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Synthesis, conformational and pharmacological studies of glycosylated chimeric peptides of Met-enkephalin and FMRFa

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Abstract

Our previous study showed that a chimeric peptide of Met-enkephalin and FMRFamide, YFa (YGGFMKKKFMRFa) not only caused antinociception and potentiated morphine analgesia but also blocked the development of tolerance and physical dependence. In the continuation of that study three chimeric analogues of YFa, [Ser^5]YFa, [*O*-Glu-Ser^5]YFa and [*O*-Gal-Ser^5]YFa, were synthesized. To increase the bioavailability and penetration of blood brain barrier (BBB), glycosylated analogues, [*O*-Glu-Ser^5]YFa and [*O*-Gal-Ser^5]YFa, have been synthesized by solid phase peptide synthesis by building block method using anomeric acetate activation method. Circular dichroism studies showed that all the three chimeric peptides are stable and have a propensity for adopting helical conformation in the presence of membrane mimicking solvent. In comparison of parent chimeric peptide YFa, helicity of [Ser^5]YFa, [*O*-Glu-Ser^5]YFa and [*O*-Gal-Ser^5]YFa has decreased. Pharmacological studies using tail-flick latency in mice showed that [*O*-Glu-Ser^5]YFa have increased analgesia and bioavailability in comparison of [*O*-Gal-Ser^5]YFa and non-glycosylated analogue [Ser^5]YFa. Exhibition of enhanced analgesia by [*O*-Glu-Ser^5]YFa as compared to [*O*-Gal-Ser^5]YFa seems to be due to preference of GLUT-1 transporter system for glucose.

Keywords: Chimeric peptides; Met-enkephalin; FMRFa; Bioavailability; Glycosylated peptides

1. Introduction

Bioavailability of peptide-based drugs to the brain is limited due to poor metabolic stability, or inability to cross the blood brain barrier (BBB) [5]. Peptide-based drugs and neuromodulators, administered peripherally, fail significantly to affect their target cells within the brain [17] because BBB that is characterized by high electrical resistance and low paracellular diffusion [11], excludes most of the peptides from reaching the brain [16]. A number of strategies have been used to improve the uptake of peptides through the BBB [2,22] and one of them is conjugation of the peptide with glucose moiety that may function as transport vector [17,4].

Glycosylation has proven to be a useful methodology for enhancing biodistribution of peptides to the brain. Improved analgesia has been reported for glycosylated deltrophin [14] and morphine [15]. A number of different sugar moieties have been investigated including glucose, galactose and xylose [4]. The improved analgesia exhibited by glycosylated-opioids may be due to increased bioavailability [18], reduced clearance [6] or improved BBB transport [4].

The role of opioids and antiopioids in modulation of antinociception becomes intriguing by endogenously present naloxone sensitive opioid Methionine-enkephalin-Arg^6-Phe^7 (MERF) [10]. MERF consists of sequence of opioid Met-enkephalin (at N-terminal) and Arg^6-Phe^7, a dipeptide that is an integral part of FMRFa/NPFF family of antiopioid peptides (at C-terminal) [7] and it binds to multiple opioid binding sites [1,12]. Based on MERF, two chimeric peptides of Met-enkephalin and FMRFa, YGGFMKKKFMRFamide (YFa) and [D-Ala^2]YAGFMKKKFMRFamide ([D-Ala^2]YFa) were designed and synthesized.

In our previous studies [8,9], it was demonstrated that intraperitoneal (i.p.) administration of YFa and intracerebroventicular (i.c.v.) administration of [D-Ala^2]YFa induced a naloxone reversible dose-dependent increase in tail-flick latency

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in mice, showing an antinociceptive effect due to involvement of opioid receptors. The chimeric peptides also potentiated morphine-induced antinociception and attenuated the development of tolerance to the antinociceptive action of morphine. This suggested that, besides opioid receptors, YFa and [D-Ala2]YFa are probably behaving as putative antagonists and not letting endogenous antiopioids bind to their receptors or they are down regulating antiopioid receptors by activating Gi proteins.

Polt et al. [17] showed that L-serinyl β-D-glucoside analogues of [Met^5] enkaphalin are transported across the BBB to bind to the opioid receptors and show enhanced analgesia as compared to non-glycosylated peptides. So to enhance the biodistribution and to study the effect of glycosylation on chimeric peptides, three serine-based analogues of YFa were designed in which Met^5 has been replaced by Ser^5 since position five can tolerate change without loss of activity [13]. Three analogues of YFa are as follows:

(i) YGGFSKKKFMRFamide ([Ser^5]YFa), wherein the Met^5 position has been changed with Ser^5 that can act as a site for glycosylation.
(ii) [*O*-Glu-Ser^5]YFa in which β-D-glucose is attached to Ser^5 so as to utilize GLUT-1 transport system for the transport of chimeric peptide across the BBB.
(iii) [*O*-Gal-Ser^5]YFa in which β-D-glucose is replaced by β-D-galactose at Ser^5 so that a comparison can be made between the two glycosylated analogues of chimeric peptides on their peripheral administration.

These analogues were characterized by MALDI-Tof and conformations of three analogues of YFa were compared by circular dichroism (CD) studies. Pharmacological studies were performed by tail-flick method via intra peritoneal (i.p.) administration of all the three analogues in mice.

2. Materials and methods

2.1. Synthesis of glycosylated chimeric peptides

2.1.1. Chemical synthesis of building block of *N*-α-(9-fluorenylmethylcarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine [or *N*-α-(9-fluorenylmethyl-carbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-serine] by activation of anomeric acetate

The Lewis acid [$BF_3{\cdot}Et_2O$, 7.2 mmol] was added to a solution of pentaacetate glucose [2.3 mmol] and Fmoc-Serine [3 mmol] in CH_2Cl_2. The mixture was stirred for 1 h and the solution was then washed with aqueous HCl (1 M), dried and concentrated (Scheme 1). The residue was purified by HPLC.

2.1.2. Solid-phase synthesis of glycopeptides

Peptides [Ser^5]YFa, [*O*-Glu-Ser^5]YFa and [*O*-Gal-Ser^5]YFa were assembled on the Rink amide methylbenzylhydramine resin (0.56 mmol/g substitution). All the solid phase reactions were carried out manually in a sintered glass tube. Peptide condensation was facilitated by using excess Fmoc amino acid (4 eq.) activated with DIPCDI, HOBt in *N*,*N*′-dimethylformamide. At the fifth position instead of Fmoc-Ser-OH, *N*-α-(9-Fmoc)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine [or *N*-α-(9-Fmoc)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-L-serine], synthesized in previous step, was introduced for the synthesis of glycopeptides. Stepwise removal of *N*-Fmoc protecting group from growing chain of peptides was achieved by 20% piperidine in DMF. Synthesized glycopeptides were released from resin by treating with TFA (95%) and the reaction was quenched with ethanedithiol and crystalline phenol. After cleavage glycosylated chimeric peptides were deacetylated by dissolving the peptides in dry methanol (10 ml) followed by addition of methanolic sodium methoxide (6 M) till the pH of the solution reached 10. After 30 min at room temperature, the peptide solution was concentrated under reduced pressure. Products were purified by HPLC on semi preparative reverse phase column using isocratic gradient of acetonitrile (0.05%TFA):water (0.05%TFA)::70:30. The correct peptide sequences were confirmed by automated peptide sequencing (490 Applied Biosystems).

2.2. Mass spectrometry

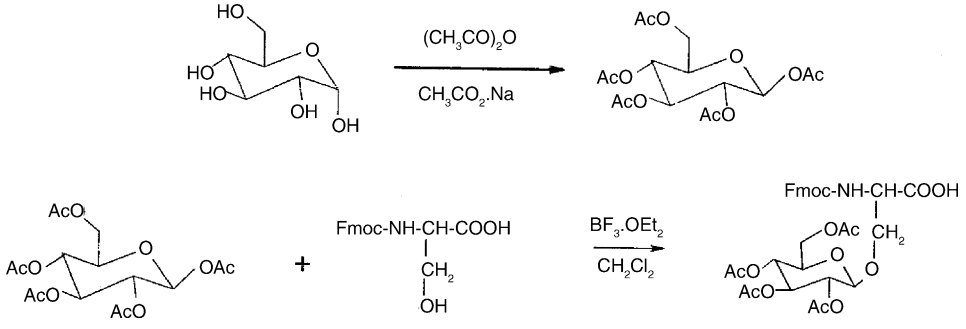
The mass analysis of three chimeric peptides [Ser^5]YFa, [*O*-Glu-Ser^5]YFa and [*O*-Gal-Ser^5]YFa was carried out using MALDI-Tof (Kratos) in the positive ion mode using α-cyano-4-hydroxycinnamic acid as the matrix.

2.3. Circular dichroism studies

The conformational studies were done by CD (Jasco J-715). CD spectra of YFa, [Ser^5]YFa, [*O*-Glu-Ser^5]YFa and [*O*-Gal-Ser^5]YFa (1 mmole) were recorded in 3.3 mM Sodium chloride-Sodium citrate buffer with varying concentrations of trifluoroethanol (TFE) by using spectropolarimeter calibrated with D-10-camphorsulfonic acid in a cell of 0.5 pathlength at room temperature. CD band intensities are represented as mean residue ellipticity.

2.4. Pharmacological studies

For study of antinociception, a locally made analgesiometer was used. The inhibition of tail-flick response was expressed as percentage maximum possible effect (%MPE) that was calculated as $[(T_1 - T_0)/(T_2 - T_0)] \times 100$ where T_0 and T_1 were the tail-flick latency before and after the injection of peptide and T_2 was the cut-off time. The antinociceptive response was measured by the radiant tail-flick test as described previously [8,9]. At the beginning of the study, the intensity of heat stimulus in the tail-flick apparatus was adjusted so as to elicit a response in control or untreated animal within 3–5 s. To minimize tail skin tissue damage, the cut-off was set at 10 s. Seven mice were used for each treatment



Scheme 1. Synthesis of *N*-α-(9-fluorenylmethylcarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine by anomeric acetate activation method.