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In vitro and *in vivo* biotransformation of **WMS-1410**, a potent GluN2B selective NMDA receptor antagonist

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ABSTRACT

Structural modification of the GluN2B selective NMDA receptor antagonist ifenprodil led to the 3-benzazepine **WMS-1410** with similar GluN2B affinity but higher receptor selectivity. Herein the *in vitro* and *in vivo* biotransformation of **WMS-1410** is reported. Incubation of **WMS-1410** with rat liver microsomes and different cofactors resulted in four hydroxylated phase I metabolites, two phase II metabolites and five combined phase I/II metabolites. With exception of catechol **4**, these metabolites were also identified in the urine of a rat treated with **WMS-1410**. However the metabolites **7**, **8** and **12** clearly show that the catechol metabolite **4** was also formed *in vivo*. As shown for ifenprodil the phenol of **WMS-1410** represents the metabolically most reactive structural element. The biotransformation of **WMS-1410** is considerably slower than the biotransformation of ifenprodil indicating a higher metabolic stability. From the viewpoint of metabolic stability the bioisosteric replacement of the phenol of **WMS-1410** by a metabolically more stable moiety should be favourable.

1. Introduction

The NMDA (N-methyl-D-aspartate) receptor is one of the three ionotropic glutamate receptors and responsible for excitatory reactions in the central nervous system (CNS) and the periphery. Seven proteins are known, which form the heterotetrameric ion channel associated NMDA receptor. These proteins are termed GluN1 (with eight splice variants GluN1a–h), GluN2A–D and GluN3A–B. A functional NMDA receptor contains at least one GluN1 and one GluN2 subunit [1,2].

Four subunits form the NMDA receptor associated ion channel being permeable for Ca^{2+}, Na^{+} and K^{+} ions. In addition to the physiological agonists glutamate and glycine, various endogenous and exogenous ligands are able to modulate the opening state of the ion channel pore. Whereas the binding sites for glutamate, glycine, Zn^{2+} ions, protons, ifenprodil and polyamines are located extracellularly, Mg^{2+} ions and open channel blockers inhibit the passage of ions by interacting with binding sites within the ion channel pore [3,4].

In this study the ifenprodil binding site is of particular interest, since this binding site allows the modulation of the opening state by reaction with appropriate ligands. Originally this binding site was supposed to be located at the GluN2B subunit, but in recent results it was identified at the interface between the GluN1 and the GluN2B subunit [5,6]. The GluN2B subunit has a distinct distribution in the CNS with high density in the cortex, hippocampus and striatum and only very low density in the cerebellum. Therefore compounds blocking the NMDA receptor by interaction with the ifenprodil binding site (GluN2B selective) can address selectively only some parts of the CNS and thus minimize side effects.

The neuroprotective potential of GluN2B selective NMDA receptor antagonists can be exploited for the treatment of acute damage of brain (*e.g.* stroke, injury) and chronic neurodegenerative diseases like Alzheimer's and Parkinson's Disease [7,8].

The aminoalcohol ifenprodil represents the first ligand with high selectivity for NMDA receptors containing the GluN2B subunit. Although the affinity of ifenprodil towards GluN2B containing NMDA receptors is very high (K_i = 10 nM) [9], its selectivity is rather poor, since it interacts with some further receptors including α_1, σ_1, σ_2, 5-HT_{2A}, and 5-HT_{2C} receptors [10]. The fast biotransformation leading to a low bioavailability is a further disadvantage of ifenprodil. Very recently we have identified the main metabolites of ifenprodil, which result from reactions of the phenolic OH-moiety [11].

In order to improve the receptor selectivity and the metabolic stability we have designed and synthesized the 3-benzazepine

Abbreviations: BSA, bovine serum albumin; CNS, central nervous system; COMT, catechol-*O*-methyl transferase; EIC, extracted ion current; IS, internal standard; NADPH, nicotinamide adenine dinucleotide phosphate; NMDA, N-methyl-D-aspartate; PAPS, adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate; SAM, S-(5′-adenosyl)-L-methionine iodide; TIC, total ion current; UDPGA, uridine 5′-diphosphoglucuronic acid trisodium salt.

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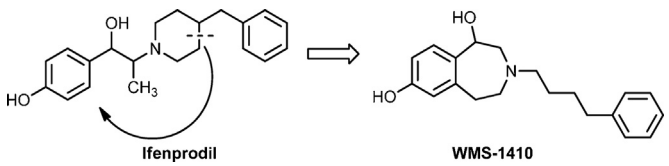

Fig. 1. Development of **WMS-1410** from ifenprodil.

WMS-1410, which is derived from ifenprodil by formal cleavage of one C—C bond of the piperidine ring and reconnection of the free ethyl chain to the benzene ring generating a seven membered ring [12,13] (Fig. 1). The distance between the basic amino moiety and the terminal phenyl ring (hydrophobic part) was not changed and thus **WMS-1410** fits nicely into the reported pharmacophore model for GluN2B selective NMDA antagonists [14]. It was shown that **WMS-1410** represents high GluN2B affinity (K_i = 13 nM), high NMDA receptor antagonistic activity (IC_{50} = 18.4 nM), and high selectivity over more than 100 relevant targets including the above mentioned off-targets of ifenprodil [12]. Additionally, in first toxicity tests and in *in vivo* experiments **WMS-1410** was highly tolerated. First metabolism experiments showed an improved stability compared to the lead compound ifenprodil [13].

Herein the detailed biotransformation of **WMS-1410** is reported. In particular the metabolically labile positions are analyzed for further improvement of the pharmacokinetic properties of the 3-benzazepine class of GluN2B antagonists. Moreover the *in vitro* and *in vivo* formed phase I and phase II metabolites as well as the rate of biotransformation are compared with the corresponding data of the lead compound ifenprodil.

2. Materials and methods

2.1. Chemicals and materials

S-(5′-adenosyl)-L-methionine iodide (SAM), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS) and Coomassie Brilliant Blue were purchased from Sigma–Aldrich (Munich, Germany). NADPH sodium salt was obtained from Carl Roth (Karlsruhe, Germany). Acetonitrile in HPLC grade was delivered by VWR (Darmstadt, Germany). Formic acid was purchased from Merck KGAA (Darmstadt, Germany). Purified water for HPLC analysis and sample dilution was generated by a Milli-Q Advantage Ultrapure Water System, Millipore (Billerica, MA, USA). All other chemicals were obtained from different suppliers in analytical grade. SPE cartridges with octadecyl stationary phase (1 mL, sorbent mass 100 mg, particle diameter 45 μm) were obtained from J.T. Baker® (Philipsburg, MT, USA). The metabolism cage (serial number 3700M071) was manufactured by Tecniplast Metabolics (Hohenpeißenberg, Germany).

2.2. Animals

For *in vivo* metabolism studies of **WMS-1410** Wistar rats weighing 200–320 g from a local strain were used (Charles River Laboratories, Sulzfeld, Germany). Rats were allowed food and water *ad libitum*. The experiment was approved by the German Animal Welfare Committee (AZ.84-02.04.2013.A148).

2.3. Preparation of rat liver microsomes

Livers of Wistar rats both sexes from a local strain were used (Charles River Laboratories, Sulzfeld, Germany). Livers (50 g) were washed with cold 1.15% (m/v) potassium chloride solution and homogenized in an Elvehjem-Potter homogenizer with equal volume of cold phosphate buffer (pH 7.4, 0.1 M) containing sodium EDTA (0.5 mM). The resulting suspension was diluted with 140 mL phosphate buffer (pH 7.4, 0.1 M) and centrifuged for 20 min at 4 °C at 9000 × *g*. The supernatant was centrifuged at 40,000 × *g* for 60 min. The resulting pellet was dissolved in phosphate buffer (pH 7.4, 0.1 M) and stored at −80 °C prior to use.

Protein concentration was determined according to Bradford [15] and Stoscheck [16]. A solution of Coomassie Brilliant Blue G 250 (5 mg) in ethanol (2.5 mL) was diluted with water (10 mL) and phosphoric acid (85%, 5 mL). The solution was diluted with water to a final volume of 50 mL. The solution was kept in darkness for 12 h (overnight) at 4 °C and filtered. For determination of the protein concentration the prepared microsomes were diluted 20-fold (5 μL microsome suspension, 20 μL 1 M NaOH, 75 μL water), 50-fold (2 μL microsomes, 20 μL 1 M NaOH, 78 μL water) and 80-fold (5 μL microsome suspension, 80 μL 1 M NaOH, 315 μL water). Calibration was performed with BSA in six concentrations between 0.0 and 0.5 mg protein/mL.

Each standard solution was measured in triplicate, the microsome preparation in duplicate. The protein solution (10 μL) was added to the Bradford-solution (190 μL). After 5 min, the concentration was determined by measuring the absorption at 595 nm.

2.4. Microsomal incubations (phase I)

WMS-1410 (300 μg) was dissolved in phosphate buffer (250 μL, pH 7.4, 0.1 M) containing magnesium chloride (100 μL, 45.5 mM). After adding 150 μL of the microsome preparation (7 mg protein/mL) and 5 mg NADPH/H^+ the mixture was incubated in a shaker at room temperature. After 120 min, the incubation was terminated by addition of an equal volume of cold acetonitrile (−20 °C). The mixture was stored in an ice bath for 10 min. Subsequently, the sample was centrifuged at 13,000 rpm for 8 min and the resulting supernatant was analyzed by HPLC–MS without further sample pre-treatment.

2.5. Microsomal incubations (phase II)

Phase II reactions were carried out using the same incubation mixture as for phase I reactions. All experiments were performed with and without addition of NADPH/H^+. The rat liver microsome preparation contained the essential enzymes glucuronyl transferases, sulfo transferases and catechol-*O*-methyl transferases. Glucuronic acid conjugates were obtained by addition of 3.5 mg UDPGA to the incubation mixture. For the formation of sulfate conjugates 10 μL PAPS solution (2.14 μM) was added. Catechol derivatives obtained during phase I reaction were methylated *in situ* by addition of 15 μL SAM solution (18.9 μM) to the incubation mixture.

2.6. HPLC/MS-analysis

For the determination of exact masses, LC–MS system 1, consisting of an Ultimate 3000 RS LC system from Dionex (Dionex Softron, Germering, Germany) coupled with a micrOTOF-Q II (Bruker Daltonics, Bremen, Germany) with an ESI-source, was used. The LC System consisted of a solvent rack (SRD 3600), a pump (DGP-3600RS), an autosampler (WPS-3000RS), a column oven (TCC-3000RS) and a DAD-detector (DAD-3000 RS) operating at 230 nm. Control of the system and data handling were carried out using the software Hystar and DataAnalysis from Bruker Daltonics (Bremen, Germany). Calibration of the micrOTOF-Q II was performed with lithium formate. The LC separation was carried out using a SecurityGuard™ C18 Cartridge 4.0 × 2.0 as a pre-column and a Phenomenex Kinetex (2.6 μM, 100 A 50 × 2.10 mm) column with a gradient. The mobile