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Synthesis and biological evaluation of strained unusual amino acid containing tetrapeptides as potential antidepressant agents

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ABSTRACT

Strained unusual amino acid derived tetrapeptides were synthesized as mimics of GLYX-13, a clinical candidate for neuroprotective and anti-depressant properties, were studied. The synthesized compounds were screened for neurite growth and anti-depressant properties *in vitro* and *in vivo* respectively comparing with the parent GLYX-13 compound. Neurite growth property was assessed by neurite length and anti-depressant property by percentage of immobility in forced swim test, a behavioural assay. Mechanistic insights about protein–ligand interactions were obtained using molecular docking study. Based on the *in vitro* and *in vivo* screening data and molecular docking study, a new analogue of GLYX-13, Compound 11a has been found to be as good as the parent compound in all respects.

1. Introduction

Many neuroactive peptides are employed by the brain and nervous system of mammals which help in specialized signalling with central nervous system (CNS). A careful study of the signalling pathways has led to the identification of some specific receptors modulated by these neuroactive peptides which opened up an important therapeutic area in CNS disorders. The *N*-methyl-D-aspartate receptor (NMDAR) is one such receptor which is reported to be involved in various disorders such as cognitive function associated with ageing, ischemia, stroke, head trauma, spinal cord injury, epilepsy, depression, schizophrenia etc. [1]. NMDARs are widely distributed in brain and spinal cord; higher densities are found in cortex and hippocampus. NMDAR ion channels are one of the postsynaptic targets of glutamate-mediated synaptic transmission in mammalian brain. For opening, NMDAR channels require binding of two different types of agonists-glutamate and glycine [2]. Many glutamate receptors are found in CNS but strychnine-insensitive site is unique to NMDAR [3]. Drugs, which are capable of blocking NMDAR activation and glutamate binding site antagonists, create some strong side-effects. As a result, attention has shifted to the glycine site agonists for altering the NMDAR, which is a potentially important target for development of cognitive enhancing therapeutics and anticonvulsant and neuroprotective agents.

The glycine site of NMDAR complex is a co-agonist site with affinity for glycine and D-serine. It is an allosteric site and hence has been explored for identifying and developing safer drugs. The advantage of partial NMDAR agonists is that they block excessive NMDA function and also potentiate NMDAR in case of abnormally depressed NMDA-mediated neuro-transmission. D-Cycloserine [4] and glycine prodrug, milacemide [5] have shown cognitive-enhancing properties *in vivo* but result in desensitization with chronic administration.

Monoclonal antibody B6B21 has been shown to act as a partial agonist at the glycine site of NMDAR. The hyper variable region of the light chain of B6B21 was cloned and sequenced [6]. Based on this sequence information several peptides were synthesized and screened using rat hippocampal membrane preparations to measure [3H]MK-801 binding in presence of 7-chlorokynuric acid, a glycine site-specific competitive inhibitor of NMDAR. Peptides that increased the binding of [3H]MK-801, an open channel blocker, in dose dependent manner were named Glyxins [7]. Glyxins NT-1 to NT-18 were synthesized which comprised of peptides containing amino acids up to 26. Of these, GLYX-13 (NT-13) [22] (**1**, Fig. 1) was found to be the best. GLYX-13 is a tetrapeptide (Thr-Pro-Pro-Thr) [23] and is known to readily cross the blood–brain barrier. Clinical data showed that it has robust anti-depressant activity [8], the effect is immediate (within 20 min) and long lasting (at least

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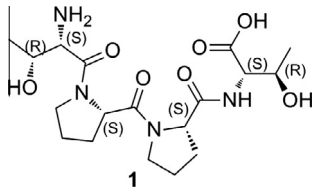

Fig. 1. Structure of parent compound GLYX-13.

for 2 weeks after single dose) with no CNS-related side-effects [9]. It was also found to enhance learning (cognitive function) [10], help in autism treatment and increase neuroprotection in cases of stroke [11]. GLYX-13 was able to modulate NMDAR in a glycine-like fashion when examined pharmacologically and electro-physiologically.

Based on the biological screening data of Glyxins, the optimal length of the peptides was found to be four. We have been working in the area of synthesis of peptides and foldamers consisting of unusual strained β-amino acids and their structural investigations [12]. In addition, the incorporation of a strained β-amino acid derived from sugar into a marine peptide natural product, azumamide, for enhanced activity [13] and many peptides with unusual amino acids have been reported [13]. Inspired by this observation, GLYX-13 skeleton was chosen as a starting point in the synthesis of β-amino acid analogues of GLYX-13, **1**, to understand the SAR. A first look at **1** revealed the presence of turn-inducing Pro–Pro dimer as the central core. This prompted us to initiate incorporation of *cis*-furan sugar amino acid, **2** [12] and *cis-exo*-β-norbornene amino acid, **3** and **4** [12] as substitutes of central Pro–Pro core of **1** (Fig. 2). These unusual amino acids have already been proven to be turn inducers as observed by us earlier [12]. The terminal amino acids in **1** are threonine and are expected to participate in hydrogen-bonding. Based on this observation we initiated incorporation of turn-inducing amino acid pairs as core and substituted the terminal amino acids, threonine, with 2-aminobenzoic acid. A preliminary molecular modelling study data indicated that insertion of sugar amino acids will not be able to induce the required turn for the expected activity as they exhibit formation of helical structures [12,13]. Thus the focus then shifted to inserting norbornene amino acid which is a known turn inducer [12]. To determine the impact of stereochemistry on the neuroprotective activity both the isomers of norbornene amino acid *viz*., (2*R*,3*S*)-*cis-exo*-β-norbornene amino acid, **3** and (2*S*,3*R*)-*cis-exo*-β-norbornene amino acid, **4**, were used.

2. Result and discussion

2.1. Chemistry

The work on the analogue synthesis began with retention of the proline–proline core with modification in the terminal amino acids. Thus a set of four analogues were synthesized incorporating glycine, **5**, 4-aminobenzoic acid **6**, β-alanine **7** and 2-aminobenzoic acid **8**, following usual peptide synthesis protocols (Fig. 3). Biological screening of these compounds did not yield encouraging results (data not shown). Therefore further attempts of analogue synthesis were shifted to modifying the turn-inducing core.

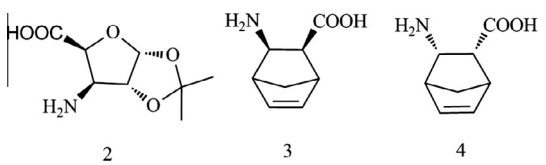

Fig. 2. *cis*-Furan sugar amino acid and *cis-exo*-norbornane amino acid.

Enantiopure norbornene amino acid (both enantiomers *exo*-(2*S*,3*R*) and *exo*-(2*R*,3*S*) individually) were used to prepare the analogues **11a**, **11b**, **13a** and **13b** [12] *N*- and *C*-Protected dimers (**9a** and **9b**) were prepared from both 2*S*,3*R*- and 2*R*,3*S*-*cis-exo*-β-norbornene amino acid, **3** and **4**, independently using our earlier protocol [12]. These dimers are used to synthesize the proposed analogues **11a**, **11b**, **13a** and **13b** by attaching the appropriately protected monomer units of L-threonine and 2-aminobenzoic acid. The trimers were prepared *via* classical peptide forming HOBt–EDCI protocol which involves coupling of amine of the dimer (readily obtained from dimer **9a** and **9b** by treating with trifluoroacetic acid) and *N*-Boc protected L-threonine *N*-Boc-2-aminobenzoic acid respectively. Similarly, tetramers were prepared from trimer acids coupled with methyl esters of L-threonine and 2-aminobenzoic acid (Scheme 1). All the analogues along with the parent compound **1** were evaluated for their biological efficacy.

2.2. Biological evaluation

GLYX-13 (**1**), a compound approved by FDA in Fast Track designation on 3rd March 2014 as an adjunctive therapy in treatment of resistant major depressive disorder. This molecule completed phase IIb clinical trials and phase III trials will start in 2015. A major problem associated with **1** is its bioavailability and hence it is administered via intraperitoneal route to undergo first-pass effect which is bypassed in intravenous route. As the molecule has shown great promise, analogues are planned which are less prone to enzymatic degradation and increase its bioavailability. The GLYX-13 (**1**) and its analogues **11a**, **11b**, **13a** and **13b** were preliminarily screened *in vitro* on Neuro-2a cell line (ATCC CCL-131) [14–16] for neurotrophic activity at 0.01, 0.1 and 1.0 μM concentrations and a concurrent MTT cell cytotoxic assay was carried out to substantiate the compounds concentrations are non-toxic (Supplementary Fig. 1 and Supplementary Table 1). All the analogues except **11b** showed noticeable neurotrophic activity at the lowest 0.01 μM concentration (Fig. 4 and Supplementary Table 2). But, the *BDNF* gene expression did not show consistent changes in analogous with the neurite length (Supplementary Fig. 4). This data suggests that the neurotrophic action of **1**, an NMDA receptor agonist, and its analogues might not be directly via activation of *BDNF* gene. Further the analogues were screened *in vivo* for their anti-depressant activity and were compared with **1** using a behavioural assay, Forced Swim Test [17,18] (Fig. 5).

This assay indicated that compounds **11a** and **11b** had activity similar to **1**. With this indication, we explored compatibility of both **11a** and **11b** to NMDA receptor which could be mediated through GRIN2B or NR2B receptor as **1**, based on the literature precedence [9]. Neuro-2a cells were treated with 1.0, 0.1, 0.01 and 0.001 μM concentrations of **11a**, **11b**, **1** and DMSO. All the three has shown increased GRIN2B gene expression levels at 0.01 μM concentration when compared to other. Further GRIN2B gene expression of **1, 11a** and **11b** at 0.01 μM were compared and **1** and **11a** induced a significant up regulation compared with DMSO treated (Fig. 6). The data suggests that **11a** and **11b** work on the same pathway as 1, viz., as NMDA receptor agonist and 11a induced more GRIN2B gene expression than others.

After analysing both *in vitro* and *in vivo* results GLYX-13 analogue **11a** seems to be the best lead molecule to consider for further development as a novel antidepressant as this molecule showed significant neurotrophic activity at 0.01 μM concentration as well with the noticeable improvement in immobility time in forced swim test as compared to vehicle treatment (Figs. 4 and 5). The present study also suggests the compound **11a** might be working as NMDA receptor agonist like parent compound, GLYX-13, in a better way at mRNA gene expression levels. A protein level work would have further substantiated the mRNA results. It also