Tryptophan Residues Play an Important Role in the Extraordinarily High Affinity Binding Interaction of UCN-01 to Human α-1-Acid Glycoprotein

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Purpose. To investigate the factors that contribute to the exceptionally high affinity binding of UCN-01 to human α_1-acid glycoprotein (hAGP).

Methods. Interactions between UCN-01, UCN-02, and staurosporine with native and chemically modified hAGPs were examined using ultracentrifugation and spectroscopic analysis.

Results. The binding affinity of staurosporine, as well as UCN-02, to hAGP was lower than that of UCN-01 by 20- and 100-fold respectively. The percentage of UCN-01 that binds to hAGP was low at acidic pH but increased with increasing pH, reaching a maximum at pH 7.4. The binding of UCN-01 to desialylated hAGP was comparable to that of hAGP. No significant difference was found for the binding of UCN-01 to F1*S and A variants of hAGP. Chemical modification of the His, Lys, Trp, and Tyr residues caused a decrease in percentage of bound UCN-01. Trp-modified hAGP showed the largest decrease in binding. Tryptophanyl fluorescence quenching results indicate that Trp residues play a prominent role in the binding of UCN-01 to hAGP.

Conclusions. A substituent at position C-7 of UCN-01 appeared to influence the binding specificity of the drug, and Trp residues in hAGP play a prominent role in the high affinity binding of UCN-01 to hAGP.

KEY WORDS: α_1-acid glycoprotein; binding site; drug disposition; UCN-01; variants.

INTRODUCTION

UCN-01 (7-hydroxystaurosporine, Fig. 1), a new anticancer drug with good inhibitory activity against protein kinase C (PKC) and cyclin dependent kinases (CDKs), is a hydroxylated derivative of staurosporine. It has been shown to inhibit the growth of various cultured human and solid tumor cells *in vitro* and *in vivo* (1–3). As a single agent, UCN-01 exhibits two key biochemical effects, namely the accumulation of cells in the G1 phase of the cell cycle and the induction of apoptosis. Both of these effects may be important for its anticancer activity. As a modulator, UCN-01 enhances the cytotoxicity of other anti-cancer drugs such as DNA-damaging agents and anti-metabolite drugs through the putative abrogation of G2 and/or S phase accumulation induced by these anti-cancer agents such as mitomycin C, cisplatin and 5-fluorouracil *in vitro* and *in vivo* (4).

Surprisingly, the pharmacokinetics of UCN-01 after administration in the form of a 72- or 3-h infusion to cancer patients displayed entirely distinctive features that would not have been predicted from the preclinical data. The pharmacokinetics of UCN-01 in experimental animals was characterized by rapid clearance with a large distribution volume. The volume of distribution at steady-state conditions (Vd_{ss}), the systemic clearance (CL_{tot}) and the half-life of elimination ($t_{1/2}$) in dogs, rats, and mice were all within the range of 6–17 L/kg, 0.6–4 L h^{-1} kg^{-1}, and 3–12 h, respectively. In contrast to the large distribution volume and rapid systemic clearance in experimental animals, the distribution volumes (0.0796–0.158 L/kg) and systemic clearance (0.0407–0.252 ml h^{-1} kg^{-1}) in the human patients were found to be extremely low. In addition, the elimination half-lives (253–1660 h) were unusually long (5).

This dramatic species difference with respect to the pharmacokinetics of UCN-01 in humans can, at least in part, be explained by specific high-affinity binding to hAGP (5). Steroid hormones and basic drugs are known to bind tightly to hAGP, with binding constants in the order of 10^6 m^{-1} (6). In comparison to the binding constant for canine AGP which is 1.3×10^7 M^{-1}, the binding constant for UCN-01 to hAGP, 8×10^8 M^{-1}, is the highest value ever reported to date. In contrast, no specific binding could be observed for rat AGP and human serum albumin (5). The results of a physiologic model, mimicking a clinical situation and using rats, suggest that the slow dissociation of UCN-01 from hAGP limits its disposition and elimination (7).

Being a member of the acute-phase protein family, the plasma concentrations of hAGP increase up to 5-fold in various acute-phase responses such as inflammation, cancer, stress, post-surgery, pregnancy and other clinical conditions (8). In addition, hAGP exists as a mixture of two or three genetic variants, namely the A and F1*S variants in the plasma of individuals (9). In the general population, three phenotypes are most frequently observed, namely, F1S/A, F1/A and S/A, depending on the presence of two or three of these variants in plasma. Figure 2 shows the amino acid sequence of the hAGP variants (10–12). The A variant and the F1*S variant differ in their amino acid sequence by at least 22 residues (8). Large differences in the binding of various drugs have been reported for the F1*S *vis-à-vis* the A variant (13), indicating a specific drug transport role for each variant.

Increases in circulating hAGP have been reported to alter the pharmacokinetic disposition and pharmacological action of numerous drugs that bind to it. For example, elevated hAGP is associated with a higher bioavailability, and lower systemic clearance and volume of distribution of HIV protease inhibitors such as saquinavir in AIDS patients (14). On the other hand, increased hAGP levels associated with advanced tumors altered the pharmacokinetics of Imatinib (STI571), a tyrosine kinase inhibitor, in leukemia patients (15). In addition to the fact that hAGP plasma concentrations are increased in certain types of cancers, changes in the expression of genetic variants of hAGP could also occur according to the specific type of cancer (16). Hence, hAGP appears to be an important modulator of drug pharmacokinetics and pharmacodynamics.

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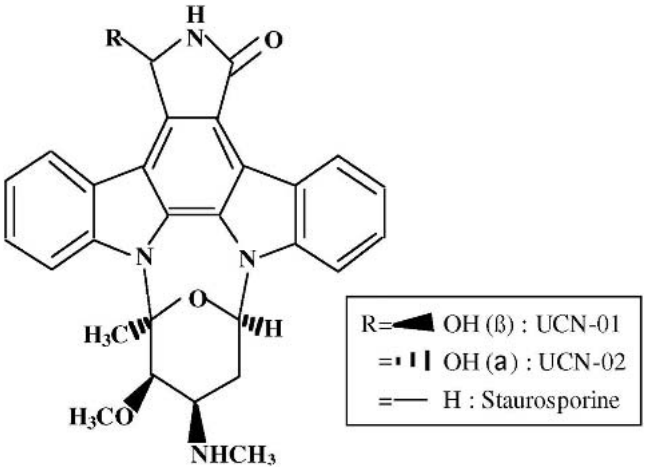

Fig. 1. Chemical structures of UCN-01, UCN-02 and staurosporine.

Since at present no crystallographic structural data for hAGP is available, it becomes important to examine the extraordinarily high affinity binding mechanism between UCN-01 and hAGP using established methods. In this study, we report on the binding interactions between UCN-01 and related compounds with native and chemically modified hAGP using ultracentrifugation and spectroscopic analysis to detect these interactions.

MATERIALS AND METHODS

Materials

UCN-01, UCN-02 and Staurosporine were supplied by Kyowa Hakko Kogyo Co. (Shizuoka, Japan). hAGP (purified from cohn fraction VI) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phenylisocyanate and 2-hydroxy-5-nitrobenzyl bromide (HNBB) were purchased from Nacalai Tesque (Kyoto, Japan). Tetranitromethane (TNM) was purchased from Aldrich Chemical Co., Milwaukee, USA. Diethylpyrocarbonate (DEP) and neuraminidase was purchased from Sigma Chemical Co. All other chemicals and solvents were of analytical grade.

Determination of Binding Constant

The binding of UCN-01 to hAGP was determined by an ultracentrifugation technique (17). A constant concentration of hAGP (4 μM) was incubated with different concentrations of UCN-01 (3.2–8 μM). Five ml of an hAGP solution containing UCN-01 was placed in siliconized tubes, along with SIGMACOATE (Sigma), incubation for 10 min on ice, and then ultracentrifuged at 225,000 × g for 24 h at 4°C. After ultracentrifugation, an aliquot (50 μl) from the top of the supernatant was used to determine the free UCN-01 concentration by HPLC, as reported previously (18). The unbound fractions were obtained from the supernatant.

Binding parameters were determined by fitting the experimental data to the following Scatchard equation using a non-linear squares program (MULTI program) (19).

$$\frac{r}{D_f} = nK - rK \quad (1)$$

where n is the number of binding sites, K the binding constant, and D_f the free drug concentration, with r denoting the moles of bound ligand per mole of total protein.

Determination of Binding for UCN-01 to hAGP

Percent binding was calculated from the following equation:

$$\text{bound}\ (\%) = \frac{[D_t] - [D_f]}{[D_t]} \times 100 \quad (2)$$

where D_t is the total concentration of UCN-01, the hAGP concentrations were set at 20 μM, and D_f is the free concentration of UCN-01.

Preparation of hAGP Derivatives

Trp-Modified hAGP

The three Trp residues of hAGP were modified by HNBB following the procedure of Fehske *et al.* (20). 40 mg of hAGP was dissolved in 10 ml deionized water adjusted to pH 4.5 with acetic acid. One milliliter of an ethanol solution of HNBB was added to 10 ml of this solution. HNBB was added (100-fold molar excess of hAGP) and the reaction mixture was shaken occasionally. After 2 h the insoluble hydrolyzed reagent was separated by centrifugation. The supernatant was dialyzed against deionized water for 60 h and then lyophilized. The degree of modification was determined using ultraviolet absorption, as shown below.

$$\text{modified}\ (\%) = \frac{A_{410} \times 44100 \times 0.4980}{13800\ (A_{280} - 0.167 \times A_{410})} \times 100 \quad (3)$$

where A_{410} is the maximal absorbance of Trp-modified hAGP, and A_{280} is the maximal absorbance of unmodified hAGP. Of the 3 Trp residues of hAGP, about 1 was modified.

Tyr-Modified hAGP

Modification of the Tyr residues was performed at 4°C according to the procedure of Sokolovsky *et al.* (21). hAGP was dissolved in 10 ml of 0.05 M Tris buffer with a pH of 8.0. A 500-fold molar excess of TNM dissolved in ethanol was added to the hAGP solution. The degree of modification was calculated from the following equation:

$$\text{modified}\ (\%) = \frac{A_{428}}{4100 \times c \times m} \times 100 \quad (4)$$

where A_{428} is the maximal absorbance of Tyr-modified hAGP, c the protein concentration, and m the number of Tyr

10	20	30	40
QIPLCANLVP	VPITNATLDQ	ITGKWFYIAS	AFRNEEYNKS
QIPLCANLVP	VPITNATLDR	ITGKWFYIAS	AFRNEEYNKS
50	60	70	80
VQEIQATFFY	FTPNKTEDTI	FLREYQTRQD	QCIYNTTYLN
VQEIQATFFY	FTPNKTEDTI	FLREYQTRQN	QCEYNSSYLN
90	100	110	120
VQRENGTISR	YVGGQEHFAH	LLILRDTKTY	MLAFDVNDEK
VQRENGTVSR	YLGGREHVAH	NLELRDTKTL	MEGSYLLDEK
130	140	150	160
NWGLSVYADK	PETTKEQLGE	FYEALDCLRI	PKSDVVYTDW
NWGLSEYADK	PETTKEQLGE	TYEALDCLCI	PRSDVMYTDW
170	180		
KKDKCEPLEK	QHEKERKQEE	GES (F1*S)	
KKDKCEPLEK	QHEKERKQEE	GES (A)	

Fig. 2. Amino acid sequence of hAGP variants. Differences in the amino acid sequence between the F1*S and A variant are underlined.

residues on an hAGP molecule. Of the 11 Tyr residues of hAGP, about 2 were modified.

Lys-Modified hAGP

Chemical modification of Lys residues was carried out using phenylisocyanate (22). 40 mg of hAGP was dissolved in 10 mL 0.067 M phosphate buffer at pH 7.4. One milliliter of an ethanol solution of phenylisocyanate was added gradually at a 300-fold molar excess over hAGP and the reaction mixture was incubated for 2 h at 4°C, dialyzed against deionized water for 40 h, and then lyophilized. The unreacted Lys residues were determined by the trinitrobenzene sulfonic acid procedure of Haynes *et al.* (23). Of the 14 Lys residues of hAGP, about 4 were modified.

His-Modified hAGP

The His residues were modified with DEP according to the procedure of Roosemont *et al.* (24). 30 mg of hAGP was dissolved in 10 ml of acetate buffer (pH 6.5, 100 mM), DEP in ethanol was then added to the hAGP solution. The ratio of DEP to hAGP was 10. The mixture was stirred for 20 min at 4°C and dialyzed against deionized water and lyophilized. The number of His residues modified was calculated using the following equation:

$$\text{modified}\,(\%) = \frac{A_{240} \times \dfrac{3\ \text{ml}}{\text{ml of test solution}}}{\Delta\varepsilon \times c \times m} \times 100 \qquad (5)$$

where $\Delta\varepsilon$ is the differential molar absorptivity for His at pH 6.0, c the protein concentration and m the number of His residues on hAGP. A_{240} is the maximal absorbance of His-modified hAGP. Of the 3 His residues in hAGP, about 2 were modified.

Separation of hAGP Genetic Variants

The hAGP genetic variants were separated using the method of Herve *et al.* (25). An iminodiacetate (IDA) Sepharose gel loaded with copper (II) ions and equilibrated in buffer 1 (20 mM sodium phosphate buffer, pH 7.0, containing 0.5 M sodium chloride) was packed into a glass column (2.0 × 30.0 cm L, Pharmacia LKB). Commercial hAGP (70 mg dissolved in 1.0 ml of buffer) was applied to the column at a flow rate of 1.0 ml/min. Fractions (10 ml) were collected, and their respective absorbance values were determined spectrometrically at 280 nm. As soon as variant A had been eluted, elution buffer 2 (buffer 1 plus 20 mM imidazole) was applied to the column to remove the bound F1 and S variants (F1/S mixture). The peak fractions of each eluent were collected, concentrated on a YM 10 membrane filter (Amicon, Danvers, MA), dialyzed against deionized water, and lyophilized. The purities of the isolated hAGP preparations were determined by isoelectric focusing (IEF) followed by incubation at 37°C for 24 h with 1 U of neuraminidase (25).

Desialylation of hAGP

hAGP was desialylated enzymatically, using the methodology described by Primozic and McNamara (26), using an immobilized neuraminidase obtained from *Clostridium perfringens*. hAGP (40 mg) was dissolved in 5 ml of 0.067 M phosphate buffer (pH 7.4) and 2 U of enzyme were added. The mixed solution was incubated at 37°C, with gentle stirring at 60 rpm for 24 h after incubation, the mixture was centrifuged and filtered to remove the immobilized enzyme. A small part of the filtrate was used to measure sialic acid by the thiobarbituric acid method. The product was dialyzed against deionized water and the dialysate was lyophilized. Approximately 95% of the sialic acid was removed, leaving an average of one sialic acid residue per protein molecule. The molecular weight of desialylated hAGP was therefore 40000.

Circular Dichroism (CD) Spectra

Circular dichroism spectra were recorded with a JASCO J-720 spectropolarimeter, using 10 μM hAGP in 20 mM buffer at 25°C. Near-UV spectra were recorded using a 10-mm path length cell, and a 0.1-mm path length cell was used for far–UV spectra. Prior to recording the spectra, samples were mixed by vortexing and then incubated for 30 min at room temperature. The mean residue ellipticity [θ], was calculated using the following equation:

$$[\theta] = \frac{\theta}{10 \times c \times 1} \qquad (6)$$

where θ (deg) is the observed ellipticity, l the cell length (cm).

$$c = nCp \qquad (7)$$

where n is the number of amino acid residues and Cp the concentration (mol/L).

Tryptophanyl Fluorescence Spectrum

Fluorescence was measured using a Jasco FP-770 fluorometer (Tokyo, Japan). The excitation wavelength of hAGP was 295 nm, and fluorescent wavelength, 340 nm. The relative fluorescence intensity was calculated using the fluorescence quenching method.

Statistical Analysis

All data are presented as means ± SD. Statistical analysis of difference was determined by one-way ANOVA followed by the modified Fisher's least sequares difference method.

RESULTS

Binding Parameter of UCN-01 Analogs to hAGP

UCN-01 is a novel derivative of staurosporine that is hydroxylated C-7, and UCN-02 is a stereoisomer at C-7 of UCN-01. UCN-01, UCN-02 and staurosporine have been shown to inhibit PKC with IC50 (μM) values of 0.0041, 0.062 and 0.0027, respectively, and to inhibit protein kinase A (PKA) with IC50 (μM) values of 0.042, 0.25, and 0.0082, respectively (27).

Table I shows the binding affinity constants, K_a, of UCN-01 analogues for hAGP. The K_a of UCN-01 for hAGP, 288 ± 75 × 10^6 M^{-1}, is the highest among the three compounds, and is in agreement with previously reported values (5). The K_a for staurosporine where hydrogen is the substituent at C-7 position is about one-twentieth. UCN-02 with an α-hydroxyl group at the C-7 position shows the smallest binding constant among the three ligands, compared to UCN-01. It is interest-

Table I. Binding Parameters of UCN-01, UCN-02, and Staurosporine to hAGP at pH 7.4

	Ligand		
	UCN-01	UCN-02	Staurosporine
K_a ($\times 10^6$ M^{-1})	288 ± 75	1.48 ± 0.11	11.33 ± 5.74
n	0.92 ± 0.04	0.93 ± 0.06	0.91 ± 0.11

All values are mean ± SD (n = 3).

ing to note that a change in the configuration of the hydroxyl group or a substitution of a hydrogen atom at the C-7 position of UCN-01 caused a 20- to 100-fold decrease in binding affinity.

Effect of pH on Binding of UCN-01 to hAGP

The binding of ligands to hAGP is known to be a pH sensitive process (28). The percentage of binding of UCN-01 to hAGP at different pHs, namely 6, 7, 7.4, and 8, was investigated (Fig. 3). The maximum binding of UCN-01 to hAGP occurs at pH 7.4 (96.33%). The binding of UCN-01 to hAGP was found to decrease in both acidic and basic pH values, where the lowest percentage binding was observed at pH 6 (69.74%).

Near-UV CD Spectra of hAGP in Various pHs

Structural profiles of hAGP at pH 6, 7, 7.4, and 8 were investigated by circular dichroism measurements. CD spectra measured at the near UV region reflects conformation or tertiary structure of a protein, while that measured at the far UV region reflects secondary structure. A change in the shape of a CD spectra measured under different conditions in comparison to a reference spectra of a particular set of conditions usually indicates a change in the conformation of the protein. The secondary structures of hAGP were essentially unchanged within the range of pH 6–8 (data not shown), but the tertiary structure changed significantly, as shown in the near UV CD spectra in Fig. 4. These results suggested that the conformational change in the tertiary structure of hAGP is pH dependent. Such a conformational change could affect the microenvironment of binding sites, thus causing a change in the binding of UCN-01 to hAGP.

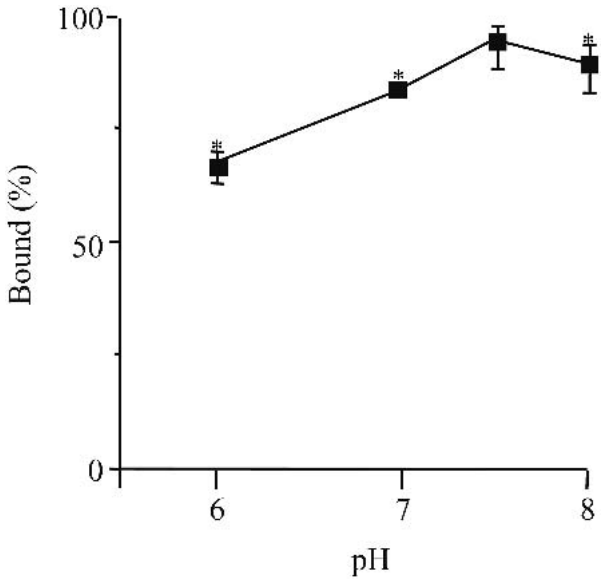

Fig. 3. Effect of pH on the binding of UCN-01 to hAGP. Concentrations are: [UCN-01] = [hAGP] = 20 μM. Each point represents the mean ± SD (n = 3). *Statistically significant compared with pH 7.4; $p < 0.01$.

Effect of Sialic Acid on the Binding of UCN-01 to hAGP at pH 7.4

The carbohydrate terminal chains of hAGP glycans are highly sialylated, containing up to 14 sialic acid residues (29). Therefore, hAGP has a low pKa (=2.6) and isoelectric point (=2.7). Sialic acid residues have been reported to influence the binding of several drugs to hAGP (30). In order to elucidate the role of sialic acid in the binding of UCN-01, we prepared desialylated hAGP and examined the binding percentage of UCN-01 to native and desialylated hAGP, which were 96.33 ± 1.26 and 95.59 ± 1.06, respectively. This suggested that the binding of UCN-01 to desialylated hAGP is comparable to that of native hAGP.

Binding Affinity of UCN-01 to hAGP Variants at pH 7.4

We separated the F1*S and A variants from the hAGP mixture and examined the percentage binding of UCN-01 to each of the variants. As shown in Table II, (the binding percentage of UCN-01 to native, F1*S and A variants are 96.33 ± 1.26, 95.08 ± 0.3 and 95.23 ± 0.45, respectively) there is no significant difference in the binding percent of UCN-01 to the F1*S and A variants of hAGP.

Near-UV CD Spectra of Chemical Modified hAGP and the Effects of the Chemical Modification on Binding of UCN-01 to hAGP

UCN-01 has a very high affinity to hAGP. This high affinity is thought to be derived from not only the hydrophobic interaction but also largely from the electrostatic interac-

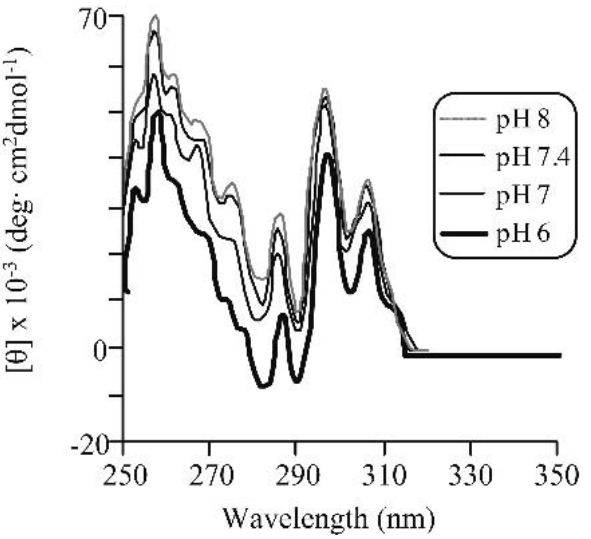

Fig. 4. Near-UV CD spectra of hAGP at various pHs The concentration is: [hAGP] = 10 μM.

Table II. Binding Percentage of UCN-01 to hAGP Variants at pH 7.4

	hAGP		
	Native	F1*S	A
Binding percentage (%)	96.33 ± 1.26	95.08 ± 0.3	95.23 ± 0.45

The concentrations are: [hAGP] = [F1*S] = [A] = [UCN-01] = 20 μM.
All values are means ± SD (n = 3).

tion between UCN-01 and hAGP. In actual fact, binding of UCN-01 to hAGP was affected by the presence of sodium chloride and fatty acid (data not shown). In order to evaluate the role of amino acid residues involved in the binding, we compared the binding of UCN-01 with chemically modified hAGPs. Chemical modification of His, Lys, Trp and Tyr residues of hAGP caused a marked decrease in the binding of UCN-01, with the modification of Trp residues showing the greatest extent of decrease (Table III). In addition, we also measured the binding percentage of staurosporine to these four chemically modified hAGPs and similar results were obtained where modification of Trp residues caused a marked decrease in the binding of staurosporine (data not shown). The CD spectra of His, Lys, Trp, and Tyr residues modified hAGP indicated that the secondary structure of all modified hAGPs hardly change (data not shown), but the tertiary structure changed significantly, particularly in the case of Trp residue modified hAGP (Fig. 5). The fluorescence quenching titration curve of UCN-01 binding to hAGP as shown in Fig. 6 indicated that tryptophan residues may play an important role in the binding of UCN-01 to hAGP.

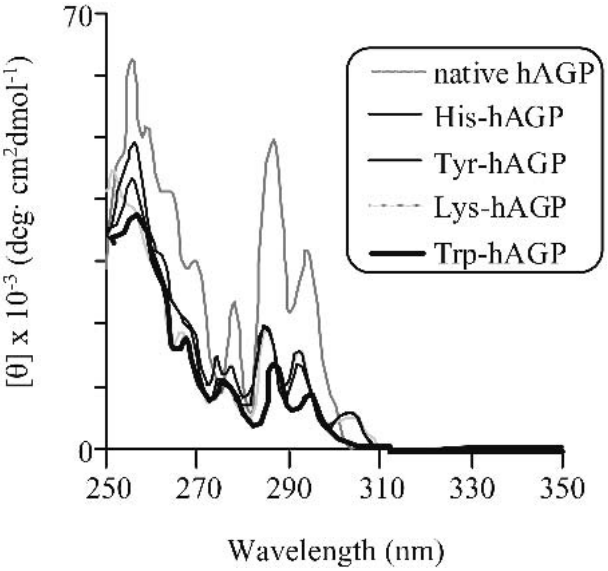

Fig. 5. Near-UV CD spectra of modified hAGPs at pH 7.4 The concentration is: [modified hAGPs] = 10 μM.

DISCUSSION

The initial treatment protocol for UCN-01 was a 72-h infusion administered at 2-week intervals. However, the clinical outcome of the first nine patients treated using this schedule demonstrated unexpectedly high concentrations of drug with a long terminal elimination half-life ($t_{1/2}$). This led to a modification of the UCN-01 administration schedule, in which the recommended phase II dose of UCN-01 is administered as a 72-h continuous infusion at 42.5 mg m^{-2} day^{-1} over a 3 day period. Subsequent courses are administered at 4-week intervals during a 36-h period (31). The extremely low clearance and small distribution volume of UCN-01 in humans could be partly due to the high degree of binding to hAGP. Although many drugs that associate with hAGP have K_a values of 10^5 to 10^6 M^{-1}, UCN-01 is unique in the tightness of its binding to hAGP, with K_a of 10^8 M^{-1}. As a consequence of this extraordinary high binding affinity, a low volume of distribution, which approximates the extracellular volume, and long $t_{1/2}$ is observed. In view of its altered pharmacokinetics due to its high interaction affinity with hAGP, the plasma levels of the hAGP are a potentially important factor in clinical treatment considerations.

hAGP is a glycoprotein that consists of 183 amino acid residues and five carbohydrate chains. The five highly sialylated complex-type-N-linked glycans contribute 45% of the molecular weight (32,33). As an acute phase reactant, hAGP's "basal" level is approximately 20 μmol/L, but it can vary from a 5- to a 10-fold range in response to stress, infection, or the effects of neoplasm in evocation of an inflammatory response. In addition, the levels of hAGP vary widely heterogeneous in cancer patients where the composition of hAGP is heterogeneous according to the type of disease, consisting of different isoforms and degrees of glycosylation. Differences in the binding of drugs to the two main genetic variants of hAGP have also been reported (25). Thus, the distribution of UCN-01 on the hAGP isoforms may also be difficult to predict.

Previous studies indicated that hAGP has one common drug binding site, which appears to be wide and flexible (34). In general, studies in our laboratory have shown that electrostatic and hydrophobic interactions are important driving forces for the binding of ligands to hAGP (35). In a study using fluorescence and ultracentrifugation experimental methods, the binding site of UCN-01 on hAGP was concluded to partly overlap with the binding site for basic drugs, acidic drugs, as well as steroid hormones (18). In order to

Table III. Binding Percentage of UCN-01 to Native and Chemically Modified hAGP at pH 7.4

	Chemically modified hAGP				
	Native	His	Lys	Trp	Tyr
Binding percentage (%)	96.33 ± 1.26	70.44 ± 2.68*	75.97 ± 1.56*	56.97 ± 2.06*	73.70 ± 1.01*

The concentrations are: [UCN-01] = [hAGP] = [chemically modified hAGP] = 20 μM.
All values are mean ± SD (n = 3).
* Statistically significant compared with native hAGP; $p < 0.01$.

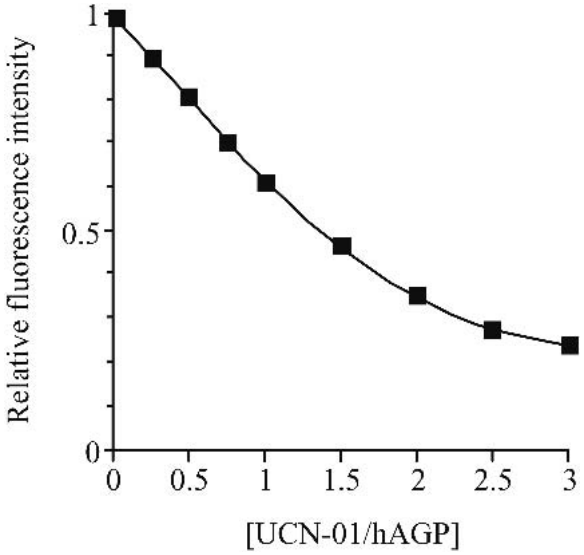

Fig. 6. Fluorescence quenching titration curve of UCN-01 binding to hAGP at pH 7.4 Concentration is: [hAGP] = 1 μM. [UCN-01] = 0 to 3 μM.

examine the ligand-binding site structural relationship of UCN-01 interactions with hAGP, the binding affinity of UCN-01, staurosporine, the "lead" compound among PKC inhibitors and UCN-02, a UCN-01 stereoisomer and weak PKC inhibitor were measured (Fig. 1). The decrease in the binding affinity followed the sequence of UCN-01 > Staurosporine > UCN-02 (Table I). It is obvious that the substituent at the C-7 position of the staurosporine molecule governs the binding affinity of UCN-01 analogues to hAGP. Interestingly, a hundred fold difference in binding affinity was recorded between UCN-01 and UCN-02, suggesting strict stereoisomeric or steric binding requirements of the UCN-01 binding site on hAGP. Meanwhile, the importance of the moiety at the C-7 position has also been reported for binding to target proteins (36,37). In a recent study, the crystal structures of staurosporine and UCN-01 in a complex with the kinase domain of PDK1 showed that, although staurosporine and UCN-01 interact with the PDK1 active site in an overall similar manner, the UCN-01 7-hydroxy group, which is not present in staurosporine, generates direct and water-mediated hydrogen bonds with active-site residues (36). On the other hand, hydrophobic interactions and hydrogen-bonding interactions were observed in the crystal structures between UCN-01 and the Chk1 kinase domain. The selectivity of UCN-01 toward Chk1 over cyclin-dependent kinases is due to the presence of a hydroxyl group in the lactam moiety, that interacts with the ATP-binding pocket. The high structural complementarity of these interactions is consistent with the potency and selectivity of UCN-01 (37). The interaction of the substituent at the C-7 position of UCN-01 to its binding protein is very important in terms of the selectivity of pharmacological activity and binding affinity, as is also the case of its binding to hAGP. It is, thus, apparent that the 7-hydroxy group of UCN-01 may play a key role in abnormally strong binding for hAGP.

The binding interactions of a series of basic ligands with hAGP were reported to increase with increasing pH. It was proposed that hydrophobic interactions dominate the high-affinity binding to hAGP (28). Meanwhile, Taheri *et al.* proposed a binding site for local anesthetics on F1*S of hAGP that is largely comprised of a structurally accommodating hydrophobic pocket, perhaps with the ability to form hydrogen bond to H-donor and -acceptor groups on the ligand and with a basic residue, mostly charged at neutral pH, that is located close to the aromatic group on bound drug molecules (38). In the present binding study, the binding of UCN-01 to hAGP also increased with increasing pH and the maximum binding of UCN-01 to hAGP was observed at pH 7.4 (96.33%). On the other hand, a low percentage of binding percentage was observed at pH 6 (69.74%) (Fig. 3). It is known that a tumor micro-environment is frequently more acidic by approximately 0.3–0.5 pH units, compared with that of its surrounding normal tissues. A decrease in the binding of UCN-01 in an acidic environment may partly contribute to the generation of free UCN-01 molecules for its anticancer activity at the cancer tissues.

hAGP exists in a variety of sialylated states which can be influenced by certain disease states. In a study investigating the effect of the sialylation state of hAGP on the binding of a model cationic drug, propranolol, the use of desialylated hAGP resulted in a modest increase in the propranolol free fraction (26). Since the binding percentage of UCN-01 to hAGP is more than 95%, an increase in the free fraction could be of clinical importance. Therefore, the binding of UCN-01 to native and desialylated hAGP was determined by ultracentrifugation. The percentage binding of desialylated hAGP was found to be comparable to that of native hAGP, suggesting that the influence of sialylation on UCN-01 binding is negligible. While asialoglycoprotein including desialylated hAGP is commonly known to be internalised by hepatocytes via receptor-mediated endocytosis, staurosporine has been reported to be an effective inhibitor of a liver uptake mechanism such as this (39). Since the liver is the major organ for the metabolism of desialylated hAGP, the significance of such protein trafficking inhibition by UCN-01 in contributing to its own low clearance deserves further investigation.

hAGP plasma concentrations differ depending on the type of cancers and such changes in the expression of the genetic variants of hAGP can be observed in various types of cancer. 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 (Fig. 2). Differences in the binding of ligands to hAGP variants have been reported. The ligand binding site of the F1*S variant is reported to be a relatively large hydrophobic pocket that is able to accommodate a variety of chemical structures, whereas the A variant binding site appears to be of smaller size and has a greater ligand specificity. The selective binding of disopyramide and methadone to the A variant and the preferential binding of dipyridamole to the F1*S variant mixture have been reported. On the other hand, lignocaine and chlorpromazine showed a slight preference for binding to the A variant and to the F1*S mixture, respectively. However, progesterone showed no selectivity with regard to any of the variants of hAGP (13). In the present study, no significant difference was detected for the binding of UCN-01 to the F1*S and A variants of hAGP (Table II). Hence, a change in the composition of the variants of hAGP may not be of clinical significance, despite the significant species difference in UCN-01 binding, in which a subsequent administration of hAGP to rats that had been infused

with UCN-01 actually caused a redistribution of the drug back to the blood stream from the peripheral tissues (40).

Chemical modification of His, Lys, Trp and Tyr residues of hAGP caused a marked decrease in the binding of UCN-01 (Table III). The binding percentage of UCN-01 to Trp-modified hAGP was also the lowest among all of the modified samples (Table III). Furthermore, modification of Trp residues also caused a marked decrease in the binding of staurosporine (data not shown). It is obvious that Trp residues are important in maintaining the tertiary structure of hAGP which is necessary for the high affinity binding of UCN-01. In addition, results of binding to chemically modified hAGP suggested that at least one Trp residue is involved in the binding of UCN-01 to hAGP. The involvement of Trp residues in UCN-01 binding could also be observed in the tryptophanyl fluorescence spectra where increasing UCN-01 concentrations led to a decrease in the fluorescence intensity of Trp residues of hAGP (Fig. 6). Interestingly, all three Trp residues are conserved in both F1*S and A variants of hAGP (Fig. 2) which showed no binding discrimination for UCN-01 (Table II).

Although we managed to identify the key factors for the unusually high binding affinity between UCN-01 and hAGP, namely the substituent at C-7 of the UCN-01 molecule and the Trp residues of hAGP, evidence is still lacking for suggesting a direct interaction between these two major factors. Among the three Trp residues of hAGP, two are relatively shielded from the bulk solvent, while the third Trp residue is located on the periphery of the domain (41). Trp^{25} has been deduced to be located deep in the binding pocket while Trp^{122} in the central hydrophobic pocket of the protein (42). Therefore, Trp^{160} could be the one that is exposed to the bulk solvent. Quantification of the chemical modification in the present study showed that, on average, about one Trp residue per mole of hAGP was modified. Since the modification of Trp residues was carried out under mild conditions without a surfactant present, the possibility of Trp^{160} being the modified residue is high. This suggests that Trp^{160} is the Trp residue that is involved in the binding of UCN-01. The identification of the specific Trp residue of hAGP involved in the high affinity binding of UCN-01 is currently underway in our laboratory. UCN-01 represents an initial candidate for a differentially selective protein kinase inhibitor. The results of this study will help in the informed design of future second generation approaches, as well as to serve as basis for further exploration of the potential of staurosporine pharmacophores which could lead to opportunities for structure-based optimization of PDK1 inhibitors.

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