Experimental and Molecular Pathology 99 (2015) 220–228

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

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

Protective effect of a novel Rho kinase inhibitor WAR–5 in experimental autoimmune encephalomyelitis by modulating inflammatory response and neurotrophic factors

Yan-hua Li [a], Jie-zhong Yu [a], Yan-le Xin [a], Ling Feng [a], Zhi Chai [b], Jian-chun Liu [b], Hong-zhen Zhang [b], Guang-Xian Zhang [d], Bao-guo Xiao [a,c,*], Cun-gen Ma [a,b,**]

[a] Institute of Brain Science, Department of Neurology, Medical School, Shanxi Datong University, Datong 037009, China
[b] "2011" Collaborative Innovation Center/Research Center of Neurobiology, Shanxi University of Traditional Chinese Medicine, Taiyuan 030619, China
[c] Institute of Neurology, Huashan Hospital, Institutes of Brain Science and State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai 200025, China
[d] Department of Neurology, Thomas Jefferson University, Philadelphia, PA 19107, USA

ARTICLE INFO

Article history:
Received 12 June 2015
Accepted 19 June 2015
Available online 23 June 2015

Keywords:
Experimental autoimmune encephalomyelitis
Rho-kinase
Y-27632 derivatives
NF–κB signaling

ABSTRACT

The Rho-kinase (ROCK) inhibitor Fasudil has proven beneficial in experimental autoimmune encephalomyelitis (EAE). Given the small safety window of Fasudil, we are looking for novel ROCK inhibitors, which have similar or stronger effect on EAE with greater safety. In this study, we report that WAR–5, a Y-27632 derivative, alleviates the clinical symptoms, attenuates myelin damage and reduces CNS inflammatory responses in EAE C57BL/6 mice at an extent similar to Fasudil, while exhibits less vasodilator and adverse reaction in vivo. WAR–5 inhibits ROCK activity, and selectively suppresses the expression of ROCK II in spleen, brain and spinal cord of EAE mice, especially in spinal cord, accompanied by decreased expression of Nogo. WAR-5 also regulates the imbalance of Th1/Th17 T cells and regulatory T cells, inhibits inflammatory microenvironment induced with NF–κB-IL-1β pathway. Importantly, WAR–5 converts M1 toward M2 microglia/macrophages that are positively correlated with BDNF and NT-3 production. Taken together, WAR–5 exhibits therapeutic potential in EAE by more selectively inhibits ROCK II, with a greater safety than Fasudil, and is worthy of further clinical study to clarify its clinical value.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system (CNS), resulting in oligodendrocyte loss, progressive destruction of myelin sheaths and axonal damage (Kutzelnigg and Lassmann, 2014). The disease-modifying drugs (DMDs) are often used for the relapsing forms of MS and have multiple benefits: (1) decreases in the frequency and severity of relapses: (2) less disability or sustained improvement; (3) well maintained or improved quality of life; and (4) less MRI lesion burden in the CNS (Goodin, 2008; Goodin et al., 2002; Pandit and Murthy, 2011).

ROCK activity is considerably increased in several neurological disorders such as stroke, spinal cord injury, MS and Parkinson's disease (Forgione and Fehlings, 2014; Mueller et al., 2005) and involved in the regulation of the cytoskeleton through downstream regulation of actin, myosin, and associated proteins (Julian and Olson, 2014). Therefore, the role of ROCK inhibition has also been studied in animal models of certain CNS diseases. The inhibition of ROCK also enhanced myelin formation in co-cultures of human OPCs and neurons and remyelination in rat cerebellar tissue for the induction of remyelination in demyelinating pathologies (Paintlia et al., 2013; Pedraza et al., 2014). In EAE models, the inhibition of ROCK ameliorates the severity of clinical score, accompanied by the protection of demyelination and decrease of neuroinflammation in the CNS (Liu et al., 2013; Sun et al., 2006; Yu et al., 2010)

A plethora of different pharmacological inhibitors of ROCK have been synthesized, including Y-27632, a member of the 4-aminopyridine series, and Fasudil, a member of the isoquinoline series (LoGrasso and Feng, 2009). Our group and others have found that Fasudil ameliorated the development of EAE, possibly by blocking inflammatory responses in the CNS, promoting neuroprotection and axonal regeneration (Hou et al., 2012; Li et al., 2014; Liu et al., 2013; Zhao et al., 2015). However, Fasudil in clinical practice has the following limitations, including a relatively narrow safety window, not suitable for long-term use and poor oral bioavailability. As a result, clinical application of Fasudil in the above-mentioned diseases, especially chronic neurodegenerative diseases, is limited. A ROCK inhibitor that effectively suppresses EAE with less side-effect is required.

* Correspondence to: B.-G. Xiao, Institute of Brain Science, Department of Neurology, Medical School, Shanxi Datong University, Datong 037009, China.
** Correspondence to: C.-G. Ma, Collaborative Innovation Center/Research Center of Neurobiology, Shanxi University of Traditional Chinese Medicine, Taiyuan 030619, China.
E-mail addresses: bgxiao@shmu.edu.cn (B. Xiao), macungen2001@163.com (C. Ma).

http://dx.doi.org/10.1016/j.yexmp.2015.06.016
0014-4800/

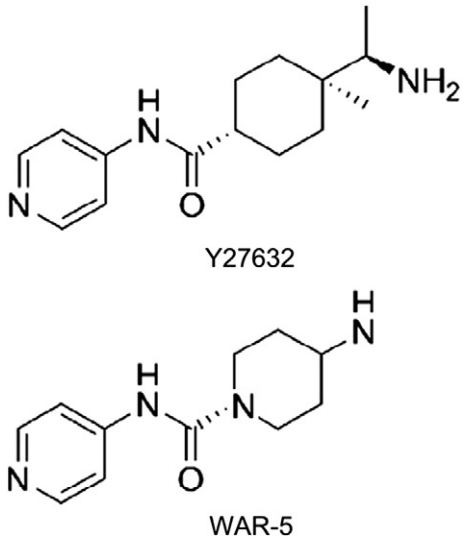

Fig. 1. The structural formula of WAR–5 and Y-27632.

In this study, we synthetized a novel ROCK inhibitor WAR–5, which may more selectively inhibit ROCK II, and investigated its therapeutic potential and possible neuroprotective and anti-inflammatory mechanisms in the treatment of EAE.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (8–10 weeks and 18–20 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice were housed under pathogen-free conditions and kept in a reversed 12:12-h light/dark cycle in a temperature-controlled room (25 ± 2 °C) for one week prior to experimental manipulation.

All animal protocol was performed according to the International Council for Laboratory Animal Science guidelines. The study was conducted by the Council for Laboratory and Ethics Committee of Shanxi Datong University, Datong, China.

2.2. Preliminary preclinical safety assessment of WAR–5 and Fasudil

Female C57BL/6 mice were injected i.p. with various doses (40, 80, 160, 240 mg/kg/d) of Fasudil or WAR–5 for 5 days. Mouse survival, appearance or general behavior was evaluated. The general locomotor activities of mice in different groups were also monitored by an open-field test. Mice were placed in the centre of an open behavioral chamber; before recording, mice were allowed to freely explore the chamber for 15 min to get used to the space in order to reduce novelty-induced stress; then their spontaneous activity was analyzed for 30 min by an automated tracking system and distance traveled were recorded.

2.3. Induction and clinical evaluation of EAE

Mouse myelin oligodendrocyte glycoprotein peptide35-55 (MOG_{35-55}, MEVGW YRSPFSRVVHLYRNGK) was synthesized in an automatic synthesizer (CL. Bio-Scientific. Company, Xi'an, China). Purity of the peptide was >95% as determined by HPLC. Complete Freund's adjuvant (CFA) consisted of incomplete Freund's adjuvant (Difco) plus 1 mg/ml heat-inactivated Mycobacterium tuberculosis (strain H37 RA; Difco).

Chronic EAE was induced by subcutaneous immunization on the upper dorsal flanks with 250 μg of MOG_{35-55} in Freund's complete adjuvant (Sigma, USA) supplemented with 1 mg/ml of M. Tuberculosis H37Ra (BD Difco, USA) (350 μg/mice). Mice were injected with 500 ng of pertussis toxin (Enzo Life Sciences, USA) via abdominal cavity at the same time of immunization and again 48 h later. Animals were evaluated for clinical score daily (from day 8 to day 28 p.i.) in a blinded fashion by at least two investigators. Clinical score of EAE was graded according to the following criteria: 0. healthy; 1. limp tail; 2. ataxia and/or paresis of hind limbs; 3. paralysis of hind limbs and/or paresis of forelimbs; 4. tetraparalysis; and 5. moribund or death. Once the clinical score of EAE reached 3, we provided special care, i.e., softening the food with water in a dish, adding nutrients such as egg, and putting the dish at the bottom of the cage, making it easy for mice to obtain food, water and nutrition.

2.4. Administration of Fasudil or WAR-5

Mice were divided into 3 groups, i.e., WAR-5-treated (WAR-5 group), Fasudil-treated (Fasudil group) and saline (NS) treated control group (EAE group) (n = 15 each group). Fasudil or WAR-5 (from Tianjin Chase Sun Pharmaceutical Co., Ltd) were dissolved in NS and injected intraperitoneally (i.p.) at 40 mg/kg/d every day on day 3 post-immunization (p.i.) till day 27 p.i. NS was injected to the control group in a similar manner.

2.5. Assay of ROCK activity

On day 28 p.i., to minimize pain, mice were sacrificed with 15 mg/kg pentobarbital, and brain, spinal cord and spleen were homogenized on ice in four volumes of an appropriate extraction buffer (50 mM Tris–HCl, pH 8.0, 0.1% triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 10 mM NaF, 10 mM beta-mercaptoethanol), and centrifuged for 30 min at 12,000 *g* to pellet the insoluble membrane/organelle fraction. ROCK activity in the supernatant fraction was measured by The CycLex Research Product ROCK assay kit (cyLex Co., Ltd, Nagano, Japan), which is used to determine ROCK activity in tissue cytosols and cell extracts following the manufacturer's instructions. Results were expressed OD value of absorbance at 450 nm.

2.6. Histology and immunohistochemistry

On day 28 p.i., mice were perfused with saline and 4% buffered paraformaldehyde. Brains and spinal cords (lower thoracic–lumbar) were sliced (10 μm), and pathological changes were detected by hematoxylin and eosin (H&E) staining and Luxol Fast Blue (LFB) staining. For immunohistochemistry, non-specific binding was blocked with 1%

Table 1
WAR–5 is safer than Fasudil in mice.
The general locomotor activities of mice in different groups were monitored by an open-field test. Statistical evaluation of mice treated with different concentration of Fasudil and WAR–5 respectively, including the percentage of survival, distance traveled (CM) for the 3 experiments (n = 3–9 mice/group/experiment) presented as mean ± SEM.

Dose	40 mg/kg/d		80 mg/kg/d		160 mg/kg/d		240 mg/kg/d	
Drug	Fasudil	WAR–5	Fasudil	WAR–5	Fasudil	WAR–5	Fasudil	WAR–5
Survival%	100	100	73 + 5.23	100	65 + 2.07	100	31 + 1.89	100
CM	1000 + 123	1300 + 267	400 + 25	1259 + 358[a]	200 + 10	1456 + 115[a]	50 + 13	1350 + 258[a]

[a] Significant ($p < 0.001$) as compared to Fasudil group with the same dose.