Fitoterapia 119 (2017) 158–167

Contents lists available at ScienceDirect

Fitoterapia

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

Ecdysterones from *Rhaponticum carthamoides* (Willd.) Iljin reduce hippocampal excitotoxic cell loss and upregulate mTOR signaling in rats

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ARTICLE INFO

Keywords:
Rhaponticum carthamoides
Neuroprotective
Ecdysterones
GRIN2B
Computational simulation

ABSTRACT

Glutamate-induced excitotoxicity is a key pathological mechanism in many neurological disease states. Ecdysterones derived from *Rhaponticum carthamoides* (Willd.) Iljin (RCI) have been shown to alleviate glutamate-induced neuronal damage; although their mechanism of action is unclear, some data suggest that they enhance signaling in the mechanistic target of rapamycin (mTOR) signaling pathway. This study sought to elucidate the mechanisms underlying ecdysterone-mediated neuroprotection. We used in silico target prediction and simulation methods to identify putative ecdysterone binding targets, and to specifically identify those that represent nodes where several neurodegenerative diseases converge. We then used histological analyses in a rat hippocampal excitotoxicity model to test the effectiveness of ecdysterones in vivo. We found that RCI-derived ecdysterones should bind to glutamatergic NMDA-type receptors (NMDARs); specifically, in vivo modeling showed binding to the GRIN2B subunit of NMDARs, which was found also to be a node of convergence in several neurodegenerative disease pathways. Computerized network construction by using pathway information from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database showed putative links between GRIN2B and mTOR pathway elements including phosphoinositide-3kinase (PI3K), mTOR, and protein kinase C (PKC); these elements are associated with neuronal survival. Brain tissue western blots of ecdysterone-treated rats showed upregulated PI3K, Akt, mTOR, and phosphorylated Akt and mTOR, and down regulated GRIN2B and the apoptotic enzyme cleaved caspase-3. Ecdysterone treatment also prevented glutamate-induced rat hippocampal cell loss. In summary, RCI-derived ecdysterones appear to prevent glutamatergic excitotoxicity by increasing mTOR/Akt/PI3K signaling activity.

1. Introduction

The prevalence of neurological diseases is growing yearly, and has become one of the most pressing problems in human health today. One of the most prominent mechanisms underlying these diseases is glutamate-induced excitotoxic damage to neurons. Glutamate is the most abundant excitatory amino acid neurotransmitter in the mammalian central nervous system (CNS). Normal glutamate signaling is important for CNS functions such as learning, memory, neuronal plasticity, and brain development [1]. However, pathologically high glutamate release is neurotoxic, especially to large excitatory neurons (e.g., hippocampal pyramidal cells). Such pathological release occurs in some nervous system diseases, including ischemic stroke, epilepsy, traumatic brain injury, and amyotrophic lateral sclerosis (ALS) [2].

Glutamate neurotransmission is mediated by two categories of ionotropic receptor, the *N*-methyl-D-aspartate receptor (NMDAR), and the non-NMDARs, including the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR) and kainic acid receptor (KAR). The receptors differ in the dynamics of their binding to glutamate, and of their ion selectivity. The accumulated evidence indicates that excessive stimulation of NMDARs causes Ca^{2+} influx that leads to oxidative stress; it can also induce the activity of hydrolases, protein kinases, and

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http://dx.doi.org/10.1016/j.fitote.2017.03.015
Received 14 December 2016; Received in revised form 24 March 2017; Accepted 30 March 2017
Available online 02 April 2017
0367-326X/

other enzymes that maybe involved in the mechanisms underlying various neurodegenerative diseases [3–5]. Therefore, NMDAR antagonists are considered potential candidate drugs for preventing excitotoxic damage. NMDARs consist of tetramers or pentamers of different subunits from three families, GRIN1, GRIN2 and GRIN3 (NR1, NR2 and NR3). In addition, there are four subtypes of GRIN2, GRIN2A/B/C/D (NR2A-NR2D). GRIN1 and GRIN2A subunits are widely distributed in mammalian brains; of these, GRIN2B subunits are mainly distributed in forebrain areas such as the hippocampus and striatum; GRIN2C subunits localize mostly to the cerebellum; and GRIN2D mostly to the midbrain. The GRIN3 subunit complexes with GRIN1 or GRIN2 [6], and forms a channel with a high permeability to Ca^{2+}. It is inhibited by dizocilpine (MK-801) and activated by glycine and glutamate [7]. However, the nonspecific inhibition of NMDARs (e.g., by MK-801) induces severe side effects, restricting its clinical utility [8]. On the contrary, GRIN2 contains the glutamate binding site, and GRIN2B subunit-specific antagonists were more neuroprotective than those of other GRIN2 subunits, suggesting that specific GRIN2B antagonists may reduce or prevent neurological damage with fewer side effects.

Rhaponticum carthamoides (Willd.) Iljin (RCI) is an herbaceous plant specifically distributed in Xinjiang, China and is used in Chinese folk medicine to treat nervous prostration, anepithymia, and hypertension [9]. Of the various compounds extracted from RCI, ecdysterones are the most abundant neurotrophic compounds [10–13]. The mechanisms underlying ecdysterone activity are only partially known, but increased Akt signaling was found to be associated with β-ecdysterone [14], and inhibition of the pro-apoptotic enzyme caspase-3 was associated with general ecdysterone activity [15].

We hypothesized that ecdysterones would mediate neuroprotection by modulating one or more components of the mTOR signaling pathway relevant to NMDAR function. To better elucidate the mechanisms underlying ecdysterone-mediated neuroprotection, we first used Chemmapper [16], an online target prediction tool, to identify putative binding targets of RCI-derived ecdysterones. We then used bioinformatic techniques to identify any potential targets that were common to several neurological diseases with possible excitotoxic mechanisms. Finally, we used a rat model of hippocampal excitotoxicity to quantify the effects of ecdysterone administration on the identified target molecules.

2. Materials and methods

2.1. Materials

The fresh roots of RCI were collected from Altai in Xinjiang, People's Republic of China, in September 2014, and were verified by Researcher Bahar-Guli, Altai Institute for Drug Control, Altai, China. RIPA tissue lysis buffer was purchased from Beyotime Institute of Biotechnology Co., Ltd. (Shanghai, China), containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4, and leupeptin. Beyotime also supplied the BCA protein assay kit (catalog no. P0010S), phenylmethanesulfonyl fluoride (PMSF; catalog no. ST506), the ECL Plus kit (catalog no. P0018). The LDH release assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (catalog no. 20160924, Jiangsu, Nanjing, China). The antibodies used were as follows: anti-GRIN2B antibody (catalog no. ab65783, Abcam, UK), anti-V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) antibody (catalog no. ab102007, Abcam, UK), anti-PIK3CA antibody (catalog no. ab183957, Abcam, UK), anti-calpain 1 antibody (catalog no. ab108400, Abcam, UK), anti-procaspase-3 and anti-cleaved caspase-3 antibody (catalog no. ab184787, Abcam, UK), anti-AKT1 antibody (catalog no. 2967, Cell Signaling Technology, USA), anti-p-AKT1 antibody (catalog no. ab81283, Abcam, UK), anti-mTOR antibody (catalog no. 2983, Cell Signaling Technology, USA), anti-p-mTOR antibody (catalog no. ab137133, Abcam, UK), anti-PKC and anti-p-PKC antibody (catalog no. ab32376 and ab76016, Abcam, UK).

2.2. Extraction of total RCI ecdysterones

Dried roots of RCI (1.5 kg) were crushed and initially refluxed with dichloromethane for 2 h. After removing the dichloromethane, the crushed root material was continuously refluxed with 10 volumes of methanol three times for 30 min each. The methanol was evaporated under vacuum to yield 70 g of extract that was stored at 4 °C for further use.

2.3. HPLC-TOF-MS analysis

The RCI-derived ecdysterones in the extracts were identified using a Waters ACQUITY UPLC system connected to a Waters LCT Premier XE time-of-flight mass spectrometer (TOF-MS, Waters, USA). An ACQUITY UPLC® BEH C18 column (2.1 × 50 mm, 1.7 μm) was used to perform the chromatography work, with the column temperature maintained at 25 °C. The mobile phase (delivered at 0.2 ml/min) consisted of solvent A (methanol) and solvent B (1% formic acid), using the following gradient elution procedure: 0–3.4 min, 20–50% A; 3.4–13.4 min, 50–70% A; 13.4–21.7 min, 70–80% A; 21.7–22 min, 80–98% A; 22–24 min, 98–98% A; 24–24.4 min, 98–20% A; and 24.4–26.7 min, 20–20% A. The mass spectrometer was optimized in V mode with the following parameters: source temperature, 100 °C with a cone gas flow of 20 L/h; desolvation gas temperature, 200 °C with a maintained gas flow of 400 L/h; capillary voltage, 2000 V; and sample cone voltage, 100 V. Finally, the raw output was processed in Masslynx 4.1 (Waters, USA).

2.4. Identification of putative ecdysterone binding target proteins

Chemmapper (http://lilab.ecust.edu.cn/chemmapper, see also [16]) was used to predict the likely targets of ecdysterone binding. This method yields predicted targets for every query molecule based on the similarity of the query molecule to sets of known ligands of a given target molecule. Ligand sets for the target are given a fitness threshold score, which the query molecule must exceed for a positive result. The similarity threshold value for a positive result in this study was ≥ 1.2. Finally, the functions of target proteins with positive results were screened in Universal Protein Resource (UNIPROT) (http://www.uniprot.org).

2.5. Identification of common elements in neurological disease-related signal pathways

We downloaded four neurodegenerative disease signal pathway charts (Parkinson's disease, Alzheimer's disease, Huntington's disease, and ALS) from the KEGG pathway database, and then compared them to identify novel common elements that may be important roles in the development or relief of the diseases. These elements were then compared with previously validated signaling cascades [17–19], and those elements not found in the previously validated cascades were excluded.

2.6. Molecular docking and molecular dynamics simulations (MDs)

The initial X-ray crystal structure of human GRIN2B with co-crystallized ligand (PDB ID: 3QEM) was downloaded from Protein Data Bank (PDB) (http://www.rcsb.org). The structures of the 20 component ecdysterones found in RCI were sketched using minimized energy conformations in Chem 3D Ultra 12.0 [20]. To identify potential antagonists of this subunit, docking ability for each ligand was analyzed in Discovery Studio (DS) 3.5 (Accelrys, USA), using the CDOCKER protocol [21]. Initially, a 5 Å grid extension was set and 500 random conformations for each ligand were generated. These conformations were then automatically translated into receptors and moved into the binding sphere to search for their appropriate binding modes. Based on