European Journal of Medicinal Chemistry 54 (2012) 439–446

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

New synthesis and promising neuroprotective role in experimental ischemic stroke of ONO-1714

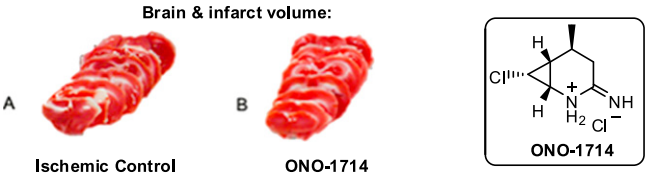
Andrea Pozo-Rodrigálvarez [b], Ana Gradillas [a], Julia Serrano [b], Ana Patricia Fernández [b], Ricardo Martínez-Murillo [b,*,1], Javier Pérez-Castells [a,**,1]

[a] Departamento de Química, Facultad de Farmacia, Universidad San Pablo CEU, Urbanización Montepríncipe, 28668 Boadilla del Monte, Madrid, Spain
[b] Neurovascular Research Group, Department of Molecular, Cellular and Developmental Neurobiology, Instituto Cajal (CSIC), Av. Doctor Arce 37, 28002 Madrid, Spain

HIGHLIGHTS

- New synthesis of selective iNOS inhibitor ONO-1714.
- ONO-1714 is potentially effective as therapeutic intervention in stroke.
- Results suggest ONO-1714 is a neuroprotective molecule in the context of brain ischemia.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 13 February 2012
Received in revised form
16 May 2012
Accepted 22 May 2012
Available online 29 May 2012

Keywords:
Focal ischemia
Infarct size
iNOS
Neuroprotection
ONO-1714

ABSTRACT

In an experimental permanent stroke model, we report here the contribution of ONO-1714 to brain damage prevention. Daily drug administration, twenty-one days prior to and two days after an experimental infarct, was performed by using mini-osmotic pumps (ALZET). Infarct volumes were assessed by image analysis of sequential coronal brain 1 mm^3 sections stained following the 2,3,5-triphenyltetrazolium chloride histological staining technique. Results of this study provide evidence of a significant reduction of the brain lesion size, suggesting ONO-1714 as a potential neuroprotective agent in stroke patients. ONO-1714 was prepared in our laboratory following a procedure which resulted in the supply of the desired compound in an easy and excellent yield.

1. Introduction

Stroke is one of the main causes of death and a major cause of long-term disability in the western world. More than 80% of all strokes are caused by cerebral ischemia [1], resulting in devastating neurological sequelae accompanied by severe morphological and molecular alterations [2]. Many advances in the understanding of the mechanisms of ischemic brain injury have been done and many pharmacological approaches to protecting the brain have been considered [3,4]. There are many evidences that inflammation, among other contributing factors, plays an important role in the outcome of ischemic stroke (IS). It is accepted that inflammatory mediators, such as nitric oxide (NO), contribute to brain damage [4]. Since stroke is common and current drug therapies for the management of stroke patients are limited, the progress toward the identification of new targets and the development of more effective new drugs has become a challenging task for prevention and early management of patients [4,5]. Actually, more than 50 neuroprotective agents have been evaluated in promising Phase III clinical trials with disappointing results. Restoration of blood flow needs to be achieved as quickly as possible. Yet, only intravenous administration of the tissue plaminogen activator (tPA), a clot-

* Corresponding author. Tel.: +34 915854714; fax: +34 915854754.
** Corresponding author. Tel.: +34 913724936; fax: +34 913510496.
E-mail addresses: r.martinez@cajal.csic.es (R. Martínez-Murillo), jpercas@ceu.es (J. Pérez-Castells).
[1] These two authors contributed equally to this work as co-directors and should be considered last authors.

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2012.05.031

dissolving agent, has been proven to be effective. However, to reap the full benefits of tPA, there is a very short window of opportunity for drug administration [4].

Studies with animals and humans provided increasing knowledge on the mechanisms of cell death following IS, including excitotoxicity, calcium ion [Ca^{2+}] overload, free-radicals, inflammation, and apoptosis [4]. In the past decade several studies have examined the role of the vasodilator mediator NO and its synthesizing enzyme system Nitric Oxide Synthase (NOS) in cerebrovascular diseases, including stroke [6]. NO is an intercellular messenger present in all vertebrates, modulating blood flow, thrombosis, and neural activity. Basically, there are three isoforms of the NOS: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS), each one involved in specific events in the brain. The vascular endothelium synthesizes NO through the action of eNOS, constitutively active, calcium dependent, that generates NO in response to shear stress and other physiological stimuli, and iNOS, transcriptionally regulated, calcium independent, which is produced in response to cytokines and endotoxin signals. Acute expression of highly reactive mediators, such as iNOS, and matrix metalloproteinase-9 (MMP9), participates in brain damage after stroke [6,7]. The reaction of NO with superoxide (O_2^-) to form the much more powerful oxidant peroxynitrite ($ONOO^-$) is a key element in resolving the contrasting roles of NO in physiology and pathology [8]. When ischemia has been developed, overproduction of NO generated by the neuronal or inducible isoforms (nNOS, iNOS) is neurotoxic, thus contributing to brain injury and responsible for the progression of brain damage [9]. This probably occurs through NO-induced formation of peroxynitrite and toxic free radicals leading to damage by lipid peroxidation [10]. NO over-expression has been also reported to stimulate the release of the neurotransmitter glutamate, thus contributing to excitotoxicity [11]. In striking contrast, NO derived from the eNOS isoform is beneficial because plays a prominent role in preventing neuronal injury by maintaining cerebral blood flow [12].

Taking together, available data define iNOS as an important player in IS, and also that pharmacological manipulations of the NO levels in the area of infarct by regulating iNOS activity might prevent neuronal injury and neurological deficits [5]. It has been found that selective iNOS inhibitors significantly reduced infarct volume, which is in striking contrast with non-selective inhibitors that were ineffective. ONO-1714, a cyclic amidine derivative and selective and highly potent inhibitor of rat and human iNOS ($Ki = 1.8$ nM) with a 10-fold selectivity over eNOS, has appeared as a newly developed competitive NOS inhibitor that has selective potency for iNOS [13], but few data has been reported regarding the neuroprotective effect of this compound [14].

ONO-1714 was prepared in our laboratory following a procedure which resulted in the supply of the desired compound in an easy an excellent overall yield. *In vitro* experiments carried out in this study assessed the efficiency of ONO-1714 on the inhibition of NO production in lipopolysaccharide (LPS) and interferon-gamma (INF-γ) stimulated mouse peritoneal macrophages, and on iNOS activity assay. In addition, the effect of ONO-1714 on the pathophysiology of stroke was explored in a model of permanent (p) middle cerebral artery (MCA) occlusion (pMCAO) in mouse. The pMCAO model is accepted as a pre-clinical experimental ischemic model for the evaluation of potential neuro-protective agents in preclinical assays [15,16]. Following this procedure, the results of this study suggest ONO-1714 as a potential neuroprotective agent in stroke patients.

The structure of ONO-1714 is shown in Scheme 1 and it was obtained following a procedure which involved an adaptation of the procedure described by Kawanaka [17], which was thwarted by our initial inability to cleavage satisfactoriously the remaining protecting group, *N*-PMB (*N*-*p*-methoxybenzil), that they proposed. All efforts for the deprotection step proved to be unsuccessful, so that we initiated a similar route shown in Scheme 1, with certain modifications with regard to Kawanaka's one, which resulted in the supply of the desired compound in an easier and better overall yield.

2. Results and discussion

2.1. Chemistry

The new synthesis proposed in this paper required the preparation of the optically active enamide (−)-**1** which synthesis is already described in the literature [18,19]. Thus, the cyclopropanation of (−)-**1** with $CHCl_3$ under alkaline conditions resulted in (−)-*anti*-**2** (45%) (the newly introduced cyclopropane moiety and the methyl moiety showed *anti*-stereochemistry) and (−)-*syn*-**2** (12%) (the newly introduced cyclopropane moiety and the methyl moiety showed *syn*-stereochemistry) respectively, as a separable mixture by column chromatography on silica gel [20].

The mayor isomer (−)-*anti*-**2** was reduced, to remove one chlorine, with tin hydride resulting in an inseparable mixture consisting of an endo-isomer, (−)-*anti-cis*-**3** and an exo-isomer, (−)-*anti-trans*-**3** (2:1) (74%). The mixture obtained was then transformed into the corresponding thioamido derivatives by treatment with Lawesson's reagent. We were pleased to obtain the corresponding mixture of isomeric thiolactams, which could be efficiently separated, by column chromatography on silica gel, to give (−)-*anti-cis*-**4** (48%) and (−)-*anti-trans*-**4** (18%) [21]. In addition, easily removal of the Dmob group from (−)-*anti-cis*-**4** with TFA provided (−)-*anti-cis*-**5** in 70% yield. The deprotected thioamide was then stirred with a saturated solution of NH_3 in methanol to yield the corresponding amidine **6** which was converted into its corresponding hydrochloride salt after acidification of the crude product with HCl—CH_3OH.

The transformation of the lactam group into the thiolactam system has revealed a convenient way to improve isomer separation and deprotection efficiency. The synthesis of amidine **6** by deprotection of (−)-**3** and afterward transformation of the amide group into thioamide was discarded as the deprotection of a mixture of 3 isomers only provided 35% yield of the corresponding amide.

In view of this efficient optimization we have extended this methodology to the synthesis of several compounds structurally related to **6** with replacement for the chloro group on the cyclopropane ring with different substituents such as $CONH_2$ [18], COOEt [19], CF_3 [18], dichloro, all in racemic and optically active forms. After being transformed into their corresponding amidine hydrochloride salts they were biologically evaluated for their ability to inhibit the iNOS. Replacement of the chloro group showed always a marked reduction of iNOS inhibition [18,19].

2.2. Biological results

2.2.1. Biological in vitro results

Endogenous iNOS inhibition was spectrophotometrically analyzed by using the Griess assay [22]. To confirm the effect of ONO-1714 on NO production, *in vitro* experiments were conducted using a LPS and INF-γ stimulated mouse peritoneal macrophage model [22]. As shown in Fig. 1A, the treatment with LPS (100 ng/mL) and INF-γ (100 ng/mL) markedly increased the production of NO from the basal level following 48 h incubation. When cells were simultaneously treated with various concentrations of ONO-1714, 100 μM, 10 μM, 1 μM and 0.1 μM, and LPS/INF-γ, NO production was significantly down-regulated in a dose-dependent manner. Characteristically, progressive reduction of nitrite-containing in