Use of Photoaffinity Labeling and Site-directed Mutagenesis for Identification of the Key Residue Responsible for Extraordinarily High Affinity Binding of UCN-01 in Human α1-Acid Glycoprotein*

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7-Hydroxystaurosporine (UCN-01) is a protein kinase inhibitor anticancer drug currently undergoing a phase II clinical trial. The low distribution volumes and systemic clearance of UCN-01 in human patients have been found to be caused in part by its extraordinarily high affinity binding to human α1-acid glycoprotein (hAGP). In the present study, we photolabeled hAGP with [3H]UCN-01 without further chemical modification. The photolabeling specificity of [3H]UCN-01 was confirmed by findings in which other hAGP binding ligands inhibited formation of covalent bonds between hAGP and [3H]UCN-01. The amino acid sequence of the photolabeled peptide was concluded to be SDVVYTDW160A and wild-type recombinant hAGP were photolabeled by [3H]UCN-01. Three recombinant hAGP mutants (W25A, W122A, and W160A) and wild-type recombinant hAGP were photolabeled by [3H]UCN-01. Only mutant W160A showed a marked decrease in the extent of photoincorporation. These results strongly suggest that Trp-160 plays a prominent role in the high affinity binding of [3H]UCN-01 to hAGP. A docking model of UCN-01 and hAGP around Trp-160 provided further details of the binding site topology.

Human α1-acid glycoprotein (hAGP)1 is an acute phase protein with a molecular mass of 41 to 43 kDa and is heavily glycosylated (45%) (1). It contains sialic acids, which cause it to be negatively charged (pI = 2.7−3.2) (2). Its glycosylation pattern can change depending on the type of inflammation (3). The biological function of hAGP is not clear, although studies using in vitro models of inflammation indicate that it plays anti-inflammatory and immunomodulating roles and has protective effects (4, 5). The “basal” level of hAGP is ~20 μmol/liter, but hAGP levels can increase by 5–10-fold in response to stress, infection, or an inflammatory response to neoplasm (6, 7). In addition to increases in hAGP plasma concentration in certain cancers, changes in the expression of genetic variants of hAGP can occur according to the specific type of cancer (8). The levels of hAGP vary widely and heterogeneously among cancer patients; according to the type of disease, the composition of hAGP consists of various isoforms and degrees of glycosylation (9). Studies have shown that increases in circulating hAGP alter the pharmacokinetic disposition and pharmacological action of numerous drugs that bind to it (10–12). For example, increased hAGP levels associated with advanced tumors alter the pharmacokinetics of Imatinib (STI571), a tyrosine kinase inhibitor, in leukemia patients (13). hAGP also appears to be an independent predictor of response and a major objective prognostic factor of survival in patients with non-small cell lung cancer treated with docetaxel chemotherapy (14). Thus, hAGP is an important modulator of drug pharmacokinetics and pharmacodynamics in anticancer therapeutics.

7-Hydroxystaurosporine (UCN-01) has an indolocarbazole moiety and was originally isolated as a selective inhibitor of a Ca2+- and phospholipid-dependent protein kinase (protein kinase C (PKC)) (15). UCN-01 is a derivative of staurosporine, which occurs naturally, inhibits numerous other kinases, and has greater selectivity for PKC than does staurosporine (16, 17). UCN-01 can mediate 3 distinct cellular effects in vitro: cell cycle arrest, induction of apoptosis, and potentiation of DNA damage-related toxicity (18–20). It exhibits anticancer activity against human and murine tumor cell lines that have aberrations in cellular signal transduction (21–24). Unlike other compounds with an indolocarbazole moiety, UCN-01 preferentially induces G1 phase accumulation in various cell lines, which corresponded to the 160th Trp residue. This strongly implies that Trp-160 was photolabeled by [3H]UCN-01. The photolabeling specificity of [3H]UCN-01 was confirmed by findings in which other hAGP binding ligands inhibited formation of covalent bonds between hAGP and [3H]UCN-01. The amino acid sequence of the photolabeled peptide was concluded to be SDVVYTDW160A and wild-type recombinant hAGP were photolabeled by [3H]UCN-01. Only mutant W160A showed a marked decrease in the extent of photoincorporation. These results strongly suggest that Trp-160 plays a prominent role in the high affinity binding of [3H]UCN-01 to hAGP. A docking model of UCN-01 and hAGP around Trp-160 provided further details of the binding site topology.

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7-Hydroxystaurosporine (UCN-01) has an indolocarbazole moiety and was originally isolated as a selective inhibitor of a Ca2+- and phospholipid-dependent protein kinase (protein kinase C (PKC)) (15). UCN-01 is a derivative of staurosporine, which occurs naturally, inhibits numerous other kinases, and has greater selectivity for PKC than does staurosporine (16, 17). UCN-01 can mediate 3 distinct cellular effects in vitro: cell cycle arrest, induction of apoptosis, and potentiation of DNA damage-related toxicity (18–20). It exhibits anticancer activity against human and murine tumor cell lines that have aberrations in cellular signal transduction (21–24). Unlike other compounds with an indolocarbazole moiety, UCN-01 preferentially induces G1 phase accumulation in various cell lines, and one of its mechanisms of action is clearly mediated by dephosphorylation of retinoblastoma protein and inhibition of cyclin-dependent kinase 2 (CDK2), an intracellular retinoblastoma protein kinase that regulates the transition from the G1 to S phase (25). In addition, UCN-01 enhances the anticancer effects of several important chemotherapeutic drugs, including mitomycin C, cisplatin, and 5-fluorouracil, in vitro and in vivo (26–28). UCN-01 is currently in the phase II study of its effects on relapsed or refractory systemic anaplastic large cell and mature T-cell lymphomas (29, 30). UCN-01 was initially administered as a 72-h continuous infusion every 2 weeks, based on data from in vitro and xenograft preclinical models. However, in the first few patients, the drug had an unexpectedly long half-life (>30 days), which was 100 times longer than the
half-life observed in preclinical models. The distribution volumes (0.0796–0.158 liter/kg) and systemic clearance (0.0407–0.252 ml/h/kg) in the human patients were found to be extremely low. This pharmacokinetic behavior of UCN-01 in humans can partly be attributed to its specific high affinity binding to hAGP, which causes slow dissociation of UCN-01 from hAGP and thereby limits its disposition and elimination (31, 32). The binding constant for UCN-01 and hAGP, $8 \times 10^8 \text{M}^{-1}$, is the highest value ever reported for protein binding studies (33).

Several protein binding studies of hAGP had been conducted using a variety of techniques including equilibrium dialysis, ultrafiltration, chemical modification, and displacement (34–37). In a recent study, we identified the key factors contributing to the unusually high binding affinity between UCN-01 and hAGP: the substituent at C-7 of the UCN-01 molecule, and the Trp residues of hAGP (38). Crystallographic structural analysis has become more common and appears to be a good method for analysis of ligand-protein interaction, but there have been no reports of crystallographic structural analysis of hAGP. Certain experimental techniques allow direct evaluation of ligand-protein complexes, which can elucidate the binding chemistry of hAGP. Photoaffinity labeling is an essential complement to modeling and mutagenesis and allows direct, unambiguous identification of the contact region between a binding protein and its specific photoactivatable ligands (39–41). There is no photoaffinity labeling study that has led to the direct determination of labeled amino acid residues in hAGP. In the present study, we used $[^3H]$UCN-01 (Fig. 1) as a photoaffinity labeling agent to characterize the binding site of hAGP. Also, single residue mutants of recombinant hAGP (W25A, W122A, and W160A) were produced in order to determine which Trp was involved in the high affinity binding of $[^3H]$UCN-01. Finally, we constructed models of the docking of UCN-01 into the binding cavity, using a three-dimensional molecular model of hAGP.

**EXPERIMENTAL PROCEDURES**

**Materials**—$[^3H]$UCN-01 (12 Ci/mmol), UCN-01, UCN-02, and ataurosporine were supplied by Kyowa Hakko Kogyo Co. (Shizuoka, Japan). hAGP (purified from cohn fraction VU) was purchased from Sigma. Sequencing grade modified trypsin was purchased from Promega. All other chemicals and solvents were of analytical grade. N-Glycosidase F (PNGase F) was purchased from Roche Applied Science. Plasma-derived AGP (pAGP), propranolol, and progesterone were purchased from Sigma. Potassium warfarin was donated by Eisai Co. (Tokyo, Japan). Restriction enzymes, Escherichia coli JM109, the DNA ligation kit, and the DNA polymerase Premix Taq® (EX Taq version) were obtained from Takara Biotechnology Co. Ltd. (Kyoto, Japan). The DNA sequencing kit was obtained from PerkinElmer Applied Biosystems (Tokyo, Japan). The Pichia expression kit was purchased from Invitrogen. DEAE Sephacel, phenyl-Sepharose Fast Flow, and Sephadex G-75 superfine were purchased from Amersham Biosciences.

**Expression and Purification of Wild-type and Mutant rhAGP**—Recombinant hAGP (rhAGP) was expressed in the methylotrophic yeast Pichia pastoris using the expression vector pPIC9, and was purified by anionic exchange, hydrophylic interaction, and gel filtration chromatography (42). The single residue mutants W22A, W122A, and W160A were prepared using a QuikChange® XL site-directed mutagenesis kit, following the procedure of Braman et al. (43).

**Purification of rhAGP**—The growth medium was separated from the yeast by centrifugation (6000 $\times g$, 10 min, 4 °C), and the secreted rhAGP was isolated from the medium as follows. The medium was brought to 60% saturation with ammonium sulfate at room temperature. The temperature was then lowered to 4 °C, and the pH was adjusted to 4.0. After shaking for 12 h, the precipitated protein was collected by centrifugation (12,000 $\times g$, 60 min, 4 °C) and resuspended in distilled water. Dialysis was performed for 48 h at 4 °C against 100 volumes of distilled water, followed by a further 24 h of dialysis against 100 volumes of 10 mM Tris-HCl buffer (pH 7.4). Then, the solution was loaded onto a column of DEAE Sephacel. rhAGP was eluted with a linear gradient of 0–1 M NaCl in 10 mM Tris-HCl buffer (pH 7.4). The eluted rhAGP was loaded onto a column of phenyl-Sepharose Fast Flow. Finally, rhAGP was purified using Sephadex G-75 superfine resin.

**Photoaffinity Labeling of hAGP—**hAGP (50 μM) was incubated with $[^3H]$UCN-01 (0.08 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 30 min. The incubation mixture was then placed on ice and irradiated for 30 min by a 100-watt black light/blue lamp (310 nm, Ultra-Violet Products, Inc., San Gabriel, CA) at a distance of 10 cm. After irradiation, the photolabeled hAGP was precipitated by adding 1 ml of acetone, followed by centrifugation at 15,000 rpm for 10 min. The pellet was washed with 1 ml of ethanol and centrifuged a second time.

**SDS-PAGE and Electroblotting—**Photolabeled hAGP was analyzed by SDS-PAGE using a 10% polyacrylamide gel (according to the method of Laemmli) and a sampling buffer (10 mM Tris-HCl, 50 mM dithiothreitol, 4 mM EDTA, and 2% (w/v) sucrose). The concentration of protein was determined by Bradford assay using bovine serum albumin as the standard (44). After electrophoresis, the gel was electrotransfered onto a PVDF membrane in a transfer buffer (25 mM Tris, 190 mM glycine, 10% methanol) using a semidyndry blot assembly. The blot was stained with Coomassie Brilliant Blue R250, followed by complete drying in air.

**Autoradiographic Analysis**—For autoradiographic analysis, the dried PVDF membrane was placed in contact with an imaging plate (BAS III, Fuji Photo Film Co.) in a cassette (BAS cassette 2040) at room temperature for 48 h. The imaging plate was scanned and analyzed using a Bio-Image Analyzer (model BAS-FLA-3000 G; Fuji Photo Film Co.) in a cassette (BAS cassette 98). The incorporation of radioactivity into individual fragments was quantified using Image Gauge V3.1 software (Fuji Film).

**Competition Experiments**—In order to determine the photolabeling specificity of the binding site of $[^3H]$UCN-01, hAGP (50 μM) was incubated with $[^3H]$UCN-01 (0.08 μM) in the presence of competitors (250 μM) prior to photolysis. The competitors were the UCN-01 analogues staurosporine and UCN-02, the basic drug propranolol, the acidic drug warfarin, and progesterone (representative steroid hormone). The photolabeled hAGP was separated by 10% gel SDS-PAGE and electroblotted onto a PVDF membrane before being subjected to autoradiographic analysis.

**Reductive Pyridylethylolation and Deglycosylation of hAGP**—After the photolabeled hAGP was precipitated by acetone, 100 μl of the buffer was added to the precipitate. Then, 10 μl of 1% SDS and 1 x 2-mercaptoethanol were added to this solution, followed by reduction at 100 °C for 10 min. For deglycosylation of hAGP, 10 μl of 10% N-octanoyl-N-methylglucamide (MEGA-8), 50 μl of deionized water and 2 units of PNGase F were added to the reduction solution, and the resulting solution was incubated for 24 h. Then, 1 μl of 4-vinylpyridine was added, the mixture was further incubated in a N2 atmosphere for 30 min at room temperature, and was then dialyzed for desalination.

**Tryptic Digestion and Purification of Photolabeled hAGP Peptide Fragments**—Tryptic digestion was performed in 50 mM NH4HCO3 (pH 7.8). After deglycosylation, deglycosylated hAGP was incubated with trypsin for 5 h at 37 °C. The ratio of trypsin to hAGP was 1:20 (w/w). Two tryptic peptides were separated by reverse-phase HPLC (4.6 × 250 mm, Vydac) high performance liquid chromatography (HPLC) using an aqueous acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The separated peptides were fractionated every 30 s; 200 μl of each fraction were added to 2.5 ml of scintillation mixture; and the radioactivity was determined using a LSC-500 liquid scintillation counter (Alkco, Tokyo, Japan). The fraction with the highest radioactivity was collected in an Eppendorf tube, and was evaporated on a SpeedVac.
evaporator until the volume of the sample was about 50 µl.

**Capillary HPLC Separation and Sequencing**—After evaporation, 10 µl of the sample was injected into an ABI 173 A MicroBlotter Capillary HPLC System (PerkinElmer Life Sciences) (45). The sample was manipulated according to the manufacturer’s instructions (User’s Manual, PerkinElmer Life Sciences). Meanwhile, the blotted membrane from the capillary HPLC separation using a C18 column (5 µm, 1.5 x 150 mm, PerkinElmer) was in contact with an imaging plate for 48 h prior to autoradiography analysis. The PVDF membrane was aligned with the chromatogram of a peptide map from the ABI 173 A MicroBlotter Capillary HPLC System. Portions of the PVDF membrane were excised for sequencing on an Applied Biosystems Procise Sequencer with reference to the autoradiogram.

**Docking of UCN-01 to hAGP**—The structure of hAGP has not previously been experimentally determined. As a model of the three-dimensional structure of hAGP for ligand docking, we used the modeled structure of hAGP obtained by Kopecky et al. (46). The initial structure of UCN-01 was taken from the crystal structure of the Chk1-UCN-01 complex (47, PDB ID 1NVQ). The docking calculation of UCN-01 to hAGP was performed using the SYBYL FlexX (48) under the condition that UCN-01 interacts with Trp-160. During the docking calculation, the structure of hAGP and the ring conformation of UCN-01 were kept rigid. The docking algorithm produced 158 different placements of UCN-01 in hAGP. All placements were evaluated by SYBYL CScore and then were ranked using AASS (Average of Auto-Scaled Scores), as follows in Equation 1.

\[
\text{AASS}_{\text{placement}} = \frac{\sum_{i} (\text{Score}_{\text{placement}} - \min(\text{Score}))}{\max(\text{Score}) - \min(\text{Score})} / n \quad (\text{Eq. 1})
\]

In the AASS calculation, \( n = 5 \) and \( i = \) F_Score (48), G_Score (49), PMP_Score (50), D_Score (51), ChemScore (52). Although the top 5 placements had nearly the same AASS values, they were classified into two types of binding modes. For each type, we chose the placement with the best AASS value as the candidate binding mode. In the type I model, UCN-01 is bound to a hydrophobic pocket formed by Val-41, Ile-44, Phe-48, and Val-156. In the type II model, UCN-01 is packed into a hydrophobic pocket consisting of Ile-28, Pro-131, Leu-138, Tyr-157, and Trp-160.

**Refinement of Docking Models**—To refine the docking models, the coordinates of the atoms of UCN-01 and the atoms of hAGP within 10 Å from UCN-01 were optimized to reduce the root mean square of the gradients of potential energy below 0.05 kcal mol\(^{-1}\)Å using SYBYL 6.9.1 (Tripos, Inc., 2003). The Tripos force field was used for the molecular energy calculation. The AMBER 7 charges (53) were used as the charges for UCN-01. The cut-off distance for the non-bonded atomic charges for hAGP. The Gasteiger-Huckel charges (54–57) were used as the charges for hAGP. The Gasteiger-Huckel charges (54–57) were used as the charges for UCN-01. The cut-off distance for the non-bonded interaction was 10 A. The distance-dependent dielectric constant of 4r was used. Due to the lack of hydrogen atoms in the modeled structure of hAGP, the initial positions of the hydrogen atoms in the hAGP were generated by the SYBYL.

**Statistical Analysis**—Statistical analysis of differences was performed by one-way ANOVA followed by the modified Fisher’s least squares difference method.

**RESULTS**

**Photolabeling of \[^{3}H\]UCN-01 to hAGP**—The autoradiogram in Fig. 2 shows that the band of radiolabeled protein appeared only upon the photoincorporation of \[^{3}H\]UCN-01. The radioactivity band indicated the incorporation of \[^{3}H\]UCN-01 to hAGP via photoradiation. No band of radiolabeled protein could be observed for the sample without irradiation indicating that no covalent attachment of \[^{3}H\]UCN-01 to hAGP occurred in the dark. Exposure to light for 30 min was sufficient for the photoincorporation of \[^{3}H\]UCN-01 to hAGP (Fig. 3). The results indicate that \[^{3}H\]UCN-01 is photoactivatable and stable in the dark.

**Competition Experiments**—In a previous study using ultracentrifugation methods, it was concluded that the binding site for UCN-01 on hAGP partly overlaps with the binding site for basic drugs, acidic drugs, and steroid hormones (58). In the present photolabeling experiment, in order to determine the photolabeling specificity of \[^{3}H\]UCN-01, we used staurosporine and UCN-02 (a stereoisomer of UCN-01), which also bind hAGP, as competitors. All staurosporine analogs significantly inhibited photoincorporation, by more than 60% (Fig. 4). Other competitors that we used were representatives of other hAGP ligands: an acidic drug (warfarin), a basic drug (propranolol), and a steroid (progesterone). Warfarin and propranolol inhibited binding by less than 30%, but to a significant degree, whereas progesterone inhibited binding by about 60% (Table I).

**Amino Acid Sequence of the Photolabeled Tryptic Peptides**—Tryptic peptides of the hAGP photolabeled with \[^{3}H\]UCN-01 were separated by reverse phase HPLC using a C18 column. The major radioactive peptides were eluted in 10.5–11.5 min (Fig. 5, A and B). The fractions eluted within this time frame were collected and concentrated with a SpeedVac evaporator for further capillary HPLC analysis. The concentrated sample from previous HPLC analysis was separated and simulta-
neously blotted onto a strip of PVDF membrane using an ABI 173 A MicroBlotter Capillary HPLC System. Autoradiographic analysis of the PVDF membrane indicated that the radioactive spot corresponded to the peak observed at 84–85 min (Fig. 6, A and B). Edman sequencing of this spot revealed an amino acid sequence of SDVVYTDXK (Fig. 7), corresponding to Ser-153 to Lys-161 of hAGP.

Photolabeling of Wild-type and Mutant rhAGP with [3H]UCN-01—Mutation of the 160th Trp residue of rhAGP to an Ala residue (W160A) caused a significant decrease in photoincorporation, by about 80%. In contrast, there was no significant difference in photoincorporation of [3H]UCN-01 between wild-type rhAGP and the rhAGP mutants W25A and W122A (Fig. 8).

Docking of UCN-01 to hAGP—We constructed models of the docking of UCN-01 into the binding cavity of hAGP around Trp-160, using the three-dimensional molecular model of hAGP published by Kopecky et al. (46), to map the possible binding sites of hAGP. Molecular modeling calculations revealed 2 potential binding sites, type I and type II, around Trp-160, both of which were located in the outer region of hAGP (Fig. 9A). Table II shows the distance and nature of interaction between donors and acceptors in the models of types I and II. In the type II model, UCN-01 is packed into a surface cleft consisting of Ile-28, Pro-131, Glu-132, Lys-135, Leu-138, Tyr-157, Trp-160, and Lys-161 (Fig. 9B). The surrounding amino acid residues within 5 Å of the UCN-01 molecule include Lys-135, Tyr-157, Trp-160, and Lys-161. The oxygen atom in the sugar ring of
UCN-01 is in contact with Lys-135. The C=O group of UCN-01 was observed to form a hydrogen bond with the amino group of Trp-160, and electrostatic interaction was observed between the amino group of Lys-161 and both the 7-OH group and C=O group of UCN-01. Furthermore, the aromatic ring of UCN-01 is adjacent to Tyr-157 and Trp-160. In contrast, these interactions were not observed in the type I model, in which UCN-01 was shown to be packed into a surface cleft consisting of Val-41, Glu-43, Ile-44, Phe-48, Tyr-50, Val-156, Thr-158, and Trp-160 (data not shown).

**DISCUSSION**

The acute phase response alters the composition of carrier proteins in plasma, which may affect the blood deposition and transport of biomediators and drugs. Understanding the interaction of drugs with plasma proteins is essential to understanding their systemic pharmacology and toxicology. Thus, information about the effects of the acute phase response on the ligand binding ability of plasma can be used to optimize drug administration protocols in clinical practice. hAGP has been reported to be a major plasma protein that predominantly binds basic drugs (60). However, protein binding studies suggest that hAGP has a wide and flexible drug binding area that accommodates not only basic but also acidic and steroidal drugs (61). A current model of the hAGP binding site depicts a buried pocket with a negatively charged region that interacts with the N termini of basic drugs (62). The tertiary structure of hAGP has proven refractory to resolution, and structure-activity studies using various approaches are needed to clarify the nature of the binding site on this important protein.

The initial treatment protocol for UCN-01 was a 72-h infusion administered at 2-week intervals, because certain cell types (e.g. MDA-MB-468 breast carcinoma cells) required 72 h of drug exposure before irreversible growth inhibition occurred (23). However, the clinical outcome of the first 9 patients treated using this schedule demonstrated unexpectedly high concentrations of the 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/m²/d over a 3-day period. Second and subsequent courses were administered for only 36 h at the same concentration and...
infusion rate, which effectively reduced the administered dose by 50% for the second and subsequent courses. In addition, the time between courses was increased from 2 weeks to 4 weeks (29). The extremely low clearance and small distribution volume of UCN-01 in humans may be partially caused by its high degree of binding to hAGP (32). Whereas many drugs that associate with hAGP have $K_a$ values of $10^5$–$10^6$ M$^{-1}$, UCN-01 is unique in its high affinity binding to hAGP, and has a $K_a$ value of $8 \times 10^6$ M$^{-1}$. The results of this extraordinarily high binding affinity include a low volume of distribution (which approximates the extracellular volume) and long $t_{1/2}$ (32). The pharmacokinetic effects of the high affinity of UCN-01 for interaction with hAGP indicate that plasma levels of hAGP should be an important consideration in planning of clinical treatment. hAGP has been reported to be a major drug binding plasma protein that interacts mainly with basic drugs (63). Previous studies indicate that hAGP has 1 common drug binding site, which appears to be wide and flexible (61). Characterization of the binding site of UCN-01 on hAGP by Kurata et al. (58) revealed partial overlap with amino acid residues implicated in binding of basic drugs, acidic drugs, and steroid hormones. In a previous attempt to further characterize the binding site, we found that Trp-160 is particularly likely to play a major role in the binding of UCN-01. However, because that conclusion was the result of deducing the location of the 3 tryptophan residues, other experimental approaches are needed to confirm it. Currently, no x-ray crystallographic data is available for hAGP, which is a heterogeneous protein consisting of different isoforms and glycosylation states that hinder crystallization. Therefore, in the present study, we examined the possibility of using UCN-01 as a photoaffinity-labeling agent. An ideal photolabeling reagent is stable not only in storage but also under the conditions in which the experiments are performed. Another problem with reagent stability is covalent attachment in the dark, either specific or nonspecific, to the protein under study. All the advantages of photoaffinity labeling are lost if such covalent attachment occurs. In the present study, a covalent bond was formed between UCN-01 and hAGP only upon photolabeling (Fig. 2).

The results of the present examination of photoinhibition by staurosporine and UCN-02 were as expected (Table I). It is interesting that a change in the configuration of the hydroxyl group of UCN-01 or substitution of a hydrogen atom at the C-7 position of UCN-01 caused a decrease in binding inhibition effects. This confirms our finding that the substituent at C-7 of the UCN-01 molecule governs the affinity of its binding to hAGP. On the other hand, the extensive photoinhibition by progesterone, but not warfarin or propranolol, suggests that the binding site of staurosporine analogs overlaps to a greater extent with the binding site of steroids. This sharing of a binding region between UCN-01 and steroids is thought to be of minimal clinical significance, given the increased hAGP concentration in cancer and the extremely high binding affinity of UCN-01 for hAGP.

Sequence analysis of the major radioactive tryptic peptides separated from hAGP photolabeled with $[^3]$HUCN-01 showed that these peptides correspond to amino acids Ser-153 to Lys-161 of hAGP. In addition, no phenylthiohydantoin (PTH) derivatives were detected at the 8th cycle, which corresponds to the 160th Trp residue (Fig. 7), indicating that the covalent bond formed upon photolabeling of $[^3]$HUCN-01 to hAGP is relatively stable under the conditions of Edman degradation, and that it is highly likely that Trp-160 was photolabeled by $[^3]$HUCN-01.

All naturally occurring genetic variants of hAGP conserve the 3 Trp residues in the protein amino acid sequence: Trp-25, Trp-122, and Trp-160 (64). It is noteworthy that there is no significant difference in the binding percentage of UCN-01 between the F1$^+$S and A variants of hAGP (38). 2 of 3 Trp residues of hAGP are relatively shielded from the bulk solvent, whereas the third Trp residue is located on the periphery of the domain. It has been deduced that Trp-25 is located deep in the binding pocket, and that Trp-122 is located in the central hydrophobic pocket of the protein (65). This suggests that Trp-160 is the Trp residue that is exposed to the bulk solvent. We also used site-directed mutagenesis to identify the key Trp residue involved in UCN-01 binding. Photolabeling of wild-type and mutant rhAGP with $[^3]$HUCN-01 revealed that photoincorporation was significantly lower for W160A than for the wild type. In contrast, the level of photoincorporation observed for the other 2 mutants, W25A and W122A, was comparable to that of the wild type. These results strongly support the hypothesis that Trp-160 is the key amino acid responsible for the extraordinarily high affinity of binding between UCN-01 and hAGP.

Previous studies have revealed the structures of UCN-01 and staurosporine bound to the active conformations of Chk1 (47), phospho-CDK2/cyclin A (66), and PDK1 (67). Coincidentally, as previously observed between staurosporine and UCN-01 complexes are the contacts involving the 7-OH group of UCN-01. Komander et al. (67) analyzed the relative affinities of staurosporine and UCN-01 for 29 different kinases, and found that binding that was potently inhibited by UCN-01 tended to involve molecules with a side chain that can directly form a hydrogen bond with the 7-OH group of UCN-01. Taking into account the experimental spectra and the unfavorable docking energy, Zsila et al. (68) suggested that it is unlikely that curcumin binds inside the central cavity of hAGP. The present docking models show that UCN-01 can interact with surface clefts of hAGP containing Trp-160. The interacting amino acid residues identified by the present type II model are consistent with results of our previous experimental studies of chemical modifications and protein binding (38), as well as those of
amino acid residues in the two latter models were similar to docking models (data not shown). In general, all interacting amino acid residues in the two latter models were similar to those of the former, except that the α-OH group at the C-7 position of UCN-02 interacts with COOH group of Glu-132, in contrast to UCN-01 where the C-7 β-OH group interacts with Lys-161. On the other hand, no interaction of any form with the amino acid side chain could be observed for the substituent at C-7 position of staurosporine where hydrogen atom exists. Another amino acid residue that deserved attention was Lys-135 as its distance from the sugar ring of UCN-02 was more than 5 Å, the greatest among the three models.

The following sequence of binding affinity for hAGP has previously been observed: UCN-01 > staurosporine > UCN-02 (38). The aromatic ring of UCN-01 is stacked on Trp-160, and the hydrophobic interaction is strengthened by the electrostatic interaction between the 7-OH group of UCN-01 and Lys-161, which are located on the same side of Trp-160. In contrast, the hydrogen bond between the 7-OH group of UCN-02 and Glu-132, which is located on the opposite side, appears to weaken the hydrophobic interaction, because the ring of UCN-02 has been diverted away from Trp-160. The aromatic ring of staurosporine is not diverted from Trp-160, due to the absence of the 7-OH group. In order to gain deeper insight on the binding mechanism of UCN-01 to hAGP, experiments using Glu-132 and Lys-161 hAGP mutants to examine the role of each mutated amino acid residue in the high affinity binding of UCN-01 is currently underway in our laboratory.

Staurosporine is a natural product derived from fermentation extracts of several bacterial species. Staurosporine was initially identified as a potent inhibitor of PKC, which is a broad-acting kinase inhibitor with little specificity or selectivity for PKC (71). Recently, the staurosporine analog N-benzoylstaurosporine (PKC412) has been reported to exhibit strong hAGP binding, and to have unusual pharmacokinetics similar to those of staurosporine, which were not predicted by animal studies (72). PKC412 is the only staurosporine inhibitor of protein kinases other than UCN-01 that has been subjected to a clinical trial. There has been a study of oral administration of PKC412 once daily (73). It is interesting that PKC412 exhibits complex pharmacology resulting from binding to hAGP. Preclinical experiments have shown extensive binding of PKC412 to human plasma proteins, with ~88–98% protein binding, depending on the drug concentration (72). Rates of binding of PKC412 to hAGP were particularly interesting. In the preclinical experiments, the plasma concentrations of PKC412 were higher, and the half-life was longer than predicted from animal studies and single dose kinetics studies with healthy volunteers (72). In contrast to UCN-01, PKC412 was metabolized to 7-hydroxy-PKC412 and an O-demethyl-PKC412, both of which also bound to hAGP. The major metabolite had a particularly long half-life (74). It is possible that PKC412 and its metabolite preferentially bind to hAGP in vivo, and this may account for the longer than anticipated plasma half-life. The dynamics of dissociation of PKC412 from plasma proteins and tissue distribution of PKC412 are likely to be complex, and plasma levels may not accurately reflect drug concentration in target tissues. Because plasma pharmacokinetic evaluation is complicated by protein binding and metabolism, studies using biologic markers of PKC inhibition can contribute to optimization of PKC412 administration.

CONCLUSIONS

Because of the potential implications of species-specific binding of UCN-01 to hAGP in human plasma for the development of staurosporine analogs, studies of analogs of UCN-01 and of PKC412, which lack hAGP binding or very weakly bind to hAGP, should be conducted along with studies of the potential usefulness of staurosporine pharmacophores. Characterization of the binding site of UCN-01 on hAGP using photoaffinity labeling and site-directed mutagenesis techniques has provided direct evidence that strongly indicates that Trp-160 plays an important role in the binding interaction between UCN-01 and hAGP. In addition to the obvious pharmacokinetic implications of the extraordinarily high affinity of binding of UCN-01 to hAGP, the present results suggest that hAGP is a suitable platform for further design of novel staurosporine analog anticancer drugs, and also for evaluation of side effects and drug interaction in clinical settings. The present results provide clues to the design of future second-generation therapeutic agents, and can serve as a basis for future studies of UCN-01 administered alone and in combination with other anticancer drugs, particularly DNA-damaging agents.

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