{50} of ~4 mg/kg (Fig. 6).

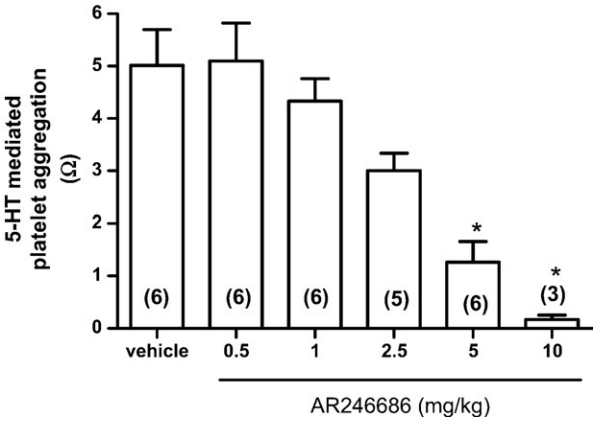

Fig. 6. Inhibition of ex-vivo platelet aggregation by AR246686. AR246686 was administered at the indicated doses by oral gavage 2 h prior to whole blood extraction by cardiac puncture. Effect of AR246686 on the amplification of platelet aggregation by 5-HT was evaluated in the whole blood impedance assay. For each blood sample collagen (1 mg/ml) stimulated aggregation served as a baseline and was subtracted from the collagen (1 mg/ml)+5-HT (1 μM) aggregation response to determine the 5-HT amplification response measure in Ohms (Ω). Values are means±S.E.M. Numbers in parentheses indicate number of animals. *$p<0.001$, as compared with vehicle by one-way ANOVA followed by Tukey's multiple comparison test.

3.7. Thrombosis and bleeding time

The aim of this experiment was to test the hypothesis that a 5-HT_{2A} receptor antagonist could preferentially reduce platelet rich thrombus formation without effects on complex clot formation seen with traumatic injury. Accordingly, we evaluated the effects of AR246686 on intravascular thrombosis and bleeding due to traumatic injury simultaneously in the same animals. Simultaneous measurements of tail transaction bleeding time and time to femoral artery occlusion were taken in each animal as described in Methods. In vehicle treated rats, the femoral artery occluded within 10 min after the initiation of endothelial injury by administration of ferric chloride to the surface of the artery. (Fig. 7B) The anti-thrombotic effect of AR246686 was observed at doses of 1 mg/kg and higher, where the time to occlusion was prolonged in a dose dependent manner. The tail bleeding time in vehicle treated animals averaged 529±50 s (Fig. 7A). However, significant prolongation of bleeding time by oral administration of AR246686 was only observed at the highest dose (20 mg/kg). The calculated therapeutic index, equal to the highest dose that did not increase bleeding time (10 mg/kg) divided by the lowest dose that delayed thrombosis (1 mg/kg), was determined to be approximately 10. For comparison, anti-thrombotic and bleeding effects of the ADP P_2Y_{12} receptor antagonist clopidogrel were measured in the same model (Fig. 7C, D). Both bleed time and time to femoral artery occlusion were significantly prolonged in rats given 10 mg/kg clopidogrel, yielding a therapeutic index of approximately 1.

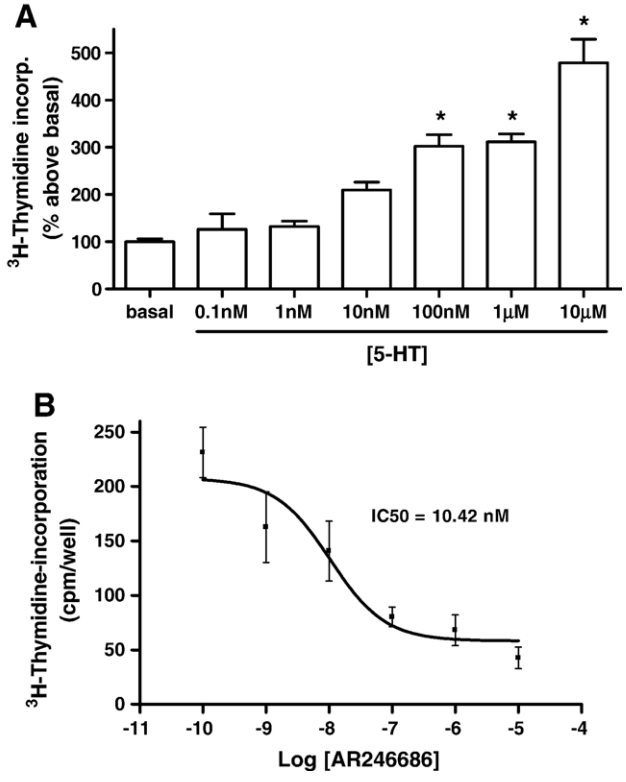

Fig. 5. A) 5-HT stimulates DNA synthesis in cultured rat aortic smooth muscle cells. DNA synthesis was analyzed by measuring the incorporation of ^{3}H-thymidine into cellular DNA. Labeling was done during the final 4 h of 24 h stimulation with the indicated doses of 5-HT. *$p<0.001$, as compared with basal by one-way ANOVA followed by Tukey's multiple comparison test. B) Effect of AR246686 on rat aortic smooth muscle cell DNA synthesis. Rat aortic smooth muscle cells were incubated in the presence of AR246686 at the concentrations shown for 20 min prior to stimulation of DNA synthesis with 10 μM 5-HT for 24 h. Labeling was done during the final 4 h of stimulation. Values for A and B are means±S.E.M. Data is representative of results from three separate experiments.

4. Discussion

In this study, we describe AR246686, a novel 5-HT_{2A} receptor antagonist and have evaluated its anti-thrombotic, anti-platelet and vascular effects. AR246686 demonstrated high affinity for both human and rat 5-HT_{2A} receptors in competitive binding assays. In addition, AR246686 inhibited intracellular signaling through the Gαq pathway at concentrations consistent with its binding affinity. This pharmacology combined with its oral bioavailablity and plasma half-life of 5.1 h allowed us to

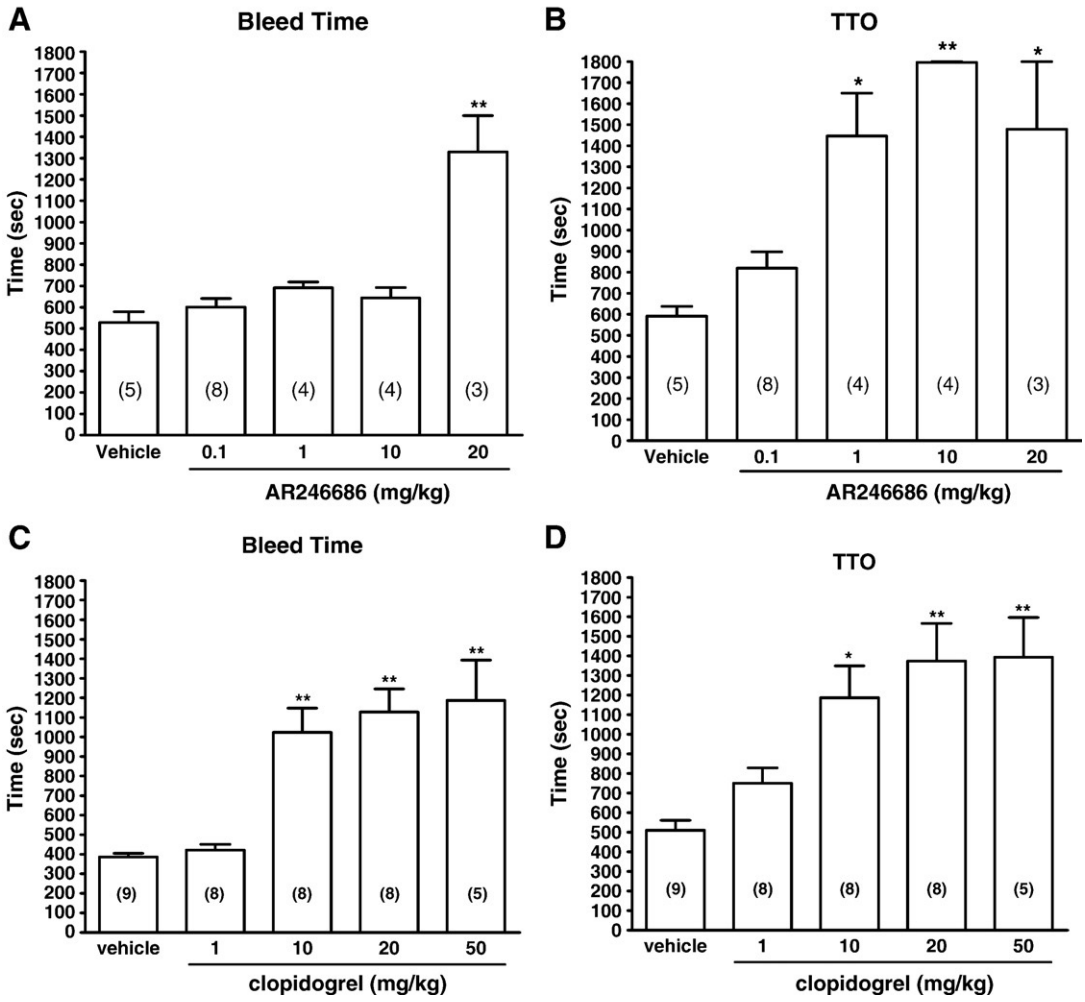

Fig. 7. Effect of AR246686 (A and B) and clopidogrel (C and D) on thrombosis and bleeding time. Tail transaction bleeding time (bleed time) and time to chemically induced femoral artery occlusion (TTO) were measured simultaneously in rats 30 (AR246686) and 120 min (clopidogrel) following oral administration of indicated doses of drug or vehicle. Values are means ± S.E.M. Numbers in parentheses indicate number of animals. TTO in non-occluded animals was taken as 1800 s. *$p<0.05$ and **$p<0.001$, as compared with vehicle by one-way ANOVA followed by Tukey's multiple comparison test.

extend our evaluation to in vivo models of thrombosis and bleeding.

AR246686 has substantial selectivity for the human 5-HT_{2A} receptor relative to other 5-HT receptor family members. We calculated an 8.7-fold lower affinity for the 5-HT_{2C} receptor based on competitive binding assay data. The activity at the 5-HT_{2C} receptor appears to be neutral antagonism since it did not increase or decrease inositol phosphate concentrations in 5-HT_{2C} transfected cells. Further, antagonist activity at the 5-HT_{2C} receptor was not likely to contribute to the platelet and smooth muscle responses since the 5-HT_{2C} receptor is expressed exclusively in the rat central nervous system and type II pneumocytes (Leysen, 2004).

Platelets express 5-HT_{2A} receptors (Drummond and Gordon, 1974), but 5-HT is only a weak stimulus for aggregation. Our data confirms that 5-HT acts to amplify platelet aggregation induced by agonists including, but not limited to, ADP, epinephrine, norepinephrine, and collagen (De Clerck and Herman, 1983). The degree of 5-HT-mediated amplification can be both agonist and donor dependent. However, under the conditions used in this study, we observed a highly reproducible and robust amplification of ADP-induced platelet aggregation in platelet rich plasma from multiple donors. While AR246686 had no effect on ADP-induced aggregation itself (not shown), it was a potent inhibitor of 5-HT-mediated amplification of human platelet aggregation. Similar results have been reported for other 5-HT_{2A} antagonists including ketanserin, sarpogrelate (M-1), R-96544, and SR 46349 (Van Nueten et al., 1981; Herbert et al., 1993;Ogawa et al., 2002).

In order to determine if AR246686 could be used to inhibit platelet function in vivo, the compound was orally dosed in rats, blood was removed and the effect on 5-HT-mediated amplification of collagen-induced platelet aggregation was measured ex-vivo. Maximal inhibition of ex-vivo platelet aggregation was seen in whole blood 2 h following an oral dose of 10 mg/kg AR246686. At this dose, we calculate plasma levels of approximately 100 ng/ml (or approximately 200 nM) based on rat pharmacokinetic data.

Simultaneous blockade of 5-HT_{2A} receptors on platelets and vascular smooth muscle cells should both contribute to the

inhibition of thrombotic arterial occlusion since, in addition to thrombosis, vasoconstriction and cell proliferation can contribute to narrowing of arterial vessels. Accordingly, we evaluated the effects of AR246686 on smooth muscle cell contraction and proliferation. We observed that AR246686 was a potent inhibitor of both processes.

To evaluate the effects of AR246686 on arterial thrombosis in vivo we used a well characterized model of platelet-dependent thrombosis ($FeCl_3$). The structure and composition of the thrombus in this experimental model is similar to that of most arterial thrombi in humans (Kurz et al., 1990). In addition, we have analyzed tail transaction bleeding time concurrently in the same animals undergoing $FeCl_3$ treatment. In this manner we were able to directly compare the effects of anti-platelet compounds on intravascular thrombus formation and traumatic bleeding simultaneously. We first tested the P_2Y_{12} ADP receptor antagonist clopidogrel. Clopidogrel significantly delayed thrombus formation in this model at a dose of 10 mg/kg. However, the same dose also resulted in a significant increase in tail transaction bleeding time, resulting in a calculated therapeutic index of approximately 1. The lack of separation between anti-thrombotic activity and bleeding time in animal models is consistent with the findings of others (Berry et al., 2001; Foster et al., 2001; Dogne et al., 2004), and the well known bleeding liability for clopidogrel in the clinical setting (Cooke and Goldschmidt-Clermont, 2006). In contrast, we observed a significant delay in thrombus formation for AR246686 at 1 mg/kg without any increase in bleeding time. A significant increase in tail bleeding time was not observed until the dose was increased to 20 mg/kg. This represents a therapeutic index of approximately 10 relative to bleeding time. The separation of anti-thrombotic activity and bleeding time has been demonstrated previously for other 5-HT_{2A} antagonists (Berry et al., 2001; Kihara et al., 2001). The mechanism of the separation for AR246686 and other 5-HT_{2A} antagonists remains unknown. The P_2Y_{12} receptor and the 5-HT_{2A} receptor couple to different G-proteins in platelets, Gi and Gq respectively (Offermanns, 2006). Thus, it is possible that the platelet signaling responses interrupted by irreversible P_2Y_{12} antagonists like clopidogrel play a primary role in formation and stabilization of a hemostatic thrombus, while interruption of the 5-HT_{2A} receptor mediated responses are more involved in the secondary amplification of platelet aggregation in the growing thrombus. The role of ADP in the coagulation cascade could also explain the lack of separation for P_2Y_{12} antagonists. The procoagulant effects of ADP on platelets have been clearly demonstrated and are thought to be mediated predominantly through the P_2Y_{12} receptor (Leon et al., 2003), while 5-HT alone does not stimulate procoagulant responses (Li et al., 1997). Thus, interruption of P_2Y_{12} mediated responses in platelets could interfere more directly with early events in hemostatic coagulation relative to 5-HT_{2A} mediated platelet responses.

In conclusion, the present study demonstrates that AR246686 is a potent, selective, and orally available 5-HT_{2A} receptor antagonist. These properties engender its ability to inhibit 5-HT-mediated amplification of platelet aggregation, vasoconstriction, and smooth muscle cell proliferation. Further, we demonstrate a pharmacological separation of the effects of AR246686 on intravascular thrombosis and traumatic bleeding time. This may provide a therapeutic advantage over current anti-platelet therapies although care should be taken when extrapolating such animal data to the clinical setting.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2007.11.056.

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