Journal of Pharmaceutical and Biomedical Analysis 117 (2016) 184–194

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

# $^1$H NMR metabolomics to study the effects of diazepam on anisatin induced convulsive seizures

Pei Li[a,1], Dan-Dan Wei[a,1], Jun-Song Wang[b,**], Ming-Hua Yang[a], Ling-Yi Kong[a,*]

[a] State Key Laboratory of Natural Medicines, Department of Natural Medicinal Chemistry, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing 210009, PR China

[b] Center for Molecular Metabolism, School of Environmental and Biological Engineering, Nanjing University of Science and Technology, 200 Xiao Ling Wei, Nanjing 210014, PR China

ARTICLE INFO

*Article history:*
Received 2 July 2015
Received in revised form 18 August 2015
Accepted 19 August 2015
Available online 1 September 2015

*Keywords:*
Diazepam
Anisatin
Convulsive seizures
NMR
Metabolomics

ABSTRACT

The anticonvulsive properties of diazepam have been extensively studied, mainly focusing on the γ-amino butyrate (GABA) system. The aim of this investigation was to integrally analyze the metabolic events related to neuroprotection of diazepam on anisatin-induced convulsive seizures by a NMR-based metabolomic approach combined with histopathological examination and behavior examination. Multivariate analysis on metabolic profiles of the piriform cortex and cerebellum of mice revealed that diazepam could relieve mice suffering from the convulsive seizures by recovering destructed neurotransmitter and neuromodulator metabolism, ameliorating oxidative stress, alleviating the disturbance in energy, amino acid and nucleic acid metabolism in anisatin intoxicated mice. This integrated metabolomics study provided a powerful and highly effective approach to elucidate therapeutic effects and assessed the safety of diazepam. This study should be helpful for our understanding of convulsive seizures, and provide a holistic view of the treatment effects of benzodiazepine on convulsive seizures.



## 1. Introduction

Seizures are one of the most common neurological serious conditions, which are essentially malfunctioning in the brain, associated with the "misfiring" of neurons [1]. Seizures are abnormal electrical discharges in the brain that result in changes of sensation, consciousness and behavior. The adverse outcomes of seizures are often brain edema and ischaemia [2], cognitive impairment [3], neurological deficits, hippocampal injury and high mortality [4]. Frequent spontaneous seizures have sufficiently high risk in developing to epilepsy, which affects approximately 65 million people worldwide [5]. Convulsive seizures have been associated with insufficient blood flow, increased energy consumption, disturbed γ-amino butyric acid (GABA) activity, and glutamate-mediated excitotoxicity [6]. Various animal models have been used to understand seizure-related brain damage, its mechanism and possible consequences in humans. Convulsant drugs, such as kainic acid, pentylenetetrazol, pilocarpine, flurothyl, and organophosphates, are mostly used in these models [7]. We have also reported a convulsion model induced by anisatin, a non-competitive GABA antagonist, and its induced metabolomic changes [8]. In this model, anisatin produced convulsive seizures through its inhibition of $GABA_A$ receptors. While enhancement of GABA receptor-mediated actions is a major strategy of antiepileptic drugs [9]. With a broad spectrum of activity, benzodiazepines (BDZs) have widely used to treat various seizure emergencies in clinic, such as partial and idiopathic generalised epilepsies, as well as status epilepticus. BDZs act through binding sites on the GABA receptor, this activity of binding leads to allosteric activation of the receptor to increase the frequency of chloride-channel opening, resulting in hyperpolarized postsynaptic membranes and then enhanced CNS depression response [10]. Diazepam is one of the most successful benzodiazepine anticonvulsant, and have definite efficacy in treating a wide-spectrum of CNS disorders. Diazepam was recommended as the standard treatment for acute convulsive seizures [11]. Despite abundant studies on the neuroprotection of diazepam, the effects of diazepam on anisatin induced convulsive seizures have not been reported.

Metabolomics has been applied to identify and quantify the variation of endogenous metabolites, with the development of analysis instrument, several analytical methods are available

* Corresponding author. Fax: +86 25 8327 1405.
** Corresponding author.
*E-mail addresses:* wang.junsong@gmail.com (J.-S. Wang), cpu_lykong@126.com (L.-Y. Kong).
[1] These authors contributed equally to the manuscript.

http://dx.doi.org/10.1016/j.jpba.2015.08.029
0731-7085/

for metabolomic research, such as nuclear magnetic resonance (NMR), gas chromatography/mass spectrometry (GC/MS), and liquid chromatography/mass spectrometry (LC/MS). As one of the main techniques for metabolomics study, NMR has the associated advantages of relatively little sample preparation, easy reference-free quantification, nondestructive analysis nature, and of the powerful ability to elucidate various metabolites, thus extremely suitable for high-throughput metabolic profiling. NMR-based metabolomics has been successfully applied in various seizures, such as pentylenetetrazole kindling model [12], a soman-induced convulsive [6] and amygdaloid-kindled rat models [13].

In this work, we applied an NMR-based metabolomics approach to evaluate the therapeutic effects of diazepam on anisatin induced convulsive seizure as an extension of our previous study. Metabolic profiles analyzed by multivariate analysis techniques, such as orthogonal signal correction-partial least square discriminate analysis (OSC-PLS-DA), revealed many small molecule metabolites alteration in brain of mice, concerning disorders of neurotransmitters and neuromodulators, stress of reactive oxygen species, disturbance in metabolism of amino acids, nucleic acids and energy substances. To our best knowledge, this is the first report of metabolomics study on diazepam-treated convulsive seizures.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Anisatin was isolated from Illicium simonsii in our lab (>97% pure as assessed by HPLC chromatography) [14]. Diazepam was bought from Sigma (St. Louis, MO, USA). Sodium 3-trimethylsilyl-1-(2, 2, 3, 3-$2H_4$) propionate (TSP) was provided by Sigma Chemical Co. (St. Louis, MO, USA). Deuterium oxide ($D_2O$, 99.9%) was purchased from Aldrich (Steinheim, Germany). Ultra-pure distilled water was prepared using a Milli-Q purification system. Acetonitrile was obtained from Merck KGaA (Darmstadt, Germany). All reagents were of analytical grade.

### 2.2. Animals and treatments

Forty-eight male ICR mice (age 6 weeks; weight 24–28 g) were purchased from Laboratory Animal Research Center, Nanjing University (Nanjing, China). The experimental animals were housed in an air-conditioned room at 25 ± 2 °C and a relative humidity of 50 ± 10%, with a 12/12 h light-dark cycle, ad libitum fed with water and standard laboratory diet throughout the experiments. The studies were approved by China Pharmaceutical University Animal Care and Use Committee (IACUC), and were carried out in accordance with National Institutes of Health guidelines. After acclimatization for 7 days, mice were randomly divided into four groups (diazepam/anisatin): in DIAN++ group, diazepam (2 mg/kg body weight) was administered by oral gavage once a day for 7 days before anisatin was injected intraperitoneally (*i.p.*) to mice at 1.0 mg/kg; in ANIA−+ group, the same volume of buffered saline was administered by oral gavage once a day for 7 days before anisatin was injected *i.p.* to mice at 1.0 mg/kg; in DIAZ+− group, diazepam was administered by oral gavage once a day for 7 days before the same volume of buffered saline injected i.p. to mice, in CON− group, mice were administered with an equivalent amount of vehicle.

After administration of anisatin, the mice were observed for 3 h to evaluate behavioral changes and assess the severity of seizures, as previously described [8]: The severity was recorded according to the following scale: 0: no change; 1: twitching of face or ears; 2: convulsant waves throughout body; 3: jerky movements of arms or legs; 4: convulsions while lying on side; 5: convulsions while lying on back.

### 2.3. Sample preparation for histopathology and NMR analysis

Toward the end of the experiment, piriform cortexes and cerebellums were rapidly removed from mice after sacrificed, flushed with ice-cold phosphate buffer solution and weighed. The tissues for histological analysis were immersed in 10% formalin and embedded in paraffin wax, sectioned, then stained with hematoxylin eosin (H&E). Those histopathological evaluations were performed as a paid service by a qualified pathologist.

Tissues for NMR analysis were homogenized in precooled acetonitrile/water (vol/vol = 1:1, 20 mL/g tissue) bathing in ice–water. The supernatants were obtained by centrifugation (12,000 rpm, 10 min, 4 °C), Each extract was lyophilized and then reconstituted into 550 μL phosphate buffer saline (0.2 mol/L $Na_2HPO_4$ and 0.2 mol/L $NaH_2PO_4$, pH 7.0), containing 0.05% TSP. Samples were vortexed and centrifuged, and ca. 500 μL of them was transferred into 5 mm NMR tubes for analysis.

### 2.4. $^1$NMR spectroscopy

NMR spectra of all piriform cortex and cerebellum samples were recorded on a Bruker AV 500 MHz spectrometer (Bruker GmbH, Karlsruhe, Germany). One-dimensional $^1$H NMR spectra were acquired with 64 scans using the nuclear Overhauser enhancement spectroscopy (NOESY)-presaturation (NOESYPR) pulse sequence (relaxation delay-90°-$t_1$-90°-tm-90°-acquire-free induction decays) (FID) with water suppression (Bruker's pulse program noesypr1d) during both the recycle delay (2 s) and mixing time (tm, 100 ms). And the delay between the first two pulses was 4 microseconds. Typically, 128 FIDs were collected into 64 K data points, using a spectral width of 10 kHz, with an acquisition time per scan of 1.36 s and a relaxation delay of 1.5 s. All $^1$H NMR spectra were manually phased, baseline corrected, aligned and referenced to TSP ($CH_3$, 0.00), using Bruker Topspin 3.0 software.

To assist metabolite assignment, a range of two-dimensional (2D) NMR experiments such as $^1$H–$^1$H total correlation spectroscopy (TOCSY), $^1$H–$^{13}$C heteronuclear single quantum correlation spectroscopy (HSQC) were carried out.

### 2.5. Data preprocessing and analysis

With the removal of signals of water and its affected neighboring regions (4.47–5.60 ppm), the NMR data were binned using an adaptive binning approach based on the code implemented in Matlab [15], affording 503 and 537 bins for cerebellum and cortex, respectively, with an average of 0.015 ppm for each bin. The binned spectra over the ranges of 0.00–4.47 ppm and 5.60–9.60 ppm was normalized to the total sum of spectral integrals to compensate for the concentration differences. The binned data were mean-centered and pareto-scaled prior to multivariate statistical analysis to simplify the interpretation of the coefficients in all models.

Multivariate statistical analysis, including unsupervised principal component analysis (PCA), orthogonal signal correction-partial least square discriminate analysis (OSC-PLS-DA) methods were performed by in-house developed scripts running in "R" software (http://cran.r-project.org/).

Principal component analysis (PCA) was firstly performed on NMR data to obtain a general overview of the metabolic pattern. Then OSC-PLS-DA was performed to reveal the differential metabolic alterations in piriform cortexes and cerebellums. The orthogonal signal correction (OSC) filter was applied to remove uninterested variation from the spectral data prior to PLS-DA.