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Characterization of benzodiazepine binding site on human α_1-acid glycoprotein using flunitrazepam as a photolabeling agent

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Abstract

The binding of flunitrazepam (FNZP) by human α_1-acid glycoprotein (hAGP) and the relationships between the extent of drug binding and desialylation and the genetic variants of hAGP were examined. The photolabeling specificity of [^{3}H]FNZP was confirmed by findings in which other hAGP-binding ligands inhibited the formation of covalent bonds between [^{3}H]FNZP and hAGP. The photolabeling of asialo-hAGP suggested that sialic acid does not involve in the binding of [^{3}H]FNZP. No difference in the labeling could be found between the F1*S variants and A variant. Similarly, FNZP did not show a difference in binding affinity to the two genetic variants of hAGP. Sequence analysis of the photolabeled peptide indicated a sequence corresponding to Tyr91-Arg105 of hAGP.

Keywords: Human alpha 1-acid glycoprotein; Flunitrazepam; Binding site; Topology analysis; Photoaffinity labeling

1. Introduction

Human α_1-acid glycoprotein (hAGP), an acute phase protein in blood, consists of 183 amino acid residues and five N-linked oligosaccharides, with a molecular weight of 41–43 kDa [1]. It is negatively charged due to the presence of sialic acids in its glycan chains [2]. hAGP can be produced as three main genetic variants, the A variant and the F1 and S variants, which are encoded by two different genes [3]. The proportion of each variant are approximately F1 50%, S 35% and A 15% [4]. The relative occurrence of the three main phenotypes of native hAGP composition in the population was found to be about 50% for F1+S+A, 35% for F1+A and 15% for S+A [5]. There is a difference of at least 22 amino acid residues between the F1*S and A variants while the F1 and S forms differ by only a few residues [3,6]. F1*S and A variants also differ in the number of Met residues.

hAGP's basal level is approximately 20 μmol/L, but it can vary from 5- to a 10-fold range in response to stress, infection, or the effects of neoplasm in evocation of an inflammatory response [7]. In addition, changes in the expression of genetic variants of hAGP could also occur apart from its glycosylation pattern depending on the type of inflammation [8]. The biological function of this protein is not clear, though it has been reported to have an anti-inflammatory and immunomodulating role as well as protective effects [9].

In the clinical practice, hAGP is a valuable diagnostic and prognostic tool. For example, increased hAGP levels associated with advanced tumors altered the pharmacokinetics of Imatinib (STI571), a tyrosine kinase inhibitor, in leukemia patients [10]. hAGP also appears to be an independent predictor of response and a major objective prognostic factor of survival in patients with non-small cell

Abbreviations: hAGP, Human α_1-acid glycoprotein; FNZP, Flunitrazepam; PSL, photo stimulated luminescence

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lung cancer treated with docetaxel chemotherapy [11]. In addition, hAGP binds and transports a number of endogenous and exogenous compounds including various basic and neutral drugs influencing their pharmacokinetics and pharmacodynamics [12].

Benzodiazepines are clinically important central nervous system depressants with anxiolytic, sedative, antiepileptic and muscle relaxant therapeutic actions. The major binding protein in plasma for benzodiazepines has been identified as serum albumin. However, hAGP had been shown to bind benzodiazepines with comparable affinity by Maruyama et al. [13]. In a previous study, we have shown that diazepam was able to inhibit the photoincorporation of FNZP to hAGP but not to albumin, suggesting that certain benzodiazepines bind more readily to hAGP instead of albumin and may involve different binding mechanism [14]. During acute phase reaction, albumin synthesis is decreased while that of hAGP may increase. Thus, the pharmacokinetics of drugs that are bound by both of these two proteins may be altered [15].

Due to the clinical significance of hAGP binding of drugs, especially during acute phase reactions, the topology of its binding site has been investigated with a variety of methods including chemical modification, quantitative structure activity relationship (QSAR) methods and molecular modeling [16–18]. In a study of the binding of nine diazepines to hAGP by means of fluorescence and CD (circular dichroism) spectroscopies at our laboratory, we found that benzodiazepines have one high affinity binding site [13,19].

X-ray crystallographic analysis and nuclear magnetic resonance are the major modern direct techniques to elucidate the structures of macromolecules and their complexes at atomic resolution [20,21]. However, the application of these methods to hAGP–drug complexes is not feasible at present due to the great difficulty in the crystallization of this serum glycoprotein which comprises a mixture of variants as well as glycoforms, and also lacking of a suitable expression system that is able to produce a recombinant hAGP with correct physiologically related glycans.

Photoaffinity labeling is one of the most powerful techniques, which can be used to study a ligand binding protein [22,23]. In a recent study, we have shown that [^{3}H]FNZP (Fig. 1) was able to form covalent bond upon photoirradiation to hAGP which can be inhibited by diazepam [14]. This paper reports further attempt using [^{3}H]FNZP as a photolabeling agent to elucidate the topography of binding site amino acid sequence of hAGP and to shed light on the possible binding mechanisms.

2. Materials and methods

2.1. Materials

[^{3}H]FNZP (71 Ci/mmol) was purchased from PerkinElmer Life Sciences. hAGP (purified from cohn fraction VI) was purchased from Sigma Chemical Co. Sequencing grade modified trypsin was purchased from Promega Co. (USA). Cyanogen bromide (CNBr), dithiotreitol, trifluoroacetic acid, ammonium bicarbonate, and imidazole were purchased from Nacalai Tesque (Kyoto, Japan). 4-Vinylpyridine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Neuraminidase, resorcinol, propranolol, lidocaine, verapamil, disopyramide, dipyridamole, testosterone, indomethacin, and salicylic acid were purchased from Sigma. *N*-glycosidase F recombinant (PNGase F) was purchased from Roche (Germany). Progesterone was from Nihon Schering. FNZP, diazepam, fludiazepam (Sumitomo Pharmaceuticals), chlorpromazine, imipramine (Mitsubishi Wellpharma), warfarin (Eisai), and phenylbutazone (Ciba-Geigy Japan) were donated by the respective companies. Acenocoumarin was a gift from Dr. Janssen from the Faculty of Pharmacy, Utrecht University, the Netherlands. All other chemicals and solvents were of analytical grade.

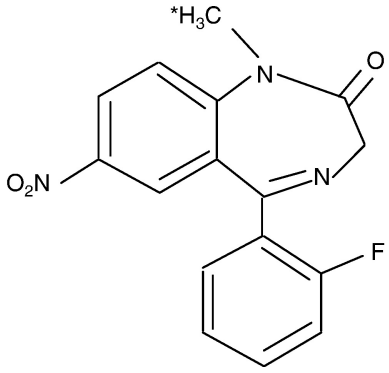

Fig. 1. Chemical structure of [^{3}H]FNZP. *: ^{3}H-labeled position.

2.2. Photoaffinity labeling of hAGP

hAGP (50 μM) was incubated with [^{3}H]FNZP (25 μM) in 100 μL of 20 mM Tris–HCl, pH 7.4, in a 1.5 mL Eppendorf tube at room temperature in the dark for 60 min. The incubation mixture was then placed on ice and irradiated for 30 min by a 100 W black light/blue lamp (Ultra-Violet Products, Inc., San Gabriel, CA, USA) at a distance of 10 cm. After irradiation, the photolabeled hAGP was precipitated by adding 1 mL of acetone, followed by centrifugation at 15×1000 rpm for 10 min. The pellet was washed with 1 mL of ethanol and centrifuged a second time.

2.3. Cyanogen bromide cleavage

For cyanogen bromide cleavage reactions, the pyridylethylated pellet was then dissolved in 100 μl of cyanogen bromide (CNBr) in 70% formic acid (CNBr:Met=400:1) and incubated under N_2 for 24 h in the dark at room temperature. One milliliter of milli-Q water was added at the end of the CNBr cleavage and the mixture was lyophilized.