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Novel Substrates for Determination of the Fibroblast Activation Protein-α Activity

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Fibroblast activation protein-α (FAP- α) is a membrane-associated serine protease of the S9b family of post-proline cleaving enzymes. It is usually expressed in reactive stromal fibroblasts in many types of diseases connected with extensive pathological alterations of the connective tissue like arthritis, fibroses, carcinomas and sarcomas. That is why the enzyme is considered a valuable marker for those entities. Design and development of specific FAP-α substrates are rather challenging due to the enzyme's structural similarity with the other proline-specific enzymes. In this paper we present the design of three novel substrates for the determination of FAP-α activity as well as the assessment of their efficacy and specificity. According to the obtained results, one of the newly developed substrates has a potential to be used as a highly specific substrate for FAP-α.

Key words: fibroblast activation protein-α, molecular modeling, enzyme substrate, substrate specificity

Introduction

Fibroblast activation protein-α (FAP-α; EC 3.4.21.B28) is a membrane-bound post-proline cleaving serine protease. It represents a 97 kDa glycoprotein existing as 170 kDa homodimer in its native form [15]. The enzyme hydrolyzes polypeptide substrates possessing Pro in P_1 position. It can act both as exo- and endopeptidase but is more efficient as an endopeptidase [14]. Some of the well-known enzyme`s natural substrates are collagen type I, neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY [9]. FAP-α is involved in normal processes like tissue remodeling during the embry-

onic development, wound healing, etc. However, normal adult human and mammalian tissues do not express FAP-α [18]. Otherwise, the enzyme is highly induced in many diseases such as rheumatoid arthritis and osteoarthritis, liver and pulmonary fibrosis and in cancer [reviewed in 11]. It is expressed mostly by reactive stromal fibroblasts but has also been found in certain types of tumor cells [5, 11]. Many studies have shown that FAP-α participates in the mechanisms of tumor growth, angiogenesis and inhibition of the antitumor immune response [12, 17]. Studies on the enzyme activity in norm and pathology need the application of highly selective substrates [6]. However, the design of FAP-α specific substrates is very difficult due to its close structural similarity with the other proline-specific enzymes [reviewed in 5].

In the present paper we describe the design and development of three novel substrates intended for the biochemical assays of FAP-α activity in tissue homogenates and/or cell lysates. Additionally, we present the assessment of these substrates' efficacy and selectivity.

Materials and Methods

Molecular modeling. Using the crystal structure of human FAP-α (Protein Data Bank ID: 1Z68), obtained by Aertgeerts et al. [1], we modeled the structure of the enzyme-substrate complex with isonicotinoyl-D-Ala-Pro-4-nitroanilide by Dreiding forcefield method [13].

FAP-α substrates. We synthesized, purified (recrystallization, high performance liquid chromatography) and analyzed (nuclear magnetic resonance, mass spectrometry) the following substrates: β-Ala-D-Ala-Pro-4-nitroanilide (AAP), β-Ala-Nle-Pro-4-nitroanilide (ANP) and isonicotinoyl-D-Ala-Pro-4-nitroanilide (IAP). The synthetic methods, substrates purification and spectral analyses will soon be published elsewhere.

Cell culturing. Three permanent cell lines were used – MCF-10A (normal immortalized human epithelial cells from mammary gland), MCF-7 (human tumor cells obtained from mammary gland carcinoma of low invasiveness) and MDA-MB-231 (human tumor cells from mammary gland carcinoma of high invasiveness). The cancer cells were cultured in 75 cm^2 tissue culture flasks in Dulbecco's Modified Eagle's Medium – high glucose 4.5‰ (DMEM), supplemented with 10% fetal calf serum and antibiotics in usual concentrations. Normal cells were cultivated in the same conditions but with the addition of 20 mg/l human epidermal growth factor (EGF), 0.5 mg/l hydrocortisone, 0.1 mg/l cholera toxin and 10 mg/l insulin. Cell cultures were maintained at 37.5 °C in a humidified atmosphere and 5% CO_2 until 95% confluence was achieved.

Biochemical assays. For the estimation of FAP-α activity towards different substrates, aliquots of human recombinant FAP-α (Enzo Life Sciences, Inc.) were incubated with 0.1 mM of the respective substrate in 0.1 M phosphate buffer (pH 7.4), containing 0.1 M NaCl and 1 mM EDTA at 37 °C. Enzyme assays were carried out in 96-well plates. Absorption of the samples at 405 nm was measured every 4 minutes on multifunctional spectrofluorimeter Varioscan. The results were statistically estimated by regression analysis and curves showing the time-dependence of the adsorption at 405 nm were built by means of EnzFitter V2. In the cases of non-linear correlation, the enzyme activity was determined from the initial rate of the reaction.

The cells were harvested by a rubber policeman and homogenized using homogenizer MSE (England) in 5 ml 0.1 M phosphate buffer (pH 7.4) with 0.1 M NaCl and 1 mM EDTA. After a spectrophotometric measurement of the protein amount [2], the samples were incubated with the above FAP-α substrates (0.1 mM) in the same buffer at 37 ºC. The enzyme reactions in the samples were followed and analysed as above.

Results and Discussion

In cancer, FAP-α is usually expressed by cancer-associated fibroblasts where it takes part in the hydrolyzes of collagen type I thus opening free spaces for tumor invasion and blood vessels formation, as well as in the inhibition of the immune system's antitumor activity [reviewed in 4, 6 and 11]. However, several studies have shown that in some types of tumors FAP-α can be a tumor suppressor, e.g. in melanomas and in non-small cells lung carcinomas [reviewed in 4 and 18]. Thus, the marker role of FAP-α needs to be clarified separately in the oncological diseases of different origin. For this purpose, highly specific substrates are required to determine the activity of the enzyme. Unfortunately, the design of selective substrates for FAP-α is a difficult task due to its close structural similarity with other proline-specific proteases. For example, FAP-α and dipeptidyl peptidase IV (DPPIV) have 50% identity in the entire amino acids sequence and 70% in the catalytic domain [3] (**Fig. 1**).

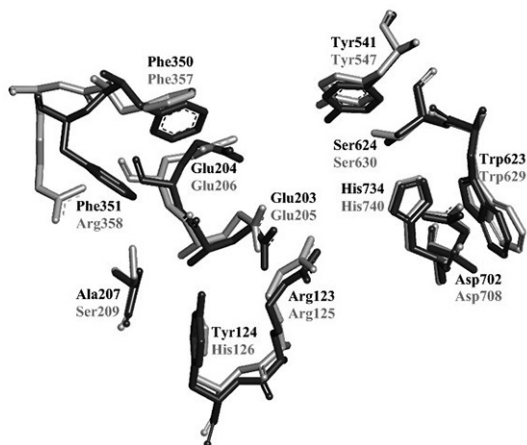

Fig. 1. Structural similarity between active centers of FAP-α (bolt) and DPPIV (pale). In the right site of the scheme, the catalytic triads of both enzymes are seen to coincide almost perfectly - Ser624, Asp702, His734 (FAP-α) and Ser630, Asp708, His740 (DPPIV). Tyr124 of FAP-α is very important for the substrates orientation. In DPPIV molecule, His126 is present in this position

The careful view of the entire active centers of the two enzymes shows that in FAP-α it is covered up mostly by non-polar amino acids, whereas in DPPIV it contains extra polar amino acids (not shown here). This fact explains why FAP-α is more efficient as an endopeptidase while DPPIV represents a typical exopeptidase. That is why, NH_2-Gly-Pro-based synthetic substrates commonly used to determine DPPIV and DPPIV-like enzymes' activities (DPP 8 and 9) are useless for the analyses of FAP-α activity. Further on, a commercial substrate for FAP-α is available – Z-Gly-Pro-7-amido-4-methylcoumarine. However, it is known to be cleaved also by other prolyl oligopeptidases (POPs).

Recently, several selective inhibitors (1, 2) and a selective substrate (3) for FAP-α were reported [7, 10, and 16] (**Fig. 2**).

All the above selective for FAP-α compounds contain isonicotinic or isoquinolinic acid residues connected with D-Ala.

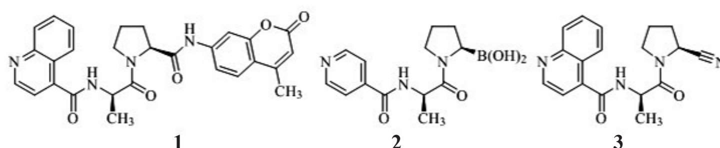

Fig. 2. FAP-α selective inhibitors N-(pyridine-4-carbonyl)-D-Ala-boroPro (1), N-(quinoline-4-carbonyl)-D-Ala-2-cyanopyrrolidine (2), and the selective substrate N-(quinoline-4-carbonyl)-D-Ala-Pro-4-methyl-7-coumaryl amide (3)

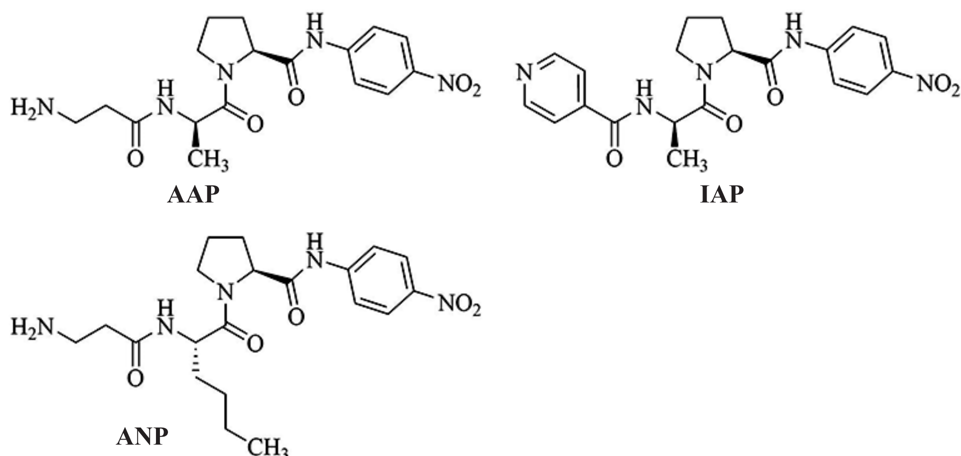

Fig. 3. Chromogenic substrates for FAP-α designed and synthesized by us: β-Ala-D-Ala-Pro-4-nitroanilide (AAP), isonicotinoyl-D-Ala-Pro-4-nitroanilide (IAP), and β-Ala-Nle-Pro-4-nitroanilide (ANP)

Based on the above studies, we designed and synthesized three novel chromogenic FAP-α substrates intended for biochemical assays of the enzyme activity (**Fig. 3**). Those substrates are as follows: isonicotinoyl-D-Ala-Pro-4-nitroanilide (IAP), which possesses the same N-(pyridine-4-carbonyl)-D-Ala - sequence as the compounds specific for FAP-α and listed above; β-Ala-D-Ala-Pro-4-nitroanilide (AAP) with a β-Ala- flexible moiety resembling the structure of isonicotinic acid and β-Ala-Nle-Pro-4-nitroanilide (ANP), possessing Nle at P_2 position which non-polar side-chain is expected to fit in the non-polar active center of FAP-α.

Molecular modeling showed that the cyclic nitrogen of the isonicotinic acid forms a hydrogen bond with the OH-group of Tyr124, thus stabilizing the enzyme-substrate complex and properly orientating the C=O-group of scissile bond towards the catalytic serine (**Fig. 4**).

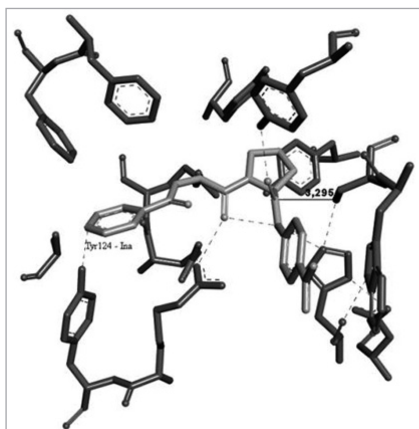

Fig. 4. Binding of the substrate isonicotinoyl-D-Ala-Pro-4-nitroanilide in the active center of FAP-α. The nitrogen atom of the cycle of isonicotinic acid forms a hydrogen bond with the OH-group of Tyr124

The principle of biochemical analyses of FAP-α activity is as follows: The enzyme cleaves the amide bond at the proline carboxyl group to liberate 4-nitroaniline, which is of yellow color and has maximum absorption at 405 nm. The enzyme activity can be estimated by the quantity of 4-nitroaniline liberated per minute per 1 mg protein at 37 ºC.

First, we studied the efficacy of every substrate to be hydrolyzed by the human recombinant FAP-α at the optimal conditions for the enzyme action (pH 7.4, 37 ºC in the presence of NaCl and EDTA). The results are given in **Fig.5**.

The results showed that FAP-α hydrolyzes all the three substrates but ANP is the most efficient, whereas IAP has the lowest efficacy. These outcomes are logical since Nle has a long non-polar side-chain which fits precisely in the enzyme active center. On the other hand, low efficacy of the substrates can be compensated by a

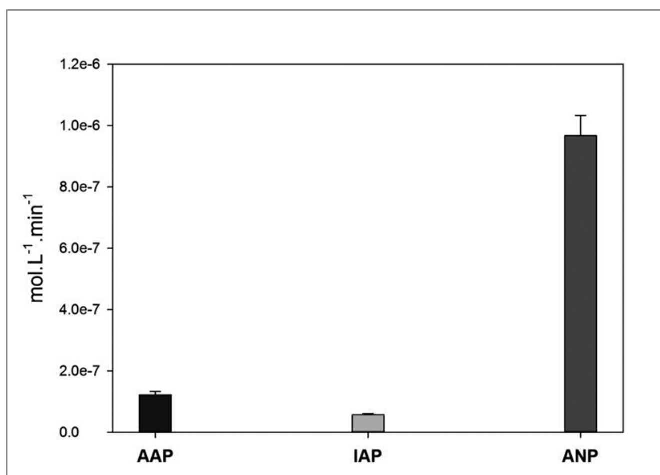

Fig. 5. Estimation of the efficacy of the newly synthesized FAP-α substrates to be cleaved by the enzyme

prolonged incubation, whereas their specificity may be crucial for the precise determination of the enzyme activity especially in heterogeneous mixtures like tissue homogenates or cell lysates.

Further, we tested the selectivity of our substrates in cell lysates using three cell lines: MCF-10A (normal immortalized human epithelial cells from mammary gland), MCF-7 (human tumor cells obtained from mammary gland carcinoma of low invasiveness), and MDA-MB-231 (human tumor cells from mammary gland carcinoma of high invasiveness). While MCF-10A has a low FAP-α activity, MCF-7 and MDA-MB-231 are usually used as negative controls since they are known to lack any enzyme activity [see e.g. 8]. According to the results (**Fig. 6**), the most efficient substrate – ANP has the lowest specificity towards FAP-α. Obviously, it is cleaved by a number of POPs and shows similar results in the three cell lines.

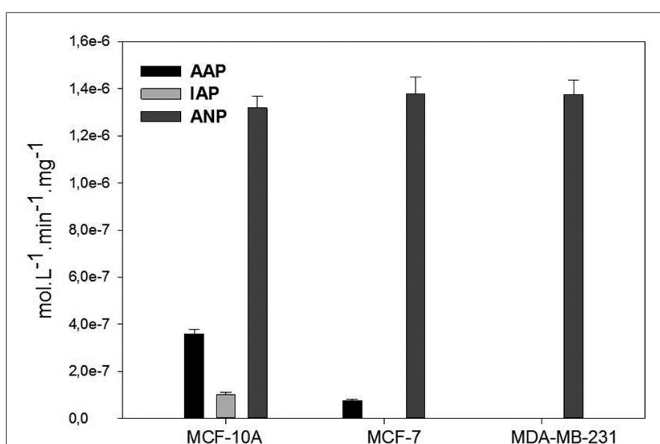

Fig. 6. Estimation of the specificity of the newly developed FAP-α substrates

The AAP substrate is more specific for it shows no FAP-α activity in MDA-MB-231. However, it demonstrates a cross-reactivity with some POP(s) in MCF-7 cell line. Finally, IAP can be considered as a highly specific substrate for the enzyme in this experiment.

Conclusion

We designed and synthesized three novel substrates for the determination of FAP-α activity – AAP, IAP and ANP. The last substrate is quickly and efficiently hydrolyzed by the enzyme and can be a useful tool to study the activity of isolated and purified FAP-α. Additionally, IAP although having a low efficacy to be cleaved by FAP-α, can be considered a highly selective substrate and can be valuable for the specific determination of the enzyme activity in heterogeneous mixtures such as tissue homogenates, plasma samples or cell lysates.

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