

RESEARCH PAPER

Cardiovascular effects of a novel potent and highly selective azaindole-based inhibitor of Rho-kinase

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Background and purpose: Rho-kinase (ROCK) has been implicated in the pathophysiology of altered vasoregulation leading to hypertension. Here we describe the pharmacological characterization of a potent, highly selective and orally active ROCK inhibitor, the derivative of a class of azaindoles, azaindole **1** (6-chloro-*N*⁴-{3,5-difluoro-4-[(3-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)oxy]-phenyl}pyrimidine-2,4-diamine).

Experimental approach: Pharmacological characterization of azaindole **1** was performed with human recombinant ROCK *in vitro*. Vasodilator activity was determined using isolated vessels *in vitro* and different animal models *in vivo*.

Key results: This compound inhibited the ROCK-1 and ROCK-2 isoenzymes with IC₅₀s of 0.6 and 1.1 nM in an ATP-competitive manner. Although ATP-competitive, azaindole **1** was inactive against 89 kinases (IC₅₀ > 10 μM) and showed only weak activity against an additional 21 different kinases (IC₅₀ = 1–10 μM). Only the kinases TRK and FLT3 were inhibited by azaindole **1** in the sub-micromolar range, albeit with IC₅₀ values of 252 and 303 nM, respectively. *In vivo*, azaindole **1** lowered blood pressure dose-dependently after i.v. administration in anaesthetized normotensive rats. In conscious normotensive and spontaneously hypertensive rats azaindole **1** induced a dose-dependent decrease in blood pressure after oral administration without inducing a significant reflex increase in heart rate. In anaesthetized dogs, azaindole **1** induced vasodilatation with a moderately elevated heart rate.

Conclusions and implications: Azaindole **1** is representative of a new class of selective and potent ROCK inhibitors and is a valuable tool for the elucidation of the role of ROCK in the cardiovascular system.

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Keywords: Rho-kinase inhibitor; *in vitro* selectivity; vasorelaxation; blood pressure; heart rate; hypertension

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; DMSO, dimethyl sulphoxide; IMAC, immobilized metal affinity chromatography; MBS, myosin-binding subunit; MLC, myosin light chain; MLCK, myosin light chain kinase; ROCK, Rho-kinase; SHR, spontaneously hypertensive rats; S6 peptide, RRLSSLRA; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; Y-27632, (+)-*R*-trans-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride; ZIP-kinase, zipper-interacting protein kinase

Introduction

The small GTP-binding proteins of the Rho family regulate various cellular functions. One effector of Rho is Rho-kinase (ROCK), a serine/threonine kinase, which is comprised of two isoenzymes, ROCK-1 (or ROKβ) and ROCK-2 (or ROKα). It is well established that ROCK plays a pivotal role in the

regulation of the tonic phase of smooth muscle cell contraction (Gong *et al.*, 1996; Christ and Wingard, 2005). The contractile effect results from ROCK-mediated phosphorylation of the myosin-binding subunit (MBS), the regulatory subunit of myosin light chain (MLC) phosphatase. Phosphorylated MBS inhibits the phosphatase activity thus allowing an increase in the level of phosphorylated MLC and contraction at a constant intracellular calcium concentration (Kimura *et al.*, 1996). This phenomenon is defined as calcium sensitization (Christ and Wingard, 2005).

ROCK activity or protein is dysregulated under certain cardiovascular pathophysiological conditions such as

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vasospasm (Masumoto *et al.*, 2002), atherosclerosis (Shimokawa *et al.*, 2001) and hypertension (Masumoto *et al.*, 2001). The ROCK locus was identified in genome-wide linkage studies as a hypertension-associated gene (Mendelsohn, 2005), and homozygous carriers of a polymorphism in the ROCK gene (ROCK2-Thr431Asn) had an increased risk of hypertension (Seasholtz *et al.*, 2006). Pharmacological inhibition of ROCK has beneficial effects in a variety of animal models of cardiovascular diseases (Christ and Wingard, 2005).

Previously, mainly two ROCK inhibitors, Y-27632 ((+)-*R*-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride) (Uehata *et al.*, 1997) and HA-1077 (fasudil), which belong to a series of homopiperazine-derived ROCK inhibitors (Ono-Saito *et al.*, 1999; Nagumo *et al.*, 2000), have been used to explore the function of ROCK in the cardiovascular system. In Japan, fasudil is already in use as a therapeutic agent to treat cerebral vascular spasm (Tachibana *et al.*, 1999). In addition, fasudil has shown benefits for the treatment of patients with angina pectoris (Shimokawa *et al.*, 2002). However, both Y-27632 and fasudil are weak inhibitors of ROCK with K_i -values of 140 and 330 nM, respectively. The selectivity profile of Y-27632 appears to be reasonable, but only 27 different kinases have been tested so far (Davies *et al.*, 2000). Fasudil shows some selectivity for ROCK inhibition. However, it is also a relatively potent inhibitor of kinases such as protein kinase A (PKA), PKG and PKC that regulate important cellular functions (Ono-Saito *et al.*, 1999; Nagumo *et al.*, 2000). Recently, it has been shown that hydroxyfasudil, the main metabolite of fasudil after oral administration, is more specific than the parent compound, but it is still a weak inhibitor of ROCK with a K_i -value of 170 nM (Shimokawa *et al.*, 1999). Another analogue of fasudil, H-1152P, although a potent ROCK-2 inhibitor ($K_i = 1.6$ nM), still retains inhibitory activity against several kinases in the submicromolar range (Tamura *et al.*, 2005).

Here, we have characterized, for the first time, the pharmacological properties of the potent and orally available ROCK inhibitor azaindole **1** (6-chloro-*N*⁴-[3,5-difluoro-4-[(3-methyl-1*H*-pyrrolo[2,3-*b*]pyridin-4-yl)oxy]phenyl]pyrimidine-2,4-diamine). This compound is a highly specific ATP-competitive inhibitor with activity in the low nanomolar range. The compound potently relaxes rabbit precontracted isolated saphenous arteries in a concentration-dependent manner, increases coronary blood flow in the rat heart Langendorff model and dose dependently lowers blood pressure in normotensive anaesthetized rats and conscious hypertensive rats without an increasing heart rate. In addition, in anaesthetized dogs, azaindole **1** induced a marked decrease in blood pressure.

Methods

Molecular cloning and expression of protein kinases

To obtain the human protein kinases ROCK-1, ROCK-2 and zipper-interacting protein kinase (ZIP-kinase) and the murine ROCK-2 for the enzymatic assays, the following primers were used for modification of the 5' and 3' ends of the respective

cDNA clones by PCR. Primer A: 5'-GGGATCCACCATGAGCCGCCCCCGCCGACGGG-3' (*Bam*HI site underlined), primer B: 5'-GGGAATTCTTATGATCCATAATTTTGC TATATG-3' (*Eco*RI site underlined), primer C: 5'-CACCA TGTCGACTGGGGACAGTTTTGAGACTCG-3', primer D: 5'-GGAATTCGCCCTTTAACTAGTTTTTCCAG-3', primer E: 5'-GGGATCCATGTCCACGTTCCAGGCAGGAGGACG-3' (*Bam*HI site underlined), primer F: 5'-GGGCTCGAGCTAGCGCA GCCCGACTCCACGCC-3' (*Xho*I site underlined). The PCR fragments containing primers A and B and primers E and F were cloned into TOPO TA. The fragments containing primers C and D were cloned into pENTR TOPO.

The murine ROCK-2 cDNA was cloned into the *Eco*RI site of pFASTBAC HTa (Invitrogen, Karlsruhe, Germany). The human ROCK-2 cDNA was modified by PCR employing primers A and B and cloned into pFASTBAC HTb after digestion with *Bam*HI and *Eco*RI. The human ROCK-1 cDNA was modified by PCR employing primers C and D and cloned into pDEST10 using GATEWAY technology (Invitrogen). Recombinant baculoviruses were prepared using the Bac-to-Bac system according to instructions from the manufacturer (Invitrogen). The human ZIP-kinase cDNA was modified by PCR employing primers E and F and cloned into the *Escherichia coli* expression vector pKa83a after digestion with *Bam*HI and *Xho*I. pKa83a is an in-house developed vector containing a kanamycin-resistance gene and an isopropyl-β-D-thiogalactopyranoside inducible promoter. From this vector, the human ZIP-kinase is produced with an N-terminal His₆ and a maltose-binding protein (MBP) tag. The final vector is transformed into the *E. coli* strain W3110.

Purification of recombinant proteins

His-tagged ROCK-1 and ROCK-2 proteins were purified from the cytosolic fraction of baculovirus-infected Sf9 insect cells. The cell pellet was resuspended in lysis buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 20 mM imidazole, 2 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 0.5% Triton X-100, 0.1 mM Pefabloc, protease inhibitor cocktail Complete, 10% glycerol, pH 7.3) at 30 °C. The cells were disrupted and homogenized with an ultraturax followed by sonification. After centrifugation (10 000 *g*, 4 °C) the proteins were purified from the supernatant by Ni-immobilized metal affinity chromatography (IMAC) on a nickel-activated chelate sepharose column. The column was washed with IMAC buffer A (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 20 mM imidazole, 2 mM TCEP, 0.5% Triton X-100, 0.1 mM Pefabloc, 10% glycerol, pH 7.3) and the bound protein was eluted at a flow rate of 1.5 ml min⁻¹ with IMAC buffer B (IMAC buffer A plus 500 mM imidazole).

The *E. coli* cell pellet containing ZIP-kinase was resuspended in lysis buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 40 mM imidazole, 2 mM TCEP, 0.1 mM Pefabloc, protease inhibitor cocktail Complete, 10% glycerol, pH 7.5) at 30 °C. Homogenization and purification of the protein was carried out as already described for ROCKs. The nickel-activated chelate sepharose was washed with IMAC buffer A, and the bound protein was eluted at a flow rate of 10 ml min⁻¹ with IMAC buffer B. The proteins were stored at -70 to -80 °C.

Kinase assays

All protein kinase activities were linear with respect to time and protein in every incubation. Human ROCK-1 and ROCK-2, murine ROCK-2, human ZIP-kinase and human MLC kinase (MLCK) (aa1425–1771) were preincubated for 10 min with test compounds in 0.05 ml of 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl₂ and 0.06% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate) (ROCK-1; ROCK-2; ZIP-kinase) or 50 mM HEPES, pH 7.4, 10 mM Mg acetate, 1 mM dithiothreitol, 0.3 mM CaCl₂, 1 μM calmodulin (MLCK). As substrate following peptides were used: RRLSSLRA (ROCK; S6 peptide), KEAKEKRQEQIAKRRRLSSL RASTSKSGGSQK (ZIP-kinase; long S6 peptide) and KKRAAR ATSNVFA (MLCK; MLC peptide). After 10 min preincubation with dimethyl sulphoxide (DMSO) (0.1% final concentration) or with increasing amounts of azaindole 1 (final concentration 0.1 nM–3 μM), the assays were initiated with [³³P]ATP (3000 mCi mmol⁻¹) (10 μM final concentration, unless stated otherwise). After 20 min incubation, the reaction was terminated by incubation at 95 °C for 10 min. After centrifugation at 10 000 g for 1 min, an aliquot of each incubation was spotted on paper matts. The papers were dried and subsequently washed twice in distilled water. After being dried, the papers were coated with scintillator and counted for radioactivity. Inhibition curves were analysed by nonlinear regression using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

The activity of azaindole 1 against 112 kinases was investigated in collaboration with Upstate (Dundee, UK) using an ATP concentration of 100 μM.

ROCK-2 autophosphorylation and myosin-binding subunit phosphorylation

Human ROCK-2 (50 ng) was preincubated (final concentration 1–30 nM in DMSO) with the test compound either alone or in the presence of human MBS (aa654–880; 100 ng) as substrate in 0.05 ml of 50 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 0.06% CHAPS for 10 min at 37 °C. The final concentration of DMSO was 0.1%. The reaction was started by the addition of [³³P]ATP (10 μM final concentration; 3000 Ci mmol⁻¹). After incubation for 30 min at 37 °C, the reaction was terminated by adding 2 × Laemmli sample buffer. The solution was boiled for 5 min at 95 °C and then applied to an SDS–polyacrylamide gel electrophoresis using a 4–15% gradient SDS-gel and the PHAST System. The gel was dried and subjected to autoradiography using Kodak XAR-5 film.

Molecular modelling

Maestro (v. 7.0, Schrödinger, Portland, OR, USA) was used for protein overlays, inhibitor docking and the generation of modelling pictures. MacroModel (v. 9.0, Schrödinger) was used to perform energy minimizations (protein coordinates fixed, inhibitor flexible; solvent water; convergence on gradient, convergence threshold = 0.2).

Rabbit isolated vessels

Chinchilla rabbits of either sex (Harlan Winkelmann, Borcheln, Germany) (about 2–3 kg) were killed by an overdose

of thiopental. The saphenous arteries were dissected and rings of the arteries (3 mm width) were suspended under an initial tension of approximately 4 g in 5 ml organ baths containing Krebs-Henseleit solution (containing 0.001% BSA) at 37 °C. Contractions were measured isometrically with Statham UC2 strain gauges connected to a DAS1802HC data acquisition board (Keithley instruments, Germering, Germany). Rings were precontracted by phenylephrine (0.15 μM; submaximal contraction) four times. Each contraction was followed by a series of 11 washing cycles and 1 resting period of 25 min. The increasing concentrations of the test compound (0.1 nM–10 μM) in DMSO, final concentration 0.1% were added to the organ baths. The concentration of the test compounds was increased by a factor of 10. Rings were subsequently contracted with phenylephrine (0.15 μM). The intact endothelium was functionally tested by the presence of the relaxant response to acetylcholine (0.5 μM).

Rat heart Langendorff preparation

The hearts of Wistar rats (200–250 g) were perfused according to Langendorff at 37 °C with a nonrecirculating system. The perfusion medium was filtered Krebs-Henseleit solution containing 11 mM glucose and 1.2 mM of CaCl₂, equilibrated with O₂ + CO₂ (95 + 5%), to give a pH of 7.4 and a pO₂ of 650–700 mm Hg. Perfusion was performed at a constant rate (10 ml min⁻¹). A latex balloon filled with saline and connected to a pressure transducer (Gould Statham, Oxnard, CA, USA) via a metal cannula was inserted into left ventricular cavity to measure the isovolumetric contractions of the left ventricle. A second pressure transducer was connected to the aortic cannula to record the perfusion pressure. Drug solutions (0.1 nM–10 μM in DMSO, final concentration 0.1%) were infused into aortic cannula at a rate of 1% of the total flow rate.

Haemodynamics in anaesthetized rats

Male Wistar rats weighing 300–350 g (Harlan Winkelmann) were anaesthetized with thiopental 100 mg kg⁻¹ intraperitoneally (i.p.). A tracheotomy was performed and catheters were inserted into the femoral artery for blood pressure and heart rate measurements (Gould pressure transducer and recorder model RS 3400) and into the femoral vein for test drug administration. The animals were ventilated with room air and their body temperature was controlled. Azaindole 1 was administered intravenously (i.v.) in doses of 0.03–0.1 mg kg⁻¹ in a solution of Transcutol/Cremophor EL/physiological saline (19/10/80 = v/v/v). The vehicle Transcutol/Cremophor EL/physiological saline (19/10/80 = v/v/v) without test drug was used as control. The volume administered was 1 ml kg⁻¹. Six animals were treated per group.

Haemodynamics in conscious rats

Female conscious normotensive and spontaneously hypertensive rats (Moellegaard, Ejby, Denmark, 220–290 g) were equipped with implantable radiotelemetry, and a data acquisition system (Data Sciences, St Paul, MN, USA)

comprising a chronically implantable transducer transmitter unit equipped with a fluid-filled catheter was used. The transmitter was implanted into the peritoneal cavity and the sensing catheter was inserted into the descending aorta. Single doses of azaindole 1 were administered to spontaneously hypertensive rats (1, 3 and 10 mg kg⁻¹) and normotensive rats (3 and 10 mg kg⁻¹), or multiple doses once daily to spontaneously hypertensive rats (10 mg kg⁻¹), as a solution (5 mg ml⁻¹) in Transcutol/Cremophor/H₂O (10/20/70 = v/v/v) orally by gavage. The volume administered by gavage was 2 ml kg⁻¹ body weight. Each dose was administered to groups of six animals. Animals in the control groups only received the vehicle. Before treatment mean blood pressure and heart rate of treated and untreated control groups was in the range of 131–142 mm Hg and 279–321 beats min⁻¹, respectively.

Haemodynamic variables in anaesthetized dogs

These studies were performed in mongrel dogs of either sex weighing between 20–30 kg. Anaesthesia was induced by thiopental and maintained with isoflurane (0.6–1.2 vol%). Fentanyl (6 µg kg⁻¹ h⁻¹) was used as an analgesic. The dogs were artificially ventilated with N₂O/O₂ (15 respiratory cycles with 30–40 ml per cycle). Azaindole 1 was dissolved in 1% DMSO, 49.5% polyethylene glycol 400 and 49.5% Transcutol and administered as an i.v. dose of 100 and 300 µg kg⁻¹ (50 µl kg⁻¹, cumulative administration). Administration of vehicle served as a control. The time interval between the two doses was 60 min.

The following parameters were investigated: systemic blood pressure (fluid-filled hollow catheter inserted via the femoral artery into the abdominal aorta and connected to a strain gauge (Braun, Melsungen, Germany), ECG (lead II), cardiac pressures and contractility via a tip catheter (PC350, Millar Instruments, Houston, TX, USA) and coronary blood flow (via a coronary flow probe (Skalar Instruments, Deeft, Netherlands). All signals were amplified with a Gould amplifier system and digitally analysed using the PONEMAH physiology platform (LDS Test&Measurement, Ismaning, Germany).

Pharmacokinetics in mice, rats and dogs

To determine the pharmacokinetics of azaindole 1, blood samples were obtained at 2, 5, 15, 30 min, 1, 2, 3, 5 and 7 h following its i.v. administration to NMRI mice (Harlan Winkelmann) (three animals per time point). In male Wistar rats and female beagle dogs, blood samples were taken at 5, 10, 15, 17, 20, 30, 45 min, 1, 2, 3, 5 and 7 h and at 5, 10, 15, 17, 20, 30, 45 min, 1, 2, 3, 4, 6, 8, 24 and 26 h after infusion, respectively. Time points for blood samples following oral administration were 15, 30 min, 1, 2, 4, 6, 8, 24 h and 15, 30 min, 1, 2, 3, 4, 5, 6, 8, 24, 26 h in rats and dogs, respectively. Oral formulation in rats and dogs was 10% EtOH; 20% Solutol; 70% H₂O and 5% EtOH; 20% polyethylene glycol 400; 75% H₂O, respectively. Pharmacokinetics in rats and dogs were determined in three animals using serial bleeding. Animals were conscious throughout the study.

After centrifugation, plasma was precipitated by the addition of acetonitrile. The supernatants were subjected to

high performance liquid chromatography performed on a 2300 HTLC system (Cohesive Technologies, Crownhire, UK) equipped with a Lichrochart Purospher Star RP-18e column (55 × 4 mm internal diameter, 3 µm particle size; Merck KGaA, Frankfurt, Germany) at a flow rate of 1 ml min⁻¹. The mobile phase consisted of 10 mM ammonium acetate (pH 3.0) and acetonitrile. A linear gradient from 20 to 80% acetonitrile (v/v) within 2 min was applied. Tandem mass spectrometry was performed on an API 3000 triple-quadrupole. Mass spectrometer (PE Sciex) connected to the high performance liquid chromatography system through a Turbolonaspay interface. Limit of quantification was 1 µg l⁻¹. Pharmacokinetic parameters were calculated from plasma concentrations with KINCALC, a validated in-house (Bayer HealthCare, Wuppertal, Germany) calculation programme.

All animal experiments were in compliance with the German guidelines for animal care/husbandry and approved by the local authorities (Regierungspräsidium Düsseldorf).

Statistics

Unless otherwise indicated, the results shown in the present publication are means ± s.e.m. from at least three independent experiments performed in duplicates or triplicates. Differences were assessed by one-way analysis of variance followed by the Bonferroni test for comparison of means or by Student's *t*-test. *P* < 0.05 was considered significant.

Chemicals

Azaindole 1 (Figure 1) was synthesized as described previously (Schirok *et al.*, 2005). pENTR TOPO was obtained from Invitrogen; Pefabloc, protease inhibitor cocktail Complete from Roche (Mannheim, Germany); nickel-activated chelate sepharose column from GE Amersham Biosciences (Freiburg, Germany); human ROCK-1 and ROCK-2, murine ROCK-2, human ZIP-kinase and human MLCK (aa1425–1771) from Upstate (Charlottesville, VA, USA); ROCK; S6 peptide from Bachem (Bubendorf, Switzerland); ZIP-kinase, long S6 peptide from Upstate; MLCK, MLC peptide from Bachem; paper mats from Wallac (Boston, MA, USA); human MBS (aa654–880) from Upstate; SDS-gel from Amersham Bioscience (Uppsala, Sweden); PHAST System from Amersham Bioscience; thiopental from Nycomed

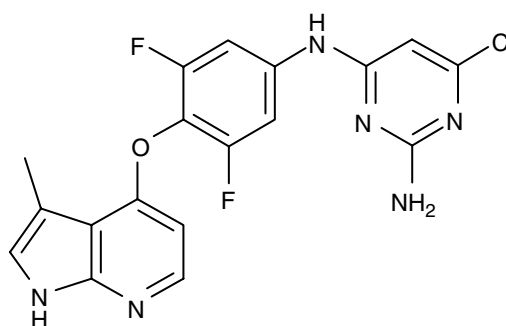


Figure 1 The chemical structure of the Rho-kinase inhibitor azaindole 1 (6-chloro-N¹-{3,5-difluoro-4-[(3-methyl-1H-pyrrolo[2,3-b]pyridin-4-yl)oxy]phenyl}pyrimidin-2,4-diamine).

(Munich, Germany); isoflurane (0.6–1.2 vol%, Isoflor) from Abbott (Chicago, IL, USA).

Results

Azaindole 1 inhibits human ROCK in vitro

To find ROCK inhibitors with novel structures, we performed a high-throughput-screening using an in-house 400 000 compound library. Chemical optimization of a lead structure resulted in the identification of azaindole 1. The compound is a highly potent inhibitor of human ROCK-1 and ROCK-2 isoenzymes with IC_{50} values of 0.6 and 1.1 nM, respectively. ROCKs from other species such as murine ROCK-2 or rat ROCK-2 were also potently inhibited by this substance with IC_{50} values of 2.4 and 0.8 nM, respectively. To demonstrate the effect of azaindole 1 in a kinase assay using a natural substrate, we tested the ROCK-dependent phosphorylation of human MBS *in vitro* (Kimura *et al.*, 1996). As shown in Figure 2, azaindole 1 concentration dependently reduced ROCK-2-mediated MBS phosphorylation. In the presence of 3 nM azaindole 1, [^{33}P]phosphate incorporation in the substrate was reduced by more than 50%, whereas phosphorylation of MBS was almost blocked at 10 nM of compound azaindole 1. In addition to substrate phosphorylation, autophosphorylation of ROCK-2 *in vitro* was also concentration-dependently reduced (Figure 2). The results correlate with the inhibitory potency of the phosphorylation of S6 substrate peptide.

To elucidate the mode of action of azaindole 1 on the enzyme, we performed kinetic analysis experiments. Human ROCK-2 was incubated with different concentrations of ATP or different concentrations of the substrate peptide S6 in the presence of increasing inhibitor concentrations. In the presence of azaindole 1, the apparent K_m values for ATP of ROCK-2 increased in a concentration-dependent manner without changes in the V_{max} value, suggesting that azaindole 1 interacts with the ATP-binding site of ROCK-2 and interferes with ATP binding in a competitive manner (Figure 3a). In contrast, varying the S6 substrate concentration in the presence of different inhibitor concentrations results in a decrease of V_{max} , whereas the apparent K_m values of the S6 substrate peptide remained unchanged (Figure 3b). Accordingly, azaindole 1-dependent inhibition of human ROCK-2 is noncompetitive with respect to the S6 substrate binding. Similar results were obtained with human ROCK-1

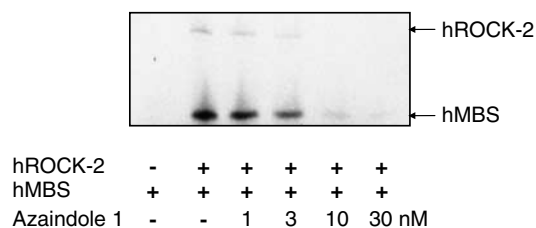


Figure 2 Inhibition of Rho-kinase isoenzymes by azaindole 1 using the physiological substrate MBS (Upstate, aa 654–880). Activity of purified recombinant human ROCK-2 was measured as described in Materials and methods. Arrows denote the autophosphorylated human ROCK-2 and phosphorylated human MBS. MBS, myosin-binding subunit; ROCK, Rho-kinase.

(data not shown). The K_i values derived from these experiments are 3.7 nM for ROCK-1 and 4.8 nM for ROCK-2.

This observation was supported by measurements of the IC_{50} values in the presence of increasing ATP concentrations. When the enzyme was assayed with 0.01, 0.1 and 1 mM ATP in the presence of the inhibitor, the IC_{50} s for inhibition of ROCK-2 shifted from 1.1 nM (0.01 mM ATP) to 12.4 (0.1 mM ATP) and 77.2 nM (1 mM ATP). However, even in the presence of 1 mM, the compound was still a potent inhibitor of human ROCK-2 with an IC_{50} of 77.2 nM. This result suggests that azaindole 1 is a competitive inhibitor of the ATP binding to ROCK-2. When azaindole 1 was preincubated with ROCK-2 for 30 min before the addition of ATP concentrations, the IC_{50} values were only slightly increased to 2.9 nM in the presence of 0.1 mM ATP and to 4.7 nM in the presence of 1 mM ATP. This result indicates that the binding of azaindole 1 is either irreversible or very slowly reversible.

Selectivity assays for azaindole 1

To exclude the possibility that the observed antagonism of ROCK is due to a nonspecific effect on vasoactive mechanisms, we examined the specificity of the azaindole 1. Firstly, we investigated the effect of azaindole 1 on the kinases involved in smooth muscle contraction. MLCK, the crucial kinase regulating calcium-dependent vasoconstriction, was only moderately inhibited by azaindole 1 with an IC_{50} of 7.4 μ M. In addition, ZIP-kinase, which has been shown to modulate myosin phosphatase activity and to participate in smooth muscle cell contraction by Ca^{2+} sensitization (Niiro and Ikebe, 2001), was only slightly inhibited by azaindole 1 (IC_{50} = 4.1 μ M).

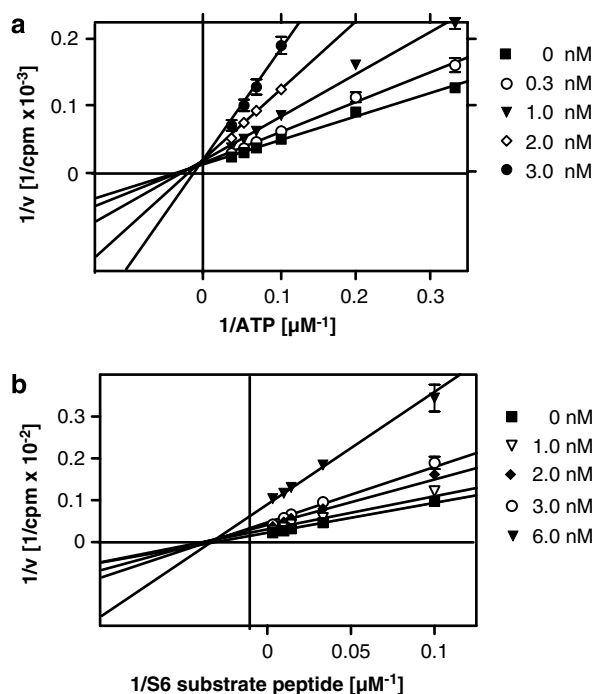


Figure 3 Double-reciprocal plots of ROCK-2 activity as a function of ATP (a) or S6 substrate peptide (b) in the presence of increasing azaindole 1 concentrations. ROCK, Rho-kinase.

The specificity against several other kinases was explored in collaboration with Upstate (Dundee, UK). Azaindole 1 was tested at 10 μM against 112 different kinases. It was inactive against 89 kinases ($\text{IC}_{50} > 10 \mu\text{M}$; data not shown) and showed only a weak activity against an additional 21 different kinases with IC_{50} s in the range of 1–10 μM (data not shown). Only the receptor tyrosine kinases TRK and FLT3 were inhibited, with IC_{50} values of 252 and 303 nM, respectively.

The selectivity of azaindole 1 against 63 cardiovascular relevant enzymes and receptors was explored in collaboration with MDS Pharma Services (Taipei, Taiwan). The compound inhibited only moderately the L-type calcium channel and the sodium channel with IC_{50} values of 6.7 and 6.8 μM , respectively. The remaining 60 receptors and enzymes were not significantly affected by azaindole 1 at 10 μM (data not shown).

Docking azaindole 1 in a ROCK-model

Compared to the other 491 human kinases investigated ROCK (ROCK-1 and ROCK-2) is unique with respect to the combination of the AGC characteristic residue Phe360 (in ROCK-1 corresponds to Phe327 in PKA, which has 37% sequence identity to ROCK) and mutations Ile82 (Leu49 in PKA), Met156 (Val123 in PKA), Ala215 (Thr183 in PKA), and Asp160 (Glu127 in PKA) (Breitenlechner *et al.*, 2003; Takami *et al.*, 2004). These residues are important shape determinants for the ATP-binding cavity of ROCK. Azaindole 1 fits nicely into the X-ray crystal structure of ROCK-1 (co-crystal with fasudil, 2esm.pdb, subunit A (Jacobs *et al.*, 2006)), while it cannot be docked well into PKA (co-crystal with H89, 1ydt.pdb, (Engl *et al.*, 1996)). The inability of azaindole 1 to bind strongly to PKA can be attributed to four key mutations (Figure 4), three of which are part of the residue combination that makes ROCK unique (see above). The finding that azaindole 1 shows a good selectivity profile compared to other human kinases is therefore supported by our molecular modelling studies.

Azaindole 1 induces vasorelaxation in vitro

We investigated the effect of azaindole 1 on different vascular beds. Azaindole 1 inhibited concentration dependently the phenylephrine-induced contraction of rabbit saphenous artery with an IC_{50} value of 65 nM. In contrast, the ROCK inhibitor Y-27632 relaxed precontracted saphenous artery with an IC_{50} of 1.5 μM (data not shown). In the rat heart Langendorff preparation, azaindole 1 reduced the perfusion pressure of the coronary artery in a concentration-dependent manner at concentrations greater than 1 nM. Administration of 10, 100, 1000 nM of azaindole 1 resulted in a reduction of the maximal perfusion pressure of 50, 60, 65%, respectively. No effects on left ventricular pressure, contractility and heart rate were observed (data not shown).

Haemodynamics in anaesthetized rats

As shown in Figure 5a, azaindole 1 induced a dose-dependent and long-lasting decrease in blood pressure in

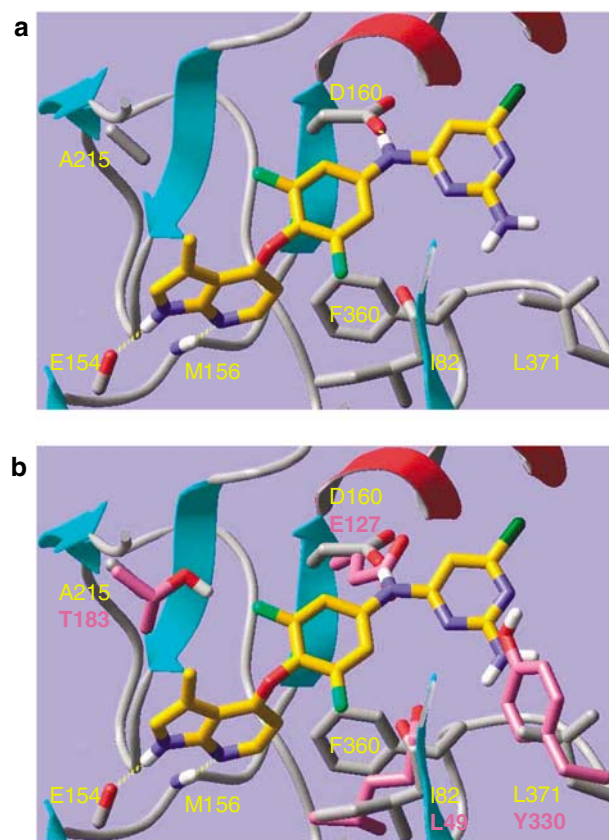


Figure 4 (a) Azaindole 1 docked and minimized into ROCK-1 (2esm.pdb, subunit a). The inhibitor is shown in tube representation (orange carbon atoms, only polar hydrogen atoms shown). Some key protein residues are labelled yellow and are partly shown in tube representation (grey carbon atoms); the remainder of the protein is shown as a ribbon diagram; (b) same as (a) plus overlaid PKA (1ydt.pdb). Only four key PKA residues are partly shown in tube representation (pink carbon atoms, pink labels). Binding mode of azaindole 1 in the Rho-kinase model. Mutated residues are coloured in magenta. PKA, protein kinase A; ROCK, Rho-kinase.

anaesthetized normotensive rats. i.v. administration of 0.03, 0.1, 0.3 mg kg^{-1} , resulted in a reduction in the maximal blood pressure of 8, 18, 35 mm Hg, respectively. Interestingly, even after the highest dose, no reflex heart rate increase was observed (Figure 5b).

Haemodynamics in conscious normotensive and hypertensive rats

In normotensive rats, azaindole 1 also induced a dose-dependent and persistent decrease in blood pressure (Figure 6a). After oral administration of 3 and 10 mg kg^{-1} , mean blood pressure dropped by 20 mm Hg (13%) and 41 mm Hg (40%), respectively. The effect lasted approximately 10 h after oral application of 3 mg kg^{-1} and more than 24 h after 10 mg kg^{-1} . A slight and transient increase of heart rate was only observed in rats treated with the highest dose of azaindole 1, 10 mg kg^{-1} (Figure 6b). In conscious spontaneously hypertensive rats, azaindole 1 induced a dose-dependent and long-lasting decrease in mean blood pressure after oral application (Figure 7a). After 1.0, 3.0 and 10 mg kg^{-1} oral application, mean blood pressure decreased

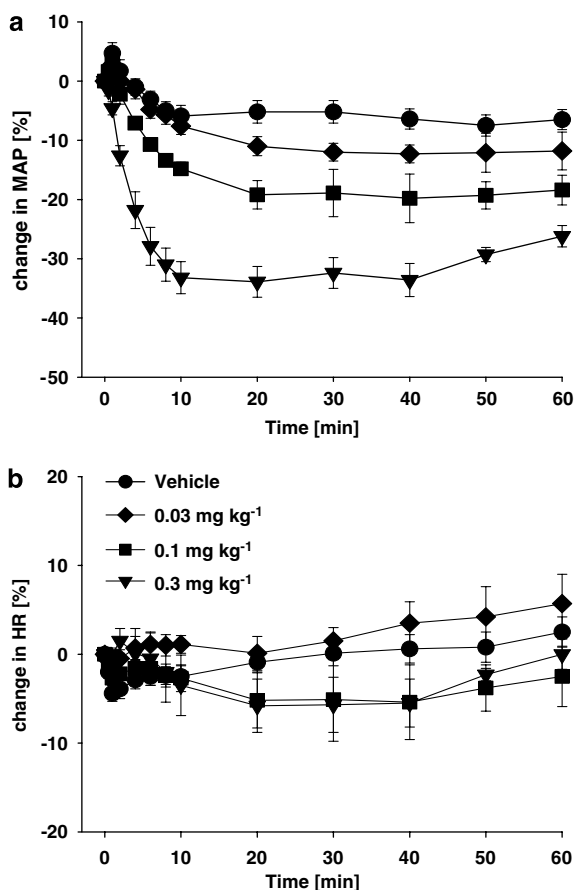


Figure 5 Effect of i.v.-administered azaindole 1 (0 (vehicle), 0.03, 0.1, 0.3 mg kg⁻¹) on mean arterial blood pressure and heart rate in normotensive anaesthetized rats ($n=6-8$ per group). Values depicted represent changes in mean arterial blood pressure and heart rate. Mean blood pressure for 0 (vehicle), 0.03, 0.1 and 0.3 mg kg⁻¹ baseline was 133 ± 4 , 129 ± 3 , 128 ± 4 and 130 ± 4 mm Hg, respectively. Mean heart rate at baseline was 337 ± 7 , 332 ± 8 , 362 ± 6 and 344 ± 12 beats min⁻¹, respectively.

maximally by 15 (10%) 32 (18%) and 68 mm Hg (40%), respectively. The onset of this lowering effect on blood pressure was fast with the maximum decrease being achieved within the first hour after administration. The effect lasted for about 10 (1.0 mg kg⁻¹), 14 (3.0 mg kg⁻¹) and 17 (10 mg kg⁻¹) h. In the vehicle-treated group, blood pressure remained unchanged. Heart rate was not significantly changed after administration of any of the doses of azaindole 1 (Figure 7b).

Multiple dosing in conscious rats

In the single-dose study, 10 mg kg⁻¹ p.o. of azaindole 1 reduced blood pressure for about 17 h. Therefore, we used this dose to investigate the effect of azaindole 1 on blood pressure after multiple dosing. The compound was given orally once daily for 4 days. On day 5, placebo and azaindole 1-treated animals only received the vehicle (Figure 8a). On days 1–4 the maximal decreases in mean arterial blood pressure were by 32, 29, 29 and 25%, respectively, whereas no significant change in heart rate was observed (Figure 8b).

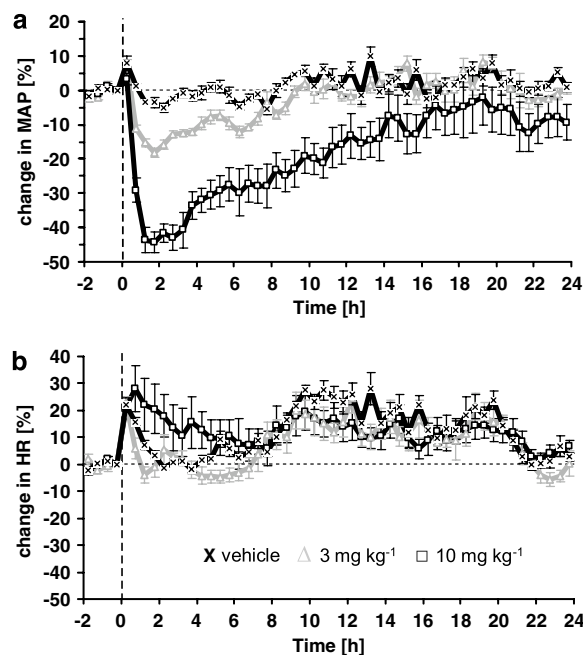


Figure 6 Effect of a single oral treatment with azaindole 1 (0 (vehicle), 3, 10 mg kg⁻¹) on mean arterial blood pressure (a) and heart rate (b) in conscious normotensive rats. The animals were treated at 0 h. Changes in blood pressure and heart rate were recorded over 24 h. Six animals per group were used. Data are given as per cent change from baseline. Mean blood pressure for 0 (vehicle), 3 and 10 mg kg⁻¹ at baseline was 97 ± 3 , 98 ± 2 and 94 ± 7 mm Hg, respectively. Mean heart rate at baseline was 319 ± 4 , 299 ± 12 and 353 ± 19 beats min⁻¹, respectively.

In the vehicle-treated group, blood pressure did not change significantly from the initial conditions. Blood pressure returned to baseline levels within 20 h after the final dose of azaindole 1. In the vehicle-treated group, the circadian profile of the heart rate did not change significantly.

Haemodynamics in anaesthetized dogs

Azaindole 1 was administered as i.v. bolus injections of 0.1 and 0.3 mg kg⁻¹. It decreased mean arterial blood pressure in a dose-related manner (Figure 9). At doses of 0.1 and 0.3 mg kg⁻¹, the maximal blood pressure lowering effect was 10 (10%) and 25 mm Hg (30%), respectively. At all doses tested, azaindole 1 caused only a moderate and dose-independent increase in heart rate. At the doses of 0.1 and 0.3 mg kg⁻¹, heart rate increased maximally by 10–15 beats min⁻¹. Despite a marked reduction in blood pressure, no change in cardiac contractility and only a slight, but statistically nonsignificant increase in coronary blood flow was observed.

Pharmacokinetic profile

The azaindole derivative azaindole 1 is a low to medium clearance drug in conscious female NMRI mice, male Wistar rats and female beagle dogs. The clearance decreased with increasing size of the animal species (Table 1). Mice had the highest volume of distribution of 2.21 kg⁻¹. However, its

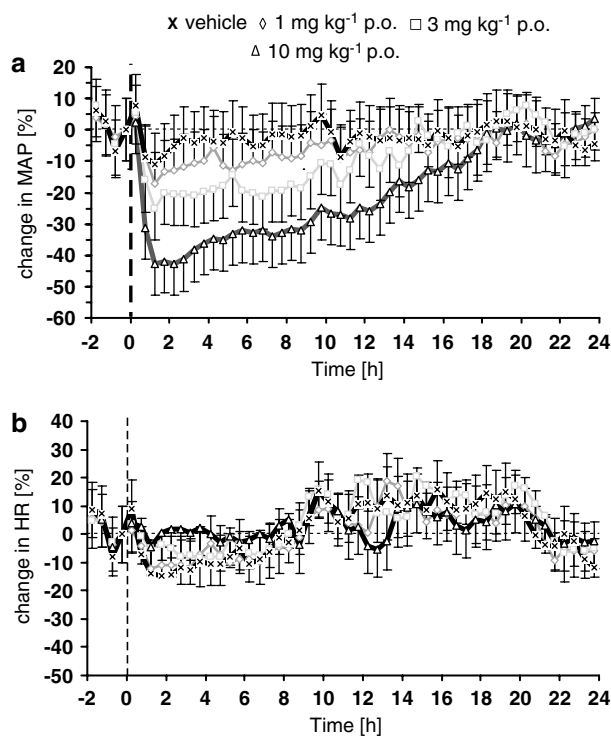


Figure 7 Effect of a single oral treatment with azaindole 1 (vehicle), 1, 3, 10 mg kg⁻¹) on mean arterial blood pressure (a) and heart rate (b) in conscious spontaneously hypertensive rats. The animals were treated at time = 0 h. Changes in blood pressure and heart rate were recorded for 24 h. Six animals per group were used. Data are given as per cent change from baseline. Mean blood pressure for 0 (vehicle), 1, 3 and 10 mg kg⁻¹ at baseline was 150 ± 11, 166 ± 10, 147 ± 13 and 161 ± 13 mm Hg, respectively. Mean heart rate at baseline was 288 ± 13, 299 ± 8, 280 ± 8 and 283 ± 12 beats min⁻¹, respectively.

half-life in dogs was longer than in rats and mice (Table 1). The oral bioavailability amounted approximately to 50% in male Wistar rats and 75% in female beagle dogs. In dogs, the half-life was significantly longer after oral administration compared to that after i.v. administration indicating a slower absorption by the oral route.

Total plasma concentration observed after oral administration of azaindole 1 correlated well with the reduction in blood pressure (data not shown). From the pharmacological study, AUC and C_{max} in spontaneously hypertensive rats following oral administration of 3 mg kg⁻¹ azaindole 1 were 0.774 mg h l⁻¹ and 0.111 mg l⁻¹, respectively. These values are slightly lower than those determined from pharmacokinetic studies (Table 1).

Discussion

In the present study, we describe the biochemical and pharmacological characterization of the structurally novel ROCK inhibitor azaindole 1. This compound was identified by high-throughput screening of an in-house 400 000 compound library and subsequent chemical optimization of the resulting lead structure using an *in vitro* ROCK assay. Azaindole 1 is one of the most potent and selective ROCK

inhibitors described so far. It inhibits human ROCK-1 and ROCK-2 isoenzymes as well as ROCK-2 from other species in the single digit nanomolar range with almost identical IC₅₀ values, which may be due to the unique and highly conserved active site of ROCKs (Okawa *et al.*, 1996; Amano *et al.*, 2000). Like most of the kinase inhibitors described so far, azaindole 1 acts as competitive inhibitor of ATP binding and as a noncompetitive inhibitor of substrate binding. Accordingly, increasing ATP to physiological relevant millimolar concentrations shifts azaindole 1-mediated ROCK-2 inhibition *in vitro* to higher IC₅₀ values. When azaindole 1 was preincubated with ROCK-2 for 30 min before the addition of ATP, the IC₅₀ values were only slightly increased. This indicates that the binding of azaindole 1 is either irreversible or very slowly reversible.

Interestingly, although competitive with ATP binding, which implicates a nonselective interaction with the conserved ATP-binding sites of other kinases, azaindole 1 is a highly specific inhibitor of ROCKs. Testing more than 110 kinases in-house and in cooperation with Upstate (UK, Dundee, Scotland) it was found that azaindole 1 weakly affected about 20 kinases with IC₅₀ values in the range of 1–10 μM. Only the receptor tyrosine kinases TRK and FLT3 were inhibited in the submicromolar range. However, azaindole 1 was at least more than 250-fold selective for ROCK. This observation is supported by two recent studies (Breitenlechner *et al.*, 2003; Jacobs *et al.*, 2006), which describe the unique structural features of the active site of ROCKs. Accordingly, docking of azaindole 1 in a model of the catalytic domain of ROCK-1 (Jacobs *et al.*, 2006) shows that the inhibitor interacts with residues at the ligand-binding site, which generates a uniquely shaped inhibitor binding pocket that confers the observed selectivity.

It is well established that the Rho/ROCK pathway plays a central role in smooth muscle cell contraction. Accordingly, inhibition of ROCK by azaindole 1-relaxed phenylephrine-precontracted strips of saphenous artery dose dependently. In addition, azaindole 1 also relaxed coronary arteries in the Langendorff-perfused rat heart model in a dose-dependent manner starting at nanomolar concentrations. The measured potency of the compound in the kinase assay *in vitro* and in the vasorelaxation experiments as well as the good selectivity profile against kinases and cardiovascular relevant receptors indicate that azaindole 1 acts via ROCK inhibition and exclude a nonspecific interaction with other vasoactive mechanisms such as MLCK, ZIP-kinase (Niuro and Ikebe, 2001) or L-type Ca²⁺ channels.

Administration of azaindole 1 in different animal models *in vivo* revealed that it has a profound ability to lower blood pressure. In anaesthetized normotensive rats, the compound induces a long-lasting decrease of blood pressure after i.v. administration from 0.03 to 0.3 mg kg⁻¹ with no reflex heart rate increase. In addition, in conscious spontaneously hypertensive rats, azaindole 1 induced a dose-dependent (1–10 mg kg⁻¹) and long-lasting reduction in the mean blood pressure after oral administration. The onset of this blood pressure lowering effect was fast with the maximum decrease attained within the first hour after administration. The effect lasted for about 10 (1.0 mg kg⁻¹), 14 (3.0 mg kg⁻¹) and 17 (10 mg kg⁻¹) h. A dose-dependent blood pressure reduction

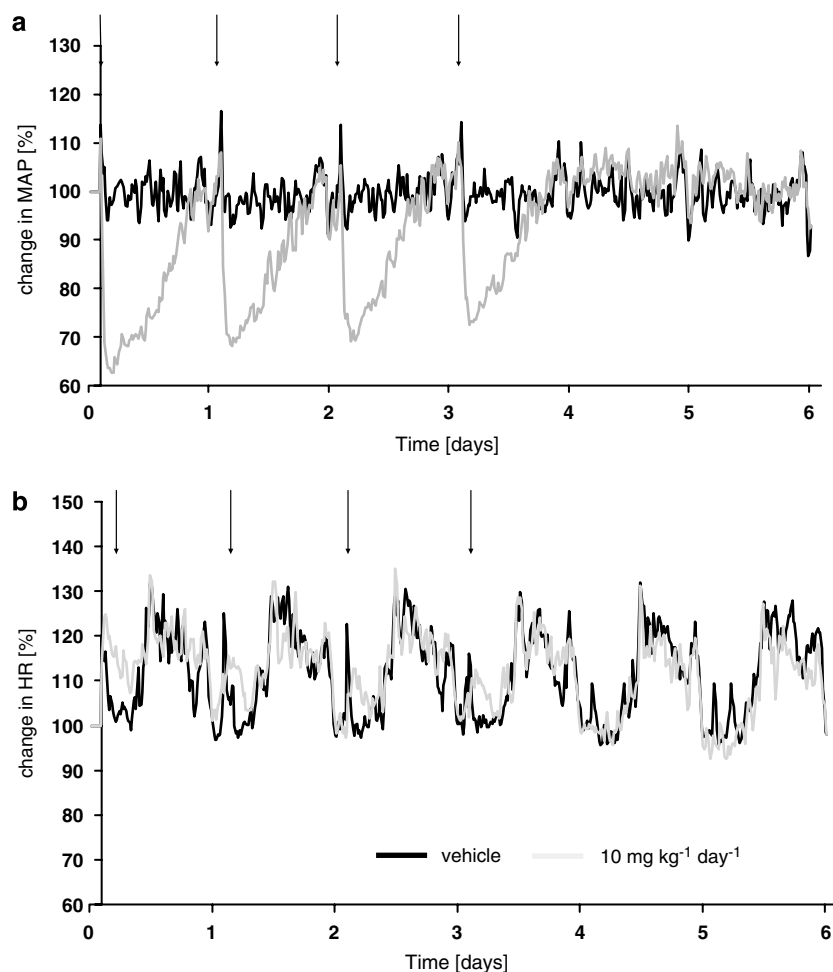


Figure 8 Continuous 24-h profile of mean arterial blood pressure (a) and heart rate (b) in conscious spontaneously hypertensive rats. Effect of azaindole 1 (10 mg kg^{-1} p.o.) after multiple treatments. Controls received the vehicle. Treatment was started at time = 0 h. Arrows denote treatment. Eight animals per group were used. Data are given as per cent change from baseline. Mean arterial blood pressure for vehicle and 10 mg kg^{-1} at baseline was 156 ± 2 and 156 ± 8 mm Hg, respectively. Mean heart rate at baseline was 306 ± 9 and 310 ± 8 beats min^{-1} , respectively.

after oral administration in spontaneously hypertensive rats has also been observed with Y-27632 (Uehata *et al.*, 1997). However, azaindole 1 was about 30-fold more potent and had distinctly longer duration of action than Y-27632. In addition, azaindole 1 induced a strong dose-dependent and persistent decrease in blood pressure in conscious normotensive rats. The effect lasted approximately 10 h after application of 3 mg kg^{-1} p.o. and more than 24 h after 10 mg kg^{-1} p.o. In contrast, Y-27632 lowered blood pressure in normotensive rats only transiently with a threshold dose of 30 mg kg^{-1} (Uehata *et al.*, 1997). The profound haemodynamic effects of azaindole 1 in the different rat models may be due to the improved potency and the reasonable pharmacokinetic profile of this compound. Interestingly, in conscious normotensive and hypertensive rats heart rate was only slightly and transiently increased at the highest dose. Recently, the vasodilator effects of two structurally different ROCK inhibitors were described (Doe *et al.*, 2007). Both ROCK inhibitors are potent and selective compounds (Doe *et al.*, 2007; Goodman *et al.*, 2007; Stavenger *et al.*, 2007), that show comparable strong blood pressure lowering effects

in different animal models of hypertension. As observed for azaindole 1, only moderate and transient increases of heart rate were observed. The reason for the absence of profound reflex tachycardia is unknown and further studies are required to elucidate this effect. Nonetheless, this observation underlines the potential use of ROCK inhibitors for the treatment of cardiovascular diseases like hypertension, where a distinct increase in heart rate is an unwanted side effect. Likewise angiotensin II receptor antagonists also show such favourable haemodynamic profiles with no reflex tachycardia *in vivo* (Stasch *et al.*, 1997).

Treatment of patients with cardiovascular disorders such as hypertension or angina requires the repeated administration of drugs to achieve constant plasma levels of the substance. In addition, the development of tolerance, as observed for organic nitrates, limits the therapeutic value of a drug. The pharmacokinetic profile of azaindole 1 shows that it has a low to medium clearance in all species investigated (NMRI mice, Wistar rats and beagle dogs). The oral bioavailability amounted to approximately 50% in male Wistar rats and 75% in female beagle dogs. In the single-dose study,

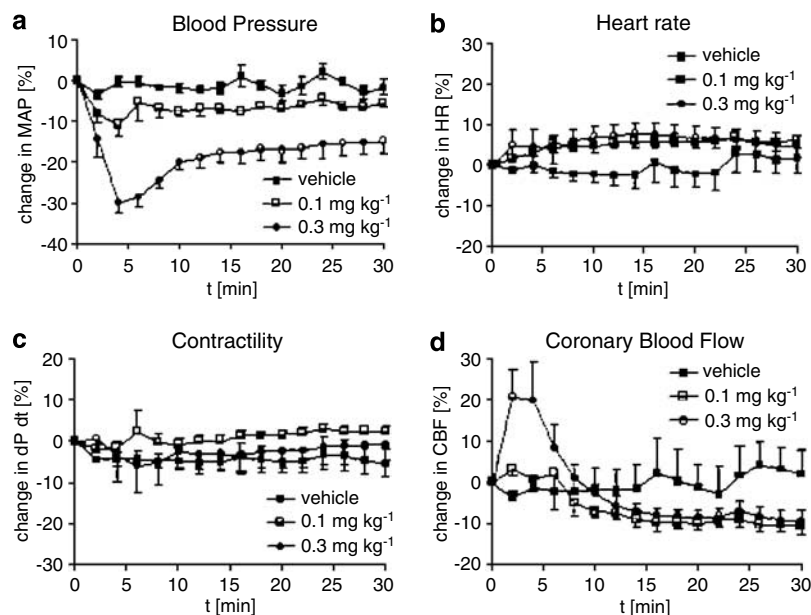


Figure 9 Effect of azaindole **1** on mean arterial blood pressure (a), heart rate (b), contractility (c) and coronary blood flow (d) in anaesthetized dogs. Data are given as per cent change from baseline. Five animals per group were used. Mean arterial blood pressure for vehicle, 0.1 and 0.3 mg kg⁻¹ at baseline was respectively 80 ± 6, 79 ± 5 and 77 ± 4 mm Hg. Mean heart rate at baseline was 106 ± 3, 110 ± 4 and 108 ± 6 beats min⁻¹. Contractility at baseline was 1598 ± 153, 1514 ± 100 and 1482 ± 77 mm Hg s⁻¹. Coronary blood flow at baseline was 43 ± 6, 46 ± 7 and 40 ± 8 ml min⁻¹.

Table 1 *In vivo* pharmacokinetic parameters of azaindole **1** in mice, rats and dogs

	NMRI mice	Wistar rat	Beagle dog
<i>i.v.</i>			
Dose	0.500	0.500	0.0500
AUC (mg h ⁻¹)	0.264	0.402	0.215
CL (lh ⁻¹ kg ⁻¹)	1.90	1.20	0.233
V _{ss} (lkg ⁻¹)	2.20	1.60	0.832
t _{1/2} (h)	1.50	1.24	2.50
<i>p.o.</i>			
Dose	Not determined	0.910	0.100
AUC (mg h l ⁻¹)		0.352	0.315
C _{max} (mg l ⁻¹)		0.0808	0.0430
T _{max} (h)		2.00	0.794
t _{1/2} (h)		1.16	7.83
Bioavailability (%)		48.0	73.2

Abbreviations: *i.v.*, intravenous; *P.O.*, per oral.

10 mg kg⁻¹ *p.o.* of azaindole **1** reduced blood pressure for about 17 h. Therefore, we used this dose to investigate the effect of azaindole **1** on blood pressure following multiple dosing in conscious spontaneously hypertensive rats. After oral administration of azaindole **1** once daily, mean arterial blood pressure was decreased significantly. As already observed in animals treated once daily, no significant increase in heart rate was observed. However, the maximal blood pressure lowering effect was gradually reduced over the 4-day observation period. At the moment, it is unclear whether multiple administration of azaindole **1** results in the development of tachyphylaxis, or whether this effect reflects variations in blood pressure due to the development of steady-state plasma levels of azaindole **1**. The blood pressure decrease was fully reversible after withdrawal of the

compound. Interestingly, rebound hypertension was not observed.

The haemodynamic profile of azaindole **1** was also investigated in anaesthetized dogs. The substance dose relatedly decreased mean arterial blood pressure after *i.v.* bolus injections of 0.1 and 0.3 mg kg⁻¹. Despite a marked blood pressure decrease, reflex tachycardia was only moderate. No decrease in cardiac contractility was observed. Although azaindole **1** efficiently lowered perfusion pressure in coronary arteries of Langendorff-perfused rat hearts, only a slight but nonsignificant increase in coronary blood flow in the dog was observed. The reason of this discrepancy is unknown and may be due to species differences and/or varying expression levels of ROCK in coronary arteries.

In summary, the results show that azaindole **1** is a potent and highly specific ROCK inhibitor. It is also an ATP-competitive kinase inhibitor that has the ability to induce vasorelaxation *in vitro* as well as in several *in vivo* models. Azaindole **1** is therefore a valuable pharmacological tool to elucidate further the physiological and pathophysiological role of ROCK and offers a novel therapeutic approach for treating cardiovascular diseases.

Conflicts of interest

The authors are employees at Bayer HealthCare with exception of Professor Ehmke, Hamburg.

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