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Phenylalanine derivatives with modulating effects on human α1-glycine receptors and anticonvulsant activity in strychnine-induced seizure model in male adult rats

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ABSTRACT

The critical role of α1-glycine receptor (α1-GLYRs) in pathological conditions such as epilepsy is well known. In the present study, structure-activity relations for a series of phenylalanine derivatives carrying selected hydrogen bond acceptors were investigated on the functional properties of human α1-GLYR expressed in *Xenopus oocytes*. The results indicate that one particular substitution position appeared to be of special importance for control of ligand activity. Among tested ligands (**1-8**), the biphenyl derivative (**2**) provided the most promising antagonistic effect on α1-GLYRs, while its phenylbenzyl analogue (**5**) exhibited the highest potentiation effect. Moreover, ligand **5** with most promising potentiating effect showed *in-vivo* moderate protection when tested in strychnine (STR)-induced seizure model in male adult rats, whereas ligand **2** with highest antagonistic effect failed to provide appreciable anti(pro)convulsant effect. Furthermore, ligands **2** and **5** with the most promising effects on human α1-GLYRs were examined for their toxicity and potential neuroprotective effect against neurotoxin 6-hydroxydopamine (6-OHDA). The results show that ligands **2** and **5** possessed neither significant antiproliferative effects, nor necrotic and mitochondrial toxicity (up to concentration of 50 μM). Moreover, ligand **2** showed weak neuroprotective effect at the 50 μM against 100 μM toxic dose of 6-OHDA. Our results indicate that modulatory effects of ligands **2** and **5** on human α1-GLYRs as well as on STR-induced convulsion can provide further insights for the design of therapeutic agents in treatment of epilepsy and other pathological conditions requiring enhanced activity of inhibitory glycine receptors.

1. Introduction

In central, as well as peripheral, nervous system, GLY functions as a neurotransmitter at inhibitory synapses, where it activates strychnine-sensitive glycine receptors (GLYRs) which are composed of five subunits (α1-4 and β) (Lynch, 2004) and belong to pentameric Cys-loop ligand-gated ion channel superfamily (Cys-loop LGICs)(Castro et al., 2012; Lester et al., 2004).

It has been well established that GLYRs play important roles in various pathological conditions such as epilepsy, hyperexcitability, motor-control, and pain sensation (Callister and Graham, 2010; Camp et al., 2010). In recent studies, the critical role of GLYRs in modulating temporal lobe epilepsy has been proposed based on clinical findings that patients suffering from temporal lobe epilepsy disease would possibly be advantaged most from facilitated glycinergic inhibition, e.g. through GLYR modulators. Accordingly, mounting evidences have accentuated the contribution of GLYRs to tonic hippocampal inhibition in response to glycine, and glycine and taurine as well as glycine uptake inhibitors were found to be of anticonvulsive role in epilepsy models (Bedet et al., 2006; Eichler et al., 2008; Meier and Grantyn, 2004; Song

Abbreviations: ANOVA, analysis of variance; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*9,*N*9-tetraacetic acid; DA, dopamine; DMSO, dimethylsulfoxide; MBS, Barth's solution; nAChR, nicotinic acetylcholine receptor; α1-GLYRs, α1-glycine receptor; ROS, radical oxygen species; 6-OHDA, 6-hydroxydopamine; ATP, adenosine triphosphate; HEK-293, human embryonic kidney cell line; DMEM, Dulbecco's Modified Eagle's Medium; STR, strychnine; VPA, valproic acid; AED, antiepileptic drug; AMPA, (RS)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid; KA, kainite; NMDA, *N*-methyl-D-aspartic acid; GluK1, homomeric kainate receptor

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et al., 2006; Zhang et al., 2008). Also, STR-sensitive glycine receptors were previously found to depress hyperexcitability in rat dentate gyrus (Eichler et al., 2008; Kirchner et al., 2003; Mori et al., 2002). Based on the aforementioned role of GLYRs in central and peripheral nervous system pathologies, and as a continuation of our research (Szymanska et al., 2011, 2009), in the present study effects of a series of unnatural racemic phenylalanine derivatives (**1–8**) on the functional properties of the cloned human α1-GLYR expressed in *Xenopus* oocytes were investigated. Moreover, selected compounds have been examined for their anti(pro)convulsant activity in STR-induced seizure model in adult rats.

In addition, the most interesting phenylalanine derivatives were tested for their toxicity using antiproliferative EZ4U test as well as for their potential neuroprotective properties in cell-death *in vitro* model using neurotoxin 6-OHDA. Cytotoxicity of 6-OHDA is believed to result from radical oxygen species (ROS) derived by 6-OHDA intraneuronal autooxidation as well as a possible direct effect of 6-OHDA on the mitochondrial respiratory chain (Rodriguez-Pallares et al., 2007), and recent studies demonstrated the direct association of mitochondrial dysfunction and oxidative stress with epileptogenesis and acquired chronic epilepsy (Blum-Degen et al., 1998; Waldbaum and Patel, 2010). In this context, the new multi-target acting anti-epileptic compounds which would act not only *via* epilepsy-relevant receptors but also possessing neuroprotective activity preventing mitochondria from ROS may contribute towards development of the therapy of epilepsy.

2. Materials and methods

2.1. Human α1-glycine receptor recordings from oocytes

Mature female *Xenopus laevis* frogs were purchased from Xenopus Express (Haute-Loire, France), housed in dechlorinated tap water at 19–21 °C with a 12/12-h light/dark cycle, and fed food pellets supplied by Xenopus Express. The procedures followed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition) of the National Institutes of Health (Bethesda, MD) and approved by the Institutional Animal Care and Use Committee at the UAEU.

As described previously by Sadek et al., 2014 (Ashoor et al., 2013a,b; Mahgoub et al., 2013; Sadek et al., 2012, 2014a, 2015a), clusters of oocytes were removed surgically under benzocaine (Sigma, St. Louis, MO) local anesthesia (0.15% w/V), and individual oocytes were dissected manually in a solution containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5). Dissected oocytes were then stored 2–7 days in modified Barth's solution (MBS) containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $CaCl_2$, 2; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5), supplemented with sodium pyruvate, 2 mM, penicillin, 10,000 IU/L, streptomycin, 10 mg/L, gentamicin, 50 mg/L, and theophylline, 0.5 mM. Briefly, oocytes were placed in a 0.2 ml recording chamber and superfused at a rate of 2–3 ml/min. The bathing solution consisted of (in mM): NaCl, 95; KCl, 2; $CaCl_2$, 2; and HEPES 5 (pH 7.5). The cells were impaled with two glass microelectrodes filled with a 3 M KCl. The oocytes were routinely voltage clamped at a holding potential of −70 mV using a GeneClamp-500 amplifier (Axon Instruments Inc., Burlingame, CA). Glycine-induced currents were evoked every 5 min by 3–4 s application of 30 μM glycine. During 10 min application time, strychnine or series of phenylalanine derivatives were applied externally by addition to the superfusate. These compounds (strychnine and series of phenylalanine derivatives) were also included in glycine containing agonist solution.All chemicals used in preparing the solutions were from Sigma-Aldrich (St. Louis, MO, USA) whereas test compounds **1–8** were synthesized as racemates in the Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen/Denmark or in the Department of Technology and Biotechnology of Drugs, Jagiellonian University Medical College, Kraków/Poland, according to previously described procedures (Szymanska et al., 2011, 2009). Stock solutions of the respective test compound used in this study were prepared in saline at a concentration of 10 mM. The cDNA plasmids for human α1-glycine receptor expression were kindly provided by Prof. Joe Lynch (The University of Queensland, Brisbane, Australia). Capped cRNA transcripts were synthesized *in vitro* using a mMESSAGE mMACHINE kit from Ambion (Austin, TX, USA) and analyzed on a 1.2% formaldehyde agarose gel to check the size and quality of the transcripts.

2.2. Data analysis

Average values were calculated as the mean of 6–8 experiments ± SEM. Throughout this study, n defines the number of oocytes or number of samples tested in each experiment. Statistical significance was analyzed using paired *t*-test. The criterion for statistical significance was set at a P value of less than 0.05.

2.3. Cell line studies

Human embryonic kidney HEK-293 cell line (ATCC CRL1573) was kindly donated by Prof. Dr. Christa Müller (Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Germany). *Neuroblastoma* IMR-32 cell line was provided by Department of Oncogenomics, Academisch Medisch Centrum, Amsterdam, Holland (Cheng et al., 1995).

2.3.1. Antiproliferative assay against HEK-293

The HEK-293 cells were seeded in 96-well flat-bottomed microtiter plate at a concentration of 1×10^4 cells/well and cultured in 200 μl/well of Dulbecco's Modified Eagle's Medium − DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 mg mL^{-1} streptomycin and 100 U mL^{-1} penicillin in a humidified atmosphere (5% CO_2, 95% air) at 37 °C for 24 h to reach 60% confluence. The stock solutions of **2** and **5** in DMSO (25 mM) were diluted into the fresh growth medium and added to the wells in the concentration range 0.1–100 μM. Final DMSO concentration did not exceed 1%. After 48 h of incubation, the EZ4U (Biomedica, Vienna, Austria) labeling mixture (20 μl) was added to each well and the cells were incubated under the same conditions for 5 h. The absorbance of the samples was measured using a microplate reader (EnSpire, PerkinElmer, Waltham, MA USA) at 492 nm. The reference compound doxorubicin (DX) was used during this study as a cytotoxic standard against HEK-293 cells.

2.3.2. Antiproliferative assay against IMR-32

The IMR-32 cell line was seeded in 96-well flat-bottomed microtiter plate at a concentration of 1×10^4 cells/well and incubated under the same conditions as described above. The stock solutions of **2**, **5** and 6-OHDA in DMSO (25 mM) were diluted into the fresh growth medium and added to the cells in concentration range 1–100 μM and incubated for 48 h. Final DMSO concentration did not exceed 1%. Next, the EZ4U assay was performed in the same way as described above.

2.3.3. Neuroprotection test

The Mitochondrial ToxGlo™ Assay and protocols were provided by Promega (Madison, WI, USA). The *neuroblastoma* IMR-32 cell line was seeded in 96-well white microtiter Plate 24 h before experiments at a concentration 100 000 cells/ml. The cells were incubated in 200 μl of DMEM (Gibco, Invitrogen Ltd, Paisley, UK) supplemented with 10% FBS in a humidified atmosphere (5% CO_2) at 37 °C. Next, the old media were removed and the **2**, **5** at the concentrations 50 μM, and 6-OHDA at the concentration 100 μM dissolved in DMEM supplemented with 10% FBS and 4.5 g/L glucose were added to the plate. The cells were incubated at 37 °C in a humidified and CO_2-supplemented incubator for 90 min. After addition of 20 μl fluorescence-based Cytotoxicity Reagent (Promega) the plate was mixed (500 rpm) and incubated at 37 °C (5%