Molecular and Cellular Probes 32 (2017) 5–12

Contents lists available at ScienceDirect

Molecular and Cellular Probes

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

Serum based fluorescent assay for evaluating dipeptidyl peptidase I activity in collagen induced arthritis rat model

Xiaoqian Liu [1], Jingjing Wang [1], Yi Chu, Xiaoying Zhou*

School of Pharmaceutical Engineering and Life Science, Changzhou University, 213164, Jiangsu, China

ARTICLE INFO

Article history:
Received 10 September 2016
Received in revised form
18 October 2016
Accepted 18 October 2016
Available online 19 October 2016

Keywords:
DPPI activity
Rheumatoid arthritis
Serum based assay
Fluorescent probe

ABSTRACT

Dipeptidyl peptidase I (DPPI) is a lysosomal cysteine protease and derived from immune granule cells. It has been suggested playing an important role in the development of rheumatoid arthritis. In this study, a coumarin based fluorescent probe (GF-AFC) was designed and synthesized to evaluate DPPI activity in serum or tissue homogenates of collagen-induced arthritis (CIA) rats, an inflammatory arthropathy model. It was revealed that the fluorescent intensity was significantly increased in a very short time after specific substrate GF-AFC reacted with the DPPI. The fluorophore (AFC) was released to shine after the cleavage reaction which was examined by ^{19}F NMR spectroscopy. It has been shown that DPPI hydrolyzed the GF-AFC in a robust, linear, and time dependent manner at a significant high rate. A serum-based DPPI activity assay was validated by spiking and gradient dilution methods, there were no interferences or auto-fluorescence observed. The Coefficient of Variance calculated for serum-based DPPI activity assays indicates the good reproducibility. The good correlation has been seen between serum DPPI levels and the severity of arthritis during RA development in CIA rats. Our study has demonstrated a new serum based diagnostic assay for detecting DPPI activity using coumarin conjugated fluorescent (GF-AFC) as a substrate. The successful implementation of the case would provide beneficial experience in rheumatoid arthritis research.

1. Introduction

As a novel putative drug target for treatment of inflammatory disease, dipeptidyl peptidase I (DPPI) constitutively expressed in a variety of tissues, such as: lung, serum, liver and spleen [1,2]. It is a class of lysosomal cysteine protease which functions as a key enzyme in the activation of granule serine protease in cytotoxic T lymphocytes, mast cells and neutrophils [3–5]. DPPI can remove dipeptides from the N-termini of peptides substrates and proteins with broad specificity [6,7].

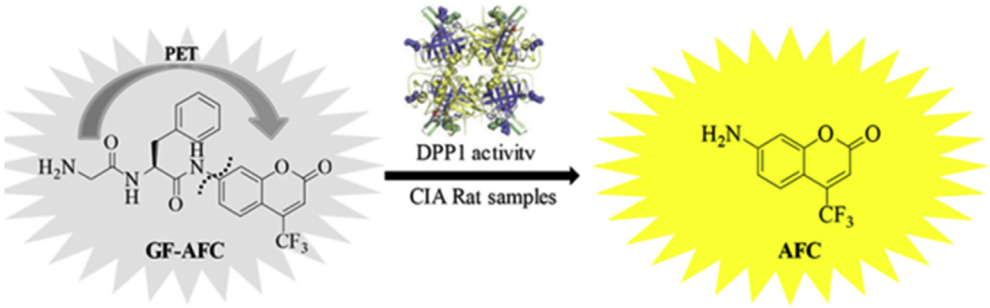
Rheumatoid arthritis (RA) is difficult to manage, although various methods have been used to treat this disease [8,9]. As a model for inflammatory arthropathy study, collagen-induced arthritis (CIA) shares many similar morphological features to human RA particularly in synovium. It has been widely used as an animal model in RA research since 1977 [10–12]. Some researches have shown that DPPI involved in the development of collagen-induced arthritis, and acted as an indicator reflecting the severity of rheumatoid arthritis [13,14].

Different synthetic probes containing fluorophores and dipeptides as target acceptors for DPPI enzyme were constructed [15–18]. Coumarin was considered as a suitable fluorophore because of its unique photo physical properties, such as: high quantum yield, high photo stability, visible excitation and emission wavelength [19–22]. Glycine-phenylalanine (GF) as dipeptide was selected to exclusively target the DPPI domain. There were a few examples reported using coumarin to construct probes for DPPI substrate to be applied for cell-based fluorescence DPPI activity assays [23]. However, to our best knowledge, there were no literature reporting to apply coumarin linked with glycine-phenylalanine dipeptides as a probe for the serum or tissue based DPPI activity assays.

In this study, a serum-based fluorescent assay has been newly developed. Unlike other different types of DPPs enzyme probe [24], a new coumarin conjugated fluorescent probe GF-AFC has been constructed specifically to evaluate DPPI activities in serum samples or different tissues of collagen-induced arthritis (CIA) rats (Scheme 1). The probe contains coumarin (AFC) as fluorophore and

* Corresponding author.
E-mail address: xiaoyingzhou@cczu.edu.cn (X. Zhou).
[1] Xiaoqian Liu and Jingjing Wang contributed equally to this work.

http://dx.doi.org/10.1016/j.mcp.2016.10.009
0890-8508/



Scheme 1. Structure of GF-AFC and schematic illustration of the method for evaluating DPPI activity.

specific dipeptide (GF) to give a "turn off" fluorescence signal; upon the cleavage by DPPI in CIA rat samples, the free coumarin (AFC) was released, resulting in a "turn-on" fluorescence signal. The more coumarin dyes released by DPPI enzyme, the higher fluorescent intensity there would be.

2. Materials and methods

2.1. Design and synthesis of GF-AFC

7-amino-4-(trifluoromethyl) coumarin, Boc-Phe-OH, dry pyridine and Boc-Gly-OH (Aladdin Industrial, Shanghai, China), I-hydroxybenzotriazole (HOBt) (Energy Chemical, Shanghai, China), Phosphorus oxychloride ($POCl_3$) and *N*, *N*-Diisopropylethylamine (DIPEA) (Sinopharm Chemical Reagent Co. Ltd., China). Trifluoroacetic acid (TFA) (Yonghua Chemical Technology Co. Ltd., Jiangsu, China), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (D&R Fine Chemical Co. Ltd., Shanghai, China) were used as received. Column chromatography was performed using 200–300 mesh silica gel (Wuse Chemical Inc., Shanghai, China). All the organic solvents were analytical grade and used without further purification. NMR spectra were recorded on NMR spectrometer (Bruker AVANCE III 400 M or Bruker AVANCE III 500 MHz, Switzerland).

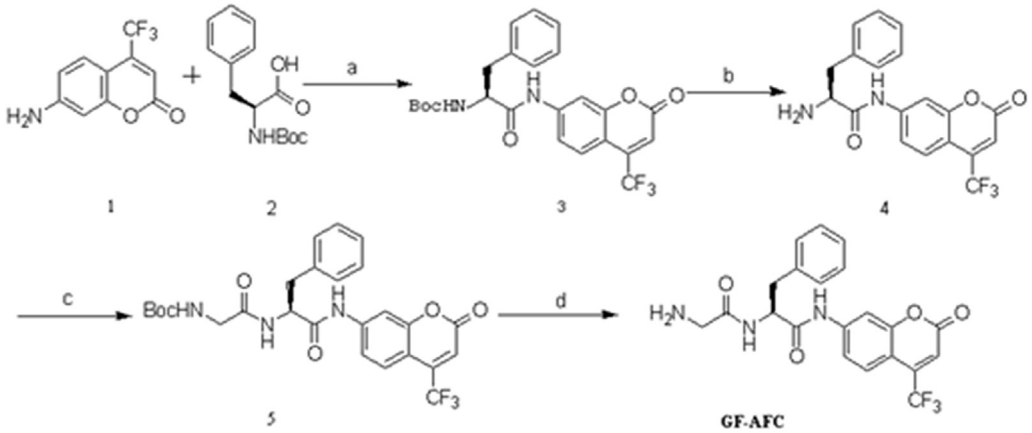
The synthetic pathway of GF-AFC probe is shown in Scheme 2. GF-AFC consists of two subunits: 7-amino-4-trifluoromethyl-coumarin (AFC) acts as a reporter group, while glycine-phenylalanine as dipeptide is targeting DPPI domain. The dipeptide sequence was suggested a much better substrate for DPPI as elucidated by the enzyme-crystal structure [17]. Also the free amino group in glycine terminus has been left deliberately as the necessary element to activate the enzyme [25,26]. GF-AFC synthesis started from amide coupling reaction between 7-amino-4-trifluoromethyl-coumarin (AFC) and Boc protected phenylalanine. After de-protection of Boc group, a second amide coupling was performed to link Boc protected glycine in the structure. The GF-AFC was finally obtained by second Boc de-protecting reaction in the acidic condition (Supplementary Scheme S1) as light yellow solid.

2.2. Absorption and fluorescence analysis in different pH conditions

All solutions for spectroscopic measurements were prepared using ultrapure water. Absorption spectra were recorded by UV spectrophotometer UH5300 (Hitachi) at room temperature with 1.0 cm quartz cells. GF-AFC was diluted with 0.5% (v/v) DMSO, then further diluted to 3 ml in different pH condition: acetic acid/sodium acetate pH 4.0; DPPI buffer pH 6.0; PBS pH 7.0; or ammonia/ammonium chloride pH 8.0 respectively at final concentration of 10 μM. AFC fluorophore alone was diluted with DPPI buffer pH 6.0–10 μM as a control. Fluorescence scan (rang 450 nm-800 nm) was carried out to determine the wavelengths for excitation and emission of AFC or GF-AFC probe. Fluorescence spectra were recorded on LS55 (PerkinElmer) and the slits for excitation and emission were both set to 10 nm.

2.3. ^{19}F nuclear magnetic resonance (^{19}F NMR) to determine the chemical shift

The GF-AFC (0.5 mM) solutions mixed with or without DPPI were prepared, and the chemical shifts between these two solutions were monitored by ^{19}F NMR. The reactions were operated in



Scheme 2. Synthesis route to GF-AFC. Reagents and conditions: a) $POCl_3$, pyridine, −15 °C, 15min; b) Trifluoroacetic acid, DCM, rt, 4 h; c) Boc-Gly-OH, HOBt, EDCI, DIPEA, DMF, rt, overnight; d) EtOAc, 6 N HCl, 4 h.