The Novel Imidazopyridine BYK191023 is a Highly Selective Inhibitor of
the Inducible Nitric Oxide Synthase

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ABBREVIATIONS

NOS, nitric oxide synthase; 2-AP, 2-amino-4-picoline; BH₄, 6R,6S-5,6,7,8-tetrahydro-L-biopterin dihydrochloride; i-, n-, eNOS, inducible, neuronal, and endothelial nitric oxide synthase; BYK191023, 2-[(2-(4-methoxy-pyridin-2-yl)-ethyl]-3H-imidazo[4,5-b]pyridine; L-NAME, N⁵-nitro-L-arginine methyl ester hydrochloride; L-NIO, N⁵-(1-iminoethyl)-L-ornithine; L-NMMA, N⁵-monomethyl-L-arginine; 1400W, (N-(3-(amino methyl)benzyl)acetamidine; L-NIL, N⁵-(1-iminoethyl)-L-lysine hydrochloride; S-ethyl-ITU, S-ethyl-isothiourea hydrobromide; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride; AG, aminoguanidine; APE, arecaidine propargyl ester.
ABSTRACT

We have identified imidazopyridine derivatives as a novel class of NO-synthase inhibitors with high selectivity for the inducible isoform. BYK191023 showed half-maximal inhibition of crudely purified human inducible, neuronal and endothelial NO-synthases at 86 nM, 17 µM and 162 µM, respectively. Inhibition of inducible NO-synthase was competitive with L-arginine, pointing to an interaction of BYK191023 with the catalytic centre of the enzyme. In radioligand and surface plasma resonance experiments BYK191023 exhibited an affinity for iNOS, nNOS and eNOS of 450 nM, 30 µM and >500 µM respectively. Inhibition of cellular NOX-synthesis in RAW, RM- and HEK293 cells after iNOS induction showed 40- to 100-fold higher IC50-values than at the isolated enzyme, in agreement with the much higher L-arginine concentrations in cell culture media and inside intact cells. BYK191023 did not show any toxicity in various rodent and human cell lines up to high micromolar concentrations. The inhibitory potency of BYK191023 was tested in isolated organ models of iNOS (LPS treated and phenylephrine precontracted rat aorta; IC50: 7 µM), eNOS (APE induced relaxation of phenylephrine precontracted rat aorta; IC50 > 100 µM) and nNOS (field stimulated relaxation of phenylephrine precontracted rabbit corpus cavernosum; IC50 > 100 µM). These data confirm the high selectivity of BYK191023 for iNOS over e- and nNOS found at isolated enzymes. All together we have identified a new highly selective iNOS-inhibitor structurally unrelated to known compounds and L-arginine. BYK191023 is a valuable tool for the investigation of iNOS mediated effects in vitro and in vivo.
Introduction

NO-synthases are enzymes responsible for the generation of nitric oxide from the amino acid L-arginine (for review see Alderton et al., 2001). There exist two classes of enzymes, which differ in their activation profile and their capacity to generate NO. Once expressed the inducible NO-synthase (iNOS) is active for prolonged periods of time and produces micromolar concentrations of NO over longer periods. The iNOS expression is stimulated in various cell types by proinflammatory signals and is involved in immune defence against invading pathogens (Stuehr et al., 1991; Schmidt and Walter, 1994; Moncada and Higgs, 1995). The constitutively expressed endothelial and neuronal isoforms are activated by elevated intracellular Ca^{2+} concentrations and generate nanomolar concentrations of NO for relatively short periods of time. These isoforms have mainly physiological functions in blood pressure regulation and neurotransmission (Marletta et al., 1998; Alderton et al., 2001). Inducible NO-synthase is considered as a proinflammatory enzyme and is activated in acute diseases such as sepsis and septic shock (Titheradge, 1999) and in various chronic inflammatory diseases such as asthma, arthritis, multiple sclerosis and inflammatory diseases of the gut and intestine (Hobbs et al., 1999; Vallance and Leiper, 2002).

A considerable number of inhibitors structurally related to the substrate L-arginine have been developed like L-NIO (McCall et al., 1991), L-NMMA (Moore et al., 1996), thiocitrullines (Furfine et al., 1994; Narayanan et al., 1995) or isothiourea derivates (Southan et al., 1995). However none of these drugs exhibit distinct isoform selectivity. Because inhibition of the endothelial isoform (eNOS) produces hypertension (Moncada and Higgs, 1995), the pharmaceutical development of highly iNOS-selective inhibitors for therapeutic use is desirable.

Selective inhibition of inducible NO-synthase seems to be a promising therapeutic approach for the treatment of the acute and chronic pathophysiological diseases mentioned above. Results of selective inhibitors in animal models of acute and chronic diseases are limited. In fact, only very few selective iNOS-inhibitors are available for research and none has reached approved clinical application (Mete and Connolly, 2003). Data from selective inhibitors available such as 1400W (GlaxoSmithKline) (Garvey et al., 1997), GW 274150 and GW273629 (GlaxoSmithKline) (Young et
al., 2000; Alderton et al., 2005), AR-C102222 (Astra Zeneca) (Beaton et al., 2001; Tinker et al., 2003), ONO1714 (Ono Pharmaceuticals) (Nishina et al., 2001), L-arginine derivatives from Pharmacia Pfizer (SC-51 or L-NIL) (Hansel et al., 2003) and the dimerization inhibitor BBS-2 (Berlex Laboratories Inc.) (McMillan et al., 2000; Blasko et al., 2002; Habisch et al., 2003) show promising results in animal models of sepsis, lung inflammation, arthritis and autoimmune diabetes. Development of some of these compounds into the clinic has been stopped due to toxicity issues (1400W) (Alderton et al., 2005). Others show only limited iNOS/eNOS-selectivity (ONO1714) (Nishina et al., 2001).

We have identified and characterized a new compound (BYK191023, Fig. 1) with imidazopyridine structure which shows potent and selective inhibition of inducible NO-synthase. BYK191023 is not only a valuable tool for preclinical research on NO-synthases but also a promising candidate for clinical development.
Material and Methods

Materials

Cell culture material, NADPH, FAD, FMN, lactate dehydrogenase, sulfanilamide, N-(1-naphtyl)ethylenediamine and lipopolysaccharide (Salmonella abortus equi) were obtained from Sigma (Dreieich, Germany). Cytokines were from Peprotec (London, U.K). NOS inhibitors were purchased from Alexis (Germany); nitrate reductase was from Roche Diagnostics (Mannheim, Germany). \[ ^{3}H \]-L-arginine (60-80 Ci/mmol) was bought from Amersham. 6R,6S-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (BH₄) was from Schircks Laboratories/Jona/Switzerland.

BYK191023 (2-[2-(4-methoxy-pyridin-2-yl)-ethyl]-3H-imidazo[4,5-b]pyridine) was synthesized at the department of medicinal chemistry of the ALTANA Pharma AG by condensation of 3-(4-methoxy-pyridin-2-yl)propionic acid with 2,3-diaminopyridine as described (Ulrich et al., 2003). \[^{3}H\]-BYK191023 and \[^{3}H\]-2AP were obtained by catalytic bromine/tritium exchange of 6-bromo-BYK191023 and 3,5-dibromo-2AP at Amersham/Pharmacia. A specific radioactivity of 21 Ci/mmol and 64 Ci/mmol was obtained. All other chemicals were from commercial suppliers with highest grade of purity.

The human isoforms of NO-synthases were obtained as essentially described (Butt et al., 2000) or obtained from TransMIT (Giessen, Germany) after transfection of Sf9 cells with the respective human cDNAs via the baculo virus system. The cytosolic fraction of homogenized Sf9 cells was used for all experiments.

Biochemical Methods

NOS Activity Assay

The enzyme reaction using isolated human NOS-isoforms was performed in 96-well microtiter F-plates essentially as described previously (Boer et al., 2000). Briefly, the assay was performed in a total volume of 100 µl in the presence of 100 nM calmodulin, 226 µM CaCl₂, 477 µM MgCl₂, 5 µM
FAD, 5 µM FMN, 1 mM NADPH, 7 mM glutathione, 10 µM BH₄, and 100 mM HEPES, pH 7.2. Final L-arginine concentrations in the assay were 3-5 µM. [³H]-L-arginine (150,000 dpm/well) was added to the mixture and the reaction was incubated for 60 min at 37 °C. Enzyme reaction was stopped by adding 10 µl of 2 M MES buffer, pH 5.0. 50 µl of the incubation mixture were transferred into a MADP N 65 filtration plate (Millipore, Eschborn, Germany) containing 50 µl of AG-50W-X8 cation exchange resin (BioRad, München, Germany). The flowthrough was collected in Pico scintillation plates (Packard, Meriden, CT) and the resin washed twice with water. The total flowthrough of 125 µl was mixed with 175 µl of Microscint-40 scintillation cocktail (Packard). Scintillation plates were counted in a Microbeta (Wallac, Turku, Finland) scintillation counter and pIC₅₀ values were calculated from concentration-response curves by means of program Prism 3.0 (GraphPAD, Sorrento Valley, CAL, U.S.A.).

Radioligand Binding Studies

Radioligand binding experiments were performed in 96-well microtiter plates as described previously (Boer et al., 2000) using ³H-labeled BYK191023 in a total volume of 100 µl in the presence of 2 mM CaCl₂, 10 µM BH₄, 1 mM dithiothreitol, 100 µM NADPH and 50 mM TRIS/HCl, pH 7.4. Radioligand competition experiments with non-labeled compounds were performed using [³H]-2AP (250,000 dpm/well) corresponding to a concentration of 18 nM as described previously (Boer et al., 2000). The assay was incubated for 60 min at 37 °C and then filtered over GF-C glass fiber filter mats (Whatman, Maidstone, UK) with a cell harvester (Skatron, Lier, Norge). Filters were rinsed with ice-cold incubation buffer without NADPH and BH₄, punched into scintillation vials and counted after adding 3.5 ml of UltimaGold scintillation cocktail (Packard).

Cytokine-induced NO Formation in RAW and RM Cells, NO Formation in HEK293/iNOS Cells

Cell culture: RAW (mouse macrophage cell line, ATCC) were cultured in DMEM, 10% FCS, 1% L-glutamine, 1% PS. After reaching confluency 2x10⁵ cells were seeded per well in 96-well microtiter F-plates and stimulated with IFN-γ (100 U/ml) and LPS (1 µg/ml) in identical serum reduced media (DMEM, 0.5% FCS, 1% L-glutamine, 1% penicillin/streptomycin without phenol red). Standard NOS inhibitors in different concentrations were added to the cells and nitrate and nitrite were
determined after 16 h in the cellular supernatant by the Griess assay. RMC (rat mesangium cell line, ATCC) were cultured in RPMI-medium 1640, 10% FCS, 1% L-glutamine, 1% PS. After reaching confluency the cell line was starved in 96 wells for 48 h in identical serum reduced media (DMEM, 0.1% FCS, 1% L-glutamine, 1% penicillin/streptomycin without phenol red) and stimulated with IL-1β (1 nM) in identical serum reduced media (DMEM, 0.5% FCS, 1% L-glutamine, 1% penicillin/streptomycin without phenol red). Nitrite was determined after 16-20 h in the cellular supernatant by the Griess assay. Culture of HEK293/iNOS cells (Su et al., manuscript submitted): EcR293 cells (human embryonic kidney 293, stably transfected with pVgRXR/Zeocin regulator vector), were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FCS (fetal calf serum), 5% penicillin and streptomycin, 5% L-glutamine, 5% Hepes buffer at 37 °C in a humidified incubator with 5% CO₂. The cDNA of iNOS was cloned into an ecdysone inducible mammalian expression vector, pIND(SP1)/Neomycin (Invitrogen Corp, CA, USA). Cells were seeded at 50% confluence, and transfected with pIND-NOS2 plasmid by lipofectamine 2000 reagent according to the supplier’s instruction. The stably transfected cells were selected in medium containing both G418 (400 µg/ml) and Zeocin (400 µg/ml). Clones growing up after about 4 weeks of selection, were picked and further analysed (Su et al., manuscript submitted). 10⁵ cells were seeded per well in 96-well microtiter F-plates and the iNOS was stimulated by the addition of Ponasterone A (SIGMA, 1 µM) for 24 h in identical serum reduced media (DMEM, 0.5% FCS, 1% L-glutamine, 1% penicillin/streptomycin without phenol red). Nitrate and nitrite were determined after 24 h in the cellular supernatant by the Griess assay.

Griess assay: Nitrate from 150 µl culture media was reduced to nitrite by adding 10 µl of nitrate reduction buffer (0.08 U/ml nitrate reductase, 530 µM FAD and 83 µM NADPH). Incubation was carried out for 15 min at 37 °C in a 96 microtiter plate. Interfering NADPH was depleted for 5 min at 37 °C by addition of mix 2 consisting of 1104 U/ml lactate dehydrogenase and 320 mM sodium pyruvate in a 10 µl volume. The reaction was abrogated by the addition of 10 µl 1% sulfanilamide in 0.1 N HCl and 10 µl 0.1% N-(1-naphtyl)ethylenediamine. Absorbance was read at 544 nm in reference to 690 nm following a 10 min room temperature incubation in a microplate reader (Wallac,
Torku, Finnland). Nitrite generated from rat mesangial cells was directly assayed in 75 µl supernatant by the addition of sulfanilamide and N-(1-naphtyl)ethylenediamine.

**Determination of Cellular Viability**

Cell viability was determined by the MTT assay as described previously (Denizot and Lang, 1986). The cells were washed with PBS before addition of MTT. Cells were incubated with 100 µl of 0.2 mg/ml MTT for 90 min at 37 °C, 5% CO₂, followed by an incubation with 100 µl of a solution containing 95% isopropanol and 5% formic acid for 10 min at room temperature. Absorbance was read at 544 nm in reference to 690 nm in a microplate reader (Wallac, Torku, Finnland).

**Pharmacological Methods**

**Isolated Rat Thoracic Aorta: iNOS and eNOS Inhibition**

Thoracic aortae were obtained from male Wistar rats (250-300 g, Charles River, Kisslegg, Germany) killed by cervical dislocation. The vessels were cleaned, cut into rings of 1.5 mm length and mounted under 0.9 to 1.0 g tension in 10 ml organ baths filled with warmed (37 °C), oxygenated (95% O₂/5% CO₂) Krebs solution of the following composition (mM): NaCl 120.0, KCl 5.5, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25.0 and glucose 11.0, additionally containing 10⁻⁵ M indomethacin for the experiments of iNOS inhibition. Isometric tension changes were measured by means of force-displacement transducers and recorded on polygraph recorders. In case of iNOS inhibition, the endothelium was previously removed by gently rubbing the intimal surface before cutting the vessel into rings which were than placed in Petri dishes containing RPMI 1640 medium containing 200 ng/ml LPS (*Salmonella abortus equi*) and incubated at 37 °C for 18 h. This incubation time was chosen from preliminary experiments showing that iNOS expression at the level of mRNA was maximal. The aortic rings were considered denuded when a maximal concentration of the muscarinic agonist, arecaidine propargyl ester (APE, 3x10⁻⁶ M), caused relaxation of less than 5%. After an initial 60 min equilibrium period the aortic rings were precontracted by 3x10⁻⁷ M phenylephrine (EC₈₀-₉₀ of its own maximal effect), after that 3x10⁻⁶ M APE (EC₁₀₀) was added to cause
rapid relaxation of the tissue within 5 min, before cumulative administration of drugs was started to evoke contraction due to inhibition of eNOS. Similarly, for measuring iNOS inhibition, LPS-treated aortic rings were first challenged with 3x10^{-7} M phenylephrine which due to iNOS-derived NO caused a partial contraction (5-10% compared to untreated tissue). Thereafter cumulative administration of test drugs restored the contraction of the hyporeactive tissue due to inhibition of iNOS. AMT (10^{-5} M) was added at the end of the cumulative test drug administration of each NOS inhibitor to define its maximal contractile effect related to that elicited by AMT (100%). All results were expressed as a percentage of the AMT-induced maximal response (Fleming et al., 1990; Eltze et al., 1998).

**Field Stimulated Rabbit Corpus Cavernosum: nNOS Inhibition**

Adult New Zealand White rabbits (Dunkin Hartley, 2.5 – 3.0 kg; Charles River, Kisslegg, Germany) were sacrificed by intravenous injection of pentobarbital (60 mg/kg) and exsanguination. The penis was dissected free in toto and immediately placed in Krebs solution. Four longitudinal strips (approximately 1.5 x 1.5 x 6 mm) were dissected from each corpus cavernosum and mounted in 10 ml organ baths under a resting tension of 1 g for measurement of isometric tension changes in Krebs solution (mM): NaCl 118.3, KCl 4.7, CaCl_2 1.9, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 25.0, Na-EDTA 0.03 and glucose 11.1, additionally containing 10^{-6} M atropine and 5x10^{-6} M guanethidine to exclude possible participation of cholinergic and adrenergic responses, respectively, to electrical field stimulation (EFS). The nutrient solution was aerated with a mixture of 95% O_2 and 5% CO_2 maintaining pH 7.4 at 37 °C. The strips were then precontracted by 10^{-5} M phenylephrine (EC_{80-90} of its own maximal response), after that relaxant nonadrenergic, noncholinergic (NANC) responses were evoked by EFS (15 V, 0.3 ms, 4 Hz, for 5 s, every 2 min) using a pair of Pt-electrodes, one located at the bottom of the organ bath and directly connected with the tissue, the other ring electrode being placed at the top of the bathing fluid. After stabilization of EFS-evoked relaxant responses within 30-45 min, the test drug was added in a cumulative manner. The potential inhibition of nNOS could be followed by an inhibition of tissue relaxation mediated by synthesis and release of neuronal NO (Ignarro et al., 1990; Eltze et al., 1999).
Inhibition of iNOS Derived NO\textsubscript{x} in Rat Aortic Rings

Rat aortic vessels were cleaned and cut into rings of 1.5 mm length. Two rings were transferred into one well in a 96-well plate and stimulated with IFN-γ (100 U/ml) and LPS (1 µg/ml) for 20 h in serum reduced media (DMEM, 0.5% FCS, 1% L-glutamine, 1% penicillin/streptomycin without phenol red). Nitrate and nitrite were determined in the supernatant by the Griess assay as described above.

Statistical Analysis

IC\textsubscript{50} values were calculated from inhibition data using Prism 3.0 (GraphPAD, Sorrento Valley, CAL, U.S.A.). Data presented are mean ± SEM.
Results

Identification of BYK191023 as a Novel and Potent Inhibitor of the Inducible Nitric Oxide Synthase with High Isoform Selectivity

The imidazopyridine BYK191023 (Fig. 1) was identified as a potent inhibitor of the inducible nitric oxide synthase (iNOS) in a biochemical screen using ALTANA Pharma’s compound library. NOS activity was measured by quantification of [3H]-L-arginine conversion to [3H]-L-citrulline. In our NOS activity assays the K_m-values for L-arginine at the three NOS-isoforms were determined as 5.7 µM (iNOS), 2.8 µM (nNOS) and 3.3 µM (eNOS) (Boer et al., 2000). BYK191023 consists of an ortho-methoxypyridine moiety linked via an ethylene bridge to an imidazopyridine residue. The compound was tested for its NOS-inhibitory potency on all three human NOS-isoforms. BYK191023 inhibited these enzymes with pIC_{50}-values of 7.03 (iNOS), 4.86 (nNOS) and 3.95 (eNOS) (Fig. 2). Mean pIC_{50}-values (n = 6) for the inhibition of NOS isoforms were 7.07 ± 0.04 (iNOS), 4.79 ± 0.08 (nNOS) and 3.81 ± 0.05 (eNOS), corresponding to IC_{50}-values of 86 nM, 17 µM and 162 µM, respectively (Table 1). Slope values of inhibition curves were near unity pointing to a homogeneous population of enzyme and an L-arginine competitive mode of action.

L-Arginine Competitive Inhibition of iNOS by BYK191023

Inhibition of iNOS in the presence of increasing L-arginine concentrations led to a shift in IC_{50}-values for BYK191023 shown in Table 2. These data and the resulting linear Schild plot (slope = 1.10; data not shown), points to a stoichiometric relationship between inhibitor and substrate interaction with the enzyme and clearly demonstrates the L-arginine competitive character of binding. To further investigate the molecular mechanism of iNOS inhibition, L-arginine saturation experiments were performed in the absence and presence of increasing concentrations of inhibitor (Fig. 3). Addition of BYK191023 resulted in a shift of the apparent L-arginine K_m-value (7.7 µM in the absence, 27 µM, 76 µM and 129 µM in the presence of 0.3 µM, 1 µM and 3 µM BYK191023, respectively), while the V_max of the reaction did not change (Fig. 3A). Analyzing the data in a Schild plot revealed a K_i for BYK191023 of 85 nM (pK_i 7.07; intercept with the abscissa; Fig. 3B). A mean K_i-value of
90 ± 16 nM was determined in three independent experiments (data not shown). Thus BYK191023 is a highly potent L-arginine competitive and selective inhibitor of the inducible NO-synthase.

### Structure Activity Relations with BYK191023 and Analogs

As can be seen from Fig. 1, BYK191023 consists of two pharmacophore moieties (methoxypyridine and imidazopyridine) linked by an ethylene bridge. To determine the contribution of both pharmacophores to the overall compound inhibitory potency, we synthesized both, the methoxypyridine moiety as well as the imidazopyridine residue and analyzed their inhibitory potential at the NO-synthases. Surprisingly, BYK205513 representing the 2-methylated imidazopyridine, exhibited no potency at the NO-synthase isoforms (Table 1). On the other hand, the 4-methoxy-2-methylpyridine compound (BYK299621) showed a micromolar inhibitory potency at the iNOS but no isoform selectivity (Table 1). Combination of both molecules led to the more potent, but also highly iNOS-selective inhibitor BYK191023 (Table 1).

While picolines and aminopicolines are well known as potent NOS inhibitors, they show only minor if any isoform selectivity (Faraci et al., 1996; Boer et al., 2000). As examples the IC₅₀ values of 2-aminopyridine (2-AP) and 6-amino-2,4-lutidine (BYK205516) are shown in Table 1. To test whether iNOS selectivity can also be conferred to the 2-aminopicoline moiety by introducing a heteroaromatic residue, we linked the imidazopyridine moiety to the 2-aminopyridine via an ethylene bridge. The resulting compound BYK237007 reveals slightly increased potency compared to BYK191023 and 100-fold selectivity versus eNOS. Selectivity versus the neuronal isoform was low (2-fold) (Table 1).

### Affinity of BYK191023 to the Inducible NOS

Subsequently we analyzed the affinity of BYK191023 towards inducible NO-synthase in radioligand binding experiments using [³H]-BYK191023 as radioligand. Saturation experiments at human iNOS using increasing concentrations of BYK191023 revealed a Kᵦ for BYK191023 of 1.08 µM (Fig. 4) and as a mean a Kᵦ of 1.19 ± 0.06 µM (n = 3). To characterize the [³H]-BYK191023 binding site radioligand competition experiments using [³H]-BYK191023 as radioligand were performed in the absence or presence of increasing concentrations of standard NOS-inhibitors. [³H]-
BYK191023 binding was inhibited dose-dependently by 1400W, AMT, S-ethyl-ITU and L-NMMA with IC$_{50}$ values of 2.2 µM, 26.3 nM, 120 nM and 5.1 µM, respectively. These binding values correspond well with the inhibition values determined in activity assays by others and us (Faraci et al., 1996; Boer et al., 2000).

To further validate the [$^3$H]-BYK191023 binding site as iNOS we used surface plasmon resonance experiments to measure direct binding of BYK191023 to the highly purified oxygenase domain of murine iNOS. Saturation experiments with BYK191023 using the murine iNOSoxy protein and Biacore technology (Biacore Int. SA, Switzerland) revealed a K$_D$ of 634 ± 81 nM for BYK191023. The association rate amounted to 7200 ± 1600 M$^{-1}$s$^{-1}$, while the dissociation rate was determined as 0.27 ± 0.07 min$^{-1}$.

As the low activity of [$^3$H]-BYK191023 against the other two isoforms, especially to eNOS precludes the use of [$^3$H]-BYK191023 in direct radioligand binding experiments, binding affinities of BYK191023 were determined in radioligand competition experiments using the nonselective NOS-ligand [$^3$H]-2-aminopicoline (Table 1). [$^3$H]-2-AP binding to nNOS and eNOS was inhibited at high concentrations with an IC$_{50}$ of 30 µM and 630 µM, respectively (Fig. 5). BYK191023 inhibited [$^3$H]-2-AP binding to iNOS in a concentration-dependent manner with an IC$_{50}$ of 450 nM (Fig. 5). Thus the iNOS selectivity of BYK191023 in binding assays reflects the selectivity previously obtained in activity assays.

As most in vivo models are performed in rodents we analyzed the inhibitory potency of BYK191023 at purified murine iNOS to exclude any species related problems. The activity assay was performed as described for the human NO-synthases using 5 nM of purified murine iNOS. BYK191023 inhibited the murine iNOS with an IC$_{50}$ of 95 nM, comparable to the potency at human iNOS (86 nM). Furthermore, a highly significant correlation of potencies with various standard inhibitors (including AMT, L-NIL, AG, 1400W, L-NIO, L-NAME and L-NMMA) at the two species was obtained (correlation coefficient $r^2 = 0.87$; $p < 0.001$; data not shown).
Inhibition of iNOS Derived Nitrite/Nitrate by BYK191023 in Various Intact Cells

The cellular potency of iNOS-inhibition by BYK191023 was tested in three cell lines from human, mouse and rat species. We used the murine macrophage cell line RAW, the rat mesangial cell line RMC and human HEK293 cells stably transfected with iNOS. Expression of iNOS in these cells was induced either by the addition of cytokines or LPS (RAW and RMC) or by the addition of Ponasteron A in the HEK293/iNOS expression system (see Materials and Methods). Nitrite generation after induction of iNOS was inhibited by BYK191023 with IC\textsubscript{50}-values of 33 µM in RMC, 3.1 µM in RAW and 13 µM in human HEK293/iNOS (Fig. 6 and Table 3). Addition of 12.5% human serum to the HEK293/iNOS-assay led to a small drop of BYK191023 potency (IC\textsubscript{50} 28 µM) possibly due to weak binding of the inhibitor to human serum. Thus BYK191023 is also a potent inhibitor of the iNOS in various cellular assay systems.

Effect of BYK191023 on Cellular Viability

Cell viability was identified by determination of succinate dehydrogenase activity using the MTT assay. Cells exposed to BYK191023 in all model systems showed no change in their viability. Even at high micromolar concentrations of BYK191023 (100 µM) cells behaved comparable to control cells in the MTT assay and in addition BYK191023 showed no toxicity in hepatocyte tissue cultures up to a concentration of 1 mM (data not shown). Therefore we conclude that BYK191023 does not exhibit any toxic effect in our cellular models.

Selective Inhibition of iNOS by BYK191023 in Isolated Tissues

We examined the potency and isoform selectivity of BYK191023 in isolated tissues. Pharmacological \textit{in vitro} models were not only established for determination of the inhibitory potency at iNOS but also at eNOS and nNOS (see Materials and Methods). \textit{In vitro} models using isolated organs proved to be suitable to determine selectivity of NOS-inhibitors. The quality of the models had previously been validated using a number of standard NOS inhibitors (Eltze \textit{et al.}, 1998; Eltze \textit{et al.}, 1999).
In LPS-treated rat aortic rings challenged with phenylephrine, cumulative administration of BYK191023 restored the contraction of the hypoactive tissue due to inhibition of iNOS, resulting in a pIC$_{50}$-value of 5.14. The inhibition curve for BYK191023 reaches a plateau at about 70 % of the maximal response related to that elicited by 10 µM AMT (Fig. 7A). To confirm and clarify the reasons for that incomplete inhibition, we measured inhibition by BYK191023 of nitrite generation from LPS treated rat aortic rings by the Griess assay. A very similar pIC$_{50}$ value of 4.88 was found (Fig. 7B). The experiment was repeated 8 times resulting in a mean pIC$_{50}$ value of 4.90 ± 0.09. High concentrations of BYK191023 caused almost complete inhibition showing that the incomplete restoration of the contraction of LPS-treated rat aorta was likely not due to a partial inhibition of inducible NO-synthase but must be related to other reasons (e.g. unspecific effects at high concentrations).

The L-arginine competitive inhibition of iNOS in rat aorta by BYK191023 is demonstrated in the original tracing (Fig. 8). BYK191023 (10 µM) inhibited iNOS and caused contraction of the hyporeactive tissue. Addition of 10 µM L-arginine antagonized the effect of BYK191023 partially, however, the presence of BYK191023 prevented a complete relaxation. A further increase in BYK191023-concentration (30 µM) was able to overcome the relaxing L-arginine effect and led to contraction again, which subsequently was attenuated by addition of a respective higher L-arginine concentration (30 µM).

Furthermore, the selectivity of iNOS-inhibition by BYK191023 was determined in isolated tissues suitable for determine eNOS and nNOS inhibition. No reversal of APE-induced vasorelaxation by BYK191023 up to 100 µM was found in phenylephrine precontracted rat aortic rings as a model for eNOS inhibition (Fig. 7A). In the neuronal NOS model of the phenylephrine precontracted rabbit corpus cavernosum, BYK191023 showed no inhibition of field stimulation-induced relaxant responses up to concentrations of 100 µM (Fig. 7A). These data confirmed the isoform selectivity of BYK191023 at the level of isolated organs. In the functional assays L-NIL showed selectivity for iNOS (pIC$_{50}$ = 6.03) over both eNOS (pIC$_{50}$ = 4.90) and nNOS (pIC$_{50}$ = 4.58), whereas L-NAME behaved as weak selective for eNOS (pIC$_{50}$ = 5.56) over both iNOS (pIC$_{50}$ = 4.01) and nNOS (pIC$_{50}$ =
5.05). As expected for a nonselective compound AMT did not differ more than a factor of 2.5 (pIC₅₀: 6.41, 6.48 and 6.11 for iNOS, eNOS and nNOS, respectively). These data are summarized in Table 4.

To further validate these functional tissue experiments as reliable iNOS and eNOS models we calculated the selectivity ratios of various standard inhibitors obtained in isolated organ models or enzymatic assays. An excellent correlation was obtained for the i-/eNOS selectivity ratios obtained from rat aortic experiments and those derived from enzymatic assays (Fig. 9). These data show that the potent inhibition of iNOS by BYK191023 at isolated enzymes translates into potent inhibition of iNOS in intact vascular tissue.
Discussion

The use of iNOS inhibitors as therapeutics in various diseases critically depends not just on the potency of the inhibitors but very much on its isoform selectivity. There is a general agreement that inhibition of the proinflammatory inducible isoform of NO-synthases is a promising approach to treat acute as well as chronic inflammatory diseases such as septic shock, rheumatoid arthritis and osteo arthritis. Inhibition of the endothelial NOS will disturb micro- and macrocirculatory homeostasis regulation and therefore lead to a serious deterioration of organ perfusion and other unwanted circulatory side effects. A clinical trial with the nonselective NOS-inhibitor L-NMMA was stopped for those reasons (Lopez et al., 2004). Therefore, a great demand for potent and highly selective iNOS inhibitors exists to examine the role of iNOS in the above mentioned pathophysiologies and animal models. Very few highly selective iNOS inhibitors have been described in the literature, but none reached the clinic. Clinical development of 1400W was discontinued due to toxicity issues (Alderton et al., 2005). Inhibitors of iNOS dimerization only act at the monomeric level of iNOS (McMillan et al., 2000) and are not suited for inhibition of already expressed and active iNOS. Other compounds (ONO1724) are struggling with selectivity problems (Nishina et al., 2001). With BYK191023 we have identified both, a potent inhibitor of the human inducible NO-synthase and an inhibitor with very high selectivity versus human nNOS (200 fold) and human eNOS (>1000 fold), respectively. A Kᵢ-value of 90 nM was determined from competition experiments using increasing concentrations of L-arginine in the absence or presence of various inhibitor concentrations. This value is in close agreement to the IC₅₀-value for inhibition of L-arginine-citrulline conversion in the presence of low L-arginine concentration as used in our standard protocol (86 nM). A detailed kinetic analysis of the binding mode of BYK191023 clearly showed a L-arginine competitive mode of interaction. This is seen from the shift of IC₅₀-values in the presence of increasing L-arginine concentrations (Table 2) and in the apparent Kₘ-values of L-arginine in the presence of increasing inhibitor concentrations (Fig. 3A). The corresponding Schild plots reveal slopes near to 1 clearly indicating a L-arginine competitive mechanism for iNOS inhibition by BYK191023. A L-arginine competitive and reversible binding of BYK191023 to iNOS was further demonstrated in a functional in vitro model using isolated rat aorta.
The concentration dependent contraction of rat aorta by BYK191023 due to iNOS inhibition could strikingly be reversed by subsequent addition of increasing L-arginine concentrations (Fig. 8).

The compound is chemically unrelated to the natural substrate L-arginine. BYK191023 consists of two chemical moieties the methoxy pyridine head group and the imidazopyridine residue linked via an ethylene bridge. It is already known that small heterocyclic compounds such as aminopicolines or imidazoles or small aliphatic compounds (isothioreas) show potent but nonselective inhibition of all NO-synthase isoforms (Faraci et al., 1996; Chabin et al., 1996). The methoxypyridine moiety is related to this structural motive, also showing potent but nonselective inhibition of NO-synthases. The introduction of the ethylimidazopyridine residue leads to slightly increased potency at the inducible isoform and to the strongly pronounced selectivity of the compound BYK191023. A similar but less dramatic increase of selectivity was seen linking the 2-aminopicoline or BYK205516 moiety to the imidazopyridine (BYK237007). The imidazopyridine residue itself shows no measurable affinity, but seems to force the inhibitor into an isoform selective position.

Both, iNOS and eNOS have been crystallized. By comparison of the reported amino acid sequence for both isoforms surrounding the active site, particularly D382 differs between iNOS and eNOS and could be responsible for the different affinities seen for iNOS and eNOS (Fischmann et al., 1999; Flinspach et al., 2004). In line with our data a recent publication describes an increase in the selectivity of iNOS inhibition especially over eNOS inhibition by N-substitution of aminopyridine (Connolly et al., 2004).

The close proximity of the substrate and BH₄ binding site could theoretically lead to an influence of BH₄ binding by our inhibitors. This seems not to be the cause of potency or isoform selectivity of BYK191023 because increasing amounts of BH₄ did not protect the NOS isoforms from inhibition by BYK191023 (data not shown). However, the molecular basis for the high isoform selectivity of BYK191023 remains unknown and further investigations using NOS crystal structures with BYK191023 will be performed. Isoform specific sequence differences of human NOS interacting with the imidazo[4,5-b]pyridine residue of BYK 191023 are considered to be potentially responsible for the remarkable isoform selectivity. In addition to the sequence differences D366 (eNOS) and D601...
(nNOS) compared with D382 (iNOS) in the region close to the imidazopyridine, S246 (eNOS) and S481 (nNOS) are different from A262 in iNOS.

Radioligand binding data with \[^{3}H\]-BYK191023 in crude cytosolic fractions from iNOS overexpressing Sf9-cells identified a single homogeneous binding site as demonstrated by the monophasic radioligand saturation curves. Association to and dissociation of \[^{3}H\]-BYK191023 from its binding site are again monophasic and in agreement with a single homogeneous binding site (data not shown). The identification of this binding site as iNOS is not only due to the fact that it is labeled specifically by the tritiated iNOS-inhibitor BYK191023, but also by the fact that the affinity of BYK191023 for its binding site is in the range of the inhibitory potency of the compound towards iNOS. Furthermore, competition experiments with selective and nonselective NOS-inhibitors clearly showed the expected behaviour for an interaction with iNOS. In addition, besides the use of a crude cytosolic fraction in radioligand binding assays, we confirmed specific binding of BYK191023 in direct interaction studies using surface plasmon resonance spectroscopy with the murine oxygenase domain of iNOS. The use of the murine oxygenase domain was necessary due to the demand of high surface density of thoroughly purified enzyme on the chip to obtain satisfactory signals.

BYK191023 showed a 5-fold weaker affinity towards iNOS (determined by radioligand experiments) than potency to inhibit enzymatic activity. Using our standard method, we have previously shown a clear correlation between inhibition of iNOS activity and inhibition of radioligand binding with \[^{3}H\]-2-aminopicoline as radioligand (Boer et al., 2000). The reason, however, for the 5-fold difference seen with BYK191023 remains unclear. An explanation for this result might be that BYK191023 inhibits the enzyme according to an irreversible mechanism or inactivation of the iNOS although the present data argue more for a reversible inhibitory mechanism. An irreversible mechanism has been shown for other iNOS inhibitors before, e.g. 1400W (Garvey et al., 1997; Zhu et al., 2005) and L-NIL (Wolff et al., 1998). Further causes for the mechanistic properties of BYK191023 are presently under investigation.

The inhibitory potency of BYK191023 at iNOS drops significantly from 86 nM in the enzymatic assay to low micromolar values in the various cellular assays. This 40 to100-fold difference is an important issue for the \textit{in vivo} use of the compound and is caused by different L-arginine
concentrations present in the radioactive enzymatic assay and in the cellular test systems. This drop in potency is in agreement with the loss of potency seen at the isolated enzyme in the presence of increasing L-arginine concentrations. The L-arginine concentrations in the enzymatic assay are approximately 3-5 µM while in cellular assays the concentration in the medium is approx. 400 µM. This 100-fold difference in L-arginine concentrations will also influence the IC₅₀ values in the cellular experiments. Increasing L-arginine concentrations in the radioactive enzymatic assay up to 100 µM led to comparable IC₅₀ values between isolated iNOS enzyme and cellular expressed iNOS (Table 2). A similar loss of potency is seen in LPS pretreated rat aorta in which the intracellular L-arginine concentration competing with an inhibitor remains unknown. Again as for the cellular systems high cellular L-arginine concentrations are probably responsible for the reduced inhibitory potency. Nevertheless, cellular potency of BYK191023 varies among different cell lines significantly. Therefore, we determined the intracellular L-arginine concentration of three cell lines used in our study. Within RAW and HEK293/iNOS cells the L-arginine concentration was significantly reduced by more than 50% during induction of iNOS. Surprisingly, the levels of L-arginine in rat mesangial cells did not change upon iNOS stimulation (data not shown). Thus the higher cellular L-arginine levels of RMCs may explain the lower potency of L-arginine competitive inhibitors such as BYK191023.

In addition, we also examined the influence of BYK191023 on cell viability using the MTT assay. However, even cells exposed to high micromolar concentrations of BYK191023 did not show any changes in dihydrogenase activity. Therefore, BYK191023 represents a valuable tool substance for in vivo studies.

Serum binding is an important issue for the development of pharmacologically active compounds designed for clinical use. Desirably drugs show only minor serum binding properties. We determined the serum binding capacity of BYK191023 in cellular assays. By adding human serum to the cellular HEK293 assay we could show that the potency of BYK191023 has insignificantly decreased. Thus, we conclude that BYK191023 has only weak serum binding.

To answer the question whether the enzymatic selectivity for iNOS over eNOS is maintained at the cellular or whole organ level we determined the potency and selectivity of BYK191023 in the LPS
treated or untreated rat aorta as highly integrated tissue models for iNOS and eNOS. An excellent correlation between iNOS/eNOS selectivity data obtained in enzymatic assays and the *in vitro* functional organ models was found. A similar comparison of iNOS/nNOS selectivity was not possible due to the weak or none existing nNOS selectivity of most NOS inhibitors.

In conclusion, BYK191023 is representative for a new class of iNOS inhibitors. BYK191023 inhibits iNOS with high selectivity in enzymatic and cellular models. Furthermore, the compound is potent in *in vitro* pharmacological models. As the compound is active *in vivo* in various rodent models (manuscript in preparation) it represents a promising new candidate for clinical development.
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References


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Footnotes

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Figure legends

Fig. 1. Chemical structure of BYK191023. 2-[2-(4-Methoxy-pyridin-2-yl)-ethyl]-3H-imidazo[4,5-b]pyridine.

Fig. 2. Inhibition of NO-synthases by BYK191023. NOS activation assay was performed with expressed human iNOS (filled circles), nNOS (filled triangles) and eNOS (open squares) in the presence of the inhibitor BYK191023. pIC50 [M] values of 7.03 (iNOS), 4.86 (nNOS) and 3.95 (eNOS) were obtained for each NOS isoform. Each data point is the mean of duplicate determinations.

Fig. 3. Substrate dependence of iNOS activity in the presence of increasing concentrations of BYK191023. NOS assay was performed with expressed human iNOS at the indicated concentrations of [3H]-L-arginine in the presence of increasing concentrations of BYK191023. A. Saturation plot. Calculated V_max values were 3.44 pmol/min in the absence of inhibitor (filled circles) and 3.43 pmol/min, 3.45 pmol/min and 2.70 pmol/min in the presence of 0.3 µM (filled squares), 1 µM (filled triangles) and 3 µM (asterisks) BYK191023, respectively. The following apparent K_m values were observed for L-arginine: 7.6 µM in the absence of inhibitor, 27 µM, 76 µM and 129 µM in the presence of 0.3 µM, 1 µM and 3 µM BYK191023, respectively. B. pK_i value determined by Schild plot. Using the Schild plot (slope = 0.80) the K_i for BYK191023 was determined as 85 nM (pK_i 7.07; intercept with the abscissa). Each data point is the mean of duplicate determinations.

Fig. 4. Saturation isotherm of [3H]-BYK191023 binding to human iNOS. The binding assay was performed with expressed human iNOS and increasing concentrations of BYK191023. An affinity constant (K_D value) of 1.08 µM was obtained for BYK191023. Each data point is the mean of duplicate determinations.

Fig. 5. Inhibition of [3H]-2-aminopicoline binding to human NOS isoforms by BYK191023. The binding assay was performed with expressed human iNOS (filled circles), nNOS (filled triangles) and...
eNOS (open squares) as described. BYK191023 inhibited [3H]-2AP binding to iNOS, nNOS and eNOS with IC$_{50}$ values of 447 nM, 30 µM and 630 µM, respectively. Each data point is the mean of duplicate determinations.

**Fig. 6.** Inhibition of iNOS in RAW, RM and HEK293 cells. The iNOS expression was stimulated and the cellular assays were performed as described using the murine macrophage cell line RAW (open squares), the rat mesangial cell line RMC (filled triangles) and the HEK/iNOS expression system (filled circles) in the presence of BYK191023. pIC$_{50}$ [M] values of 5.41 (RAW), 4.34 (RMC) and 4.97 (HEK293/iNOS) were obtained for each cell line. Each data point is the mean of duplicate determinations.

**Fig. 7.** BYK191023 inhibits selectively iNOS in isolated organ models. A. Potency and selectivity of BYK191023 in isolated tissues using rat aorta for i- and eNOS and rabbit corpus cavernosum for nNOS. iNOS (filled circles) activity was inhibited by BYK191023 with an IC$_{50}$ of 7.9 µM. In contrast BYK191023 shows no inhibition in the eNOS specific (open squares) nor in the nNOS specific (filled triangles) functional model up to 100 µM. B. Inhibition of iNOS by BYK191023 in rat aortic rings monitored by the Griess assay. The iNOS was stimulated in aortic rings as described and generation of nitrite/nitrate was inhibited by BYK191023 with an IC$_{50}$ of 13 µM. Each data point is the mean of triplicate determinations.

**Fig. 8.** iNOS inhibition at LPS-treated rat aorta. Original tracing showing the diminished contraction to phenylephrine (0.3 µM) but full contraction by added AMT (3 µM) due to iNOS inhibition (control). After their washout (W) the repeated contraction to phenylephrine could be partially restored and subsequently attenuated by repeated administration of increasing concentrations of BYK191023 (10 and 30 µM) and L-arginine (10 and 30 µM), respectively. Ordinate: mg of developed force of contraction.
Fig. 9. Correlation of i/eNOS selectively in enzymatic assays versus isolated organ models. The selectivity of various standard inhibitors in the enzymatic assay using human NOS isoforms was plotted against i/eNOS selectivity of the same standard inhibitors in the NOS-models using isolated rat aortic rings (correlation coefficient $r^2 = 0.988; p < 0.001$).
TABLE 1. Structure and potency of BYK191023 and analogs at human NOS isoforms (n = 3-6).

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (iNOS)</th>
<th>IC$_{50}$ (nNOS)</th>
<th>IC$_{50}$ (eNOS)</th>
</tr>
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<tbody>
<tr>
<td>BYK191023</td>
<td>86 nM</td>
<td>17 µM</td>
<td>162 µM</td>
</tr>
<tr>
<td>BYK205513</td>
<td>&gt; 100 µM</td>
<td>&gt; 100 µM</td>
<td>&gt; 100 µM</td>
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<td>BYK205516</td>
<td>102 nM</td>
<td>26 nM</td>
<td>43 nM</td>
</tr>
<tr>
<td>BYK237007</td>
<td>55 nM</td>
<td>98 nM</td>
<td>5.6 µM</td>
</tr>
<tr>
<td>BYK299621</td>
<td>3.5 µM</td>
<td>5.6 µM</td>
<td>2.6 µM</td>
</tr>
<tr>
<td>2-AP</td>
<td>79 nM</td>
<td>68 nM</td>
<td>112 nM</td>
</tr>
</tbody>
</table>
TABLE 2. L-arginine competitive inhibition of the iNOS by BYK191023 (n = 2).

<table>
<thead>
<tr>
<th>L-arginine [µM]</th>
<th>16</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ [µM]</td>
<td>0.15</td>
<td>0.45</td>
<td>1.07</td>
<td>2.57</td>
</tr>
<tr>
<td>BYK191023</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 3. Inhibition of iNOS in various intact cells (n = 5).

<table>
<thead>
<tr>
<th>IC$_{50}$ [µM]</th>
<th>RMC</th>
<th>RAW</th>
<th>HEK293/iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BYK191023</td>
<td>33.0 ± 8.8</td>
<td>3.1 ± 0.5</td>
<td>13.6 ± 4.2</td>
</tr>
</tbody>
</table>
TABLE 4. Potencies of drugs for inhibition of iNOS and eNOS in rat thoracic aorta and of nNOS in field stimulated rabbit corpus cavernosum. Given are pIC_{50} [M] values (with % maximal inhibitory effect in brackets) for the drugs derived from their individual half-maximal effects (means ± SEM, n = 10-16).

<table>
<thead>
<tr>
<th>Compound</th>
<th>iNOS</th>
<th>eNOS</th>
<th>nNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BYK191023</td>
<td>5.14 ± 0.04 (64)</td>
<td>&lt; 4.00</td>
<td>&lt; 4.00</td>
</tr>
<tr>
<td>L-NIL</td>
<td>6.03 ± 0.03 (90)</td>
<td>4.90 ± 0.03 (94)</td>
<td>4.58 ± 0.08 (74)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>4.01 ± 0.03 (38)</td>
<td>5.56 ± 0.02 (100)</td>
<td>5.05 ± 0.03 (96)</td>
</tr>
<tr>
<td>AMT</td>
<td>6.41 ± 0.04 (100)</td>
<td>6.48 ± 0.03 (100)</td>
<td>6.11 ± 0.04 (90)</td>
</tr>
</tbody>
</table>
Figure 4

The graph shows the binding of \[^3\text{H}\text{-BYK191023} \text{free} \, \mu\text{M}] \text{versus} \[^3\text{H}\text{-BYK191023 bound} \, \text{pmol/mg}].\]
Figure 6

NO\textsubscript{x}-production (% of control) vs log BYK191023 [M]
Figure 7

A

% inhibition of NOS (% of AMT maximum)

log BYK191023 [M]

B

NO\textsubscript{x} production (% of control)

log BYK191023 [M]