Characterization of HKI-272 Covalent Binding to Human Serum Albumin

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ABSTRACT:

The study was initiated as an observation of incomplete extraction recovery of N-(4-(3-chloro-4-(2-pyridinylmethoxy)anilino)-3-cyano-7-ethoxy-6-quinolyl)-4-(dimethylamino)-2-butenamide (HKI-272) from human plasma. The objective of this study was to 1) identify the binding site(s) of HKI-272 to human plasma protein(s); 2) characterize the nature of the binding; and 3) evaluate the potential reversibility of the covalent binding. After incubation of [14C]HKI-272 with human plasma, the mixture was directly injected on liquid chromatography/mass spectrometry (LC/MS), and an intact molecular mass of HKI-272 human serum albumin (HSA) adduct was determined to be 66,999 Da, which is 556 Da (molecular mass of HKI-272) larger than the measured molecular mass of HSA (66,443 Da). For peptide mapping, the incubation mixture was separated with SDS-polyacrylamide gel electrophoresis followed by tryptic digestion combined with LC/tandem MS. A radioactive peptide fragment, LDELREDGKASSAK [amino acid (AA) residue 182–195 of albumin], was confirmed to covalently bind to HKI-272. In addition, after HCl hydrolysis, a radioactive HKI-272-lysine adduct was identified by LC/MS. After combining the results of tryptic digestion and HCl hydrolysis, the AA residue of Lys190 of HSA was confirmed to covalently bind to HKI-272. A standard HKI-272-lysine was synthesized and characterized by NMR. The data showed that the adduct was formed via Michael addition with the ε-amine of lysine attacking to the β-carbon of the amide moiety of HKI-272. Furthermore, reversibility of the covalent binding of HKI-272 to HSA was shown when a gradual release of HKI-272 was observed from protein pellet of HKI-272-treated human plasma after resuspension in phosphate buffer, pH 7.4, at 37°C for 18 h.

Her-2 (erbB-2 or neu) is a member of the epidermal growth factor receptor family of tyrosine-protein kinases (Olayioye et al., 2000). Overexpression of Her-2 is observed in breast, ovarian, lung, prostate, and oral cancers (Ross and Fletcher, 1998). N-(4-(3-Chloro-4-(2-pyridinylmethoxy)anilino)-3-cyano-7-ethoxy-6-quinolyl)-4-(dimethylamino)-2-butenamide (HKI-272) is a potent small molecule, irreversible inhibitor of Her-2 tyrosine kinase in vitro (Kwak et al., 2005) and in vivo and is currently in development as an alternative for first- and second-line therapy in metastatic breast cancer patients who overexpress Her-2 (Minami et al., 2007). [14C]HKI-272 was administered to healthy volunteers to better understand its disposition in humans. After a single oral 200-mg dose of [14C]HKI-272 (free base) to humans, exposures based on area under the curve values of HKI-272 and known metabolites, including N-oxide of N,N-dimethyl (M7), N-oxide of pyridinyl (M3), and N-desmethyl (M6) metabolites, were measured by validated bioanalytical methods. Only approximately 26% of total plasma radioactivity area under the curve was accounted for as determined by accelerator mass spectrometer analysis. In vitro experiments conducted with mouse, rat, rabbit, dog, nonhuman primate, and human plasma showed that the extraction recovery was species-dependent, with approximately 100% recovery from rodent and rabbit plasma, 80% recovery from dog plasma, but only 50 and 45% recovery from cynomolgus monkey and human plasma after incubation at 37°C for up to 6 h.

The incomplete recovery of HKI-272 from plasma was speculated to be caused by the formation of a covalent adduct to plasma protein via its α,β-unsaturated amide group. The α,β-unsaturated amide moiety of HKI-272 was pharmacologically designed to undergo Michael addition to covalent bind to the conserved, solvent-exposed cysteine residue present in some ERB family members (Cys773 in epidermal growth factor receptor and Cys805 in Her-2) (Wissner et al., 2007). Plasma protein binding is normally considered to be a safe way to deliver drugs to their target tissues by preventing elimination from hepatic and renal pathways (Bertucci and Domenici, 2002). Furthermore, it has been reported that some types of covalent binding, such as Michael addition, are reversible (Lin et al., 2008). For example, Bailleul and Slatter (1991) showed that conjugate adducts of GSH with

ABBREVIATIONS: HKI-272, N-(4-(3-chloro-4-(2-pyridinylmethoxy)anilino)-3-cyano-7-ethoxy-6-quinolyl)-4-(dimethylamino)-2-butenamide; LC/MS, liquid chromatography/mass spectrometry; PAGE, polyacrylamide gel electrophoresis; AA, amino acid; HPLC, high-performance liquid chromatography; HSA, human serum albumin; IS, internal standard; TIC, total ion chromatogram; XIC, extract ion chromatogram; BMS-204352, (3S)-3-(5-chloro-2-methoxyphenyl)-3-fluoro-6-(trifluoromethyl)-1,3-dihydro-2H-indol-2-one.
carbamate thioester moiety could be reversible under physiological conditions. S-Linked GSH conjugates of isocyanates, isothiocyanates, α,β-unsaturated carbonyl functionalities, and others could be cleaved to regenerate the original electrophiles after transporting a distance in systemic circulation (Jochheim et al., 2002). Plöman et al. (1994) found that glutathione transferase (GST-pi) was inactivated after incubations with a 5-fold molar excess of ethacrynic acid, an α,β-unsaturated ketone, whereas adding an excess of GSH fully restored the catalytic activity of the enzyme after 120 h. Therefore, it can be speculated that if in vivo reversibility of HKI-272 protein adduct could occur, it could represent a mechanism for HKI-272 delivering and regenerating to the pharmacological target.

The combination of liquid chromatography/mass spectrometry (LC/MS) analysis with in-gel tryptic digestion after SDS-polyacrylamide gel electrophoresis (PAGE) separation is currently a universal approach in molecular biology to characterize protein property, such as determination of amino acid (AA) sequences, identification of post-translational modifications, qualitative and quantitative characterization of degradation/oxidation of AA residues during physiologic process, and so on. Recent advances in resolution, sensitivity, and mass accuracy with mass spectrometry have greatly improved its application in efficiency and quality. In this study, a state-of-the-art mass spectrometer, LTQ-Orbitrap (Thermo Fisher Scientific, Waltham, MA) (Makarov, 2000), was used to characterize covalent binding of HKI-272 to protein(s)/peptide(s). Using Orbitrap’s superior features in mass accuracy (<5 ppm with external calibration) and resolution (>30,000), the accurate molecular masses of HKI-272 peptide adducts and fragment ions were obtained.

Materials and Methods

Materials. [14C]HKI-272 maleate was synthesized by the Radiosynthesis Group, Chemical Development, Wyeth Research (Pearl River, NY). The radiochemical purity and the chemical purity (by UV detection) were 98.2 and >99%, respectively. The specific activity of [14C]HKI-272 maleate was 89.7 μCl/μg (108.4 μCi/ml as the free base). Nonradiolabeled HKI-272 was obtained from Wyeth Research, Chemical and Pharmaceutical Development (Montreal, QC, Canada) and had a chemical purity of >99%. The chemical structure of HKI-272 with the position of 14C labels is shown in Fig. 1. Plasma of rats, mice, dogs, rabbits, monkeys, and humans was purchased from Biorecognition (Liverpool, NY). ProteoExtract All-in-One Trypsin Digestion kit was purchased from Calbiochem, (San Diego, CA). β-Mercaptoethanol and the chemicals used in the synthesis of the HKI-272-lysine adduct were purchased from Sigma-Aldrich (St. Louis, MO). Solvents used for extraction and chromatographic analysis were high-performance liquid chromatography (HPLC) or American Chemical Society reagent grade and were purchased from E Merck (Gibbstown, NJ). Deuteron oxide and dimethyl sulfoxide-d6 were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Incubation of HKI-272 with Human Plasma. A stock solution of HKI-272 was made by dissolving the powder in 1 ml of 50% (v/v) dimethyl sulfoxide. The final concentration was ~18 mM. An aliquot of 5.6 μl of [14C]HKI-272 solution was added to 1 ml of human plasma to reach the final concentration of HKI-272 of 100 μM with a specific activity of 3.5 μCi/μg. The sample was incubated at 37°C for 6 h in a shaking water bath. After the incubation, the mixture was subject to several experiments to characterize HKI-272 covalent binding, including 1) direct injection onto LC/MS to determine the molecular masses of the adducts of HKI-272 proteins; 2) loading on SDS-PAGE for protein separation followed by tryptic digestion for peptide mapping; and 3) HCl (2 N) digestion.

SDS-PAGE. An aliquot of 50 μl of β-mercaptoethanol was added to 950 μl of Laemmli sample buffer (Bio-Rad Laboratories) to make loading buffer. The buffer was mixed with an equal volume of HKI-272-treated plasma followed by boiling for 3 to 5 min. The mixture was cooled to room temperature before loading. The plasma sample (~75 μg) was loaded onto the gel, whereas Bio-Rad Laboratories molecular marker (20 μl) and GE Healthcare (Little Chalfont, Buckinghamshire, UK) Rainbow [14C]methylated protein molecular mass markers (4 μl) were loaded into a separate well. In addition, several protein samples were loaded as reference, including human serum albumin (HSA) solution (40 μg/ml), HSA-depleted HKI-272-treated plasma, and HSA-depleted HSA solution. HSA was depleted from human plasma by using a POROS Affinity Depletion Anti-HSA column (Applied Biosystems, Foster City, CA).

Electrophoresis was performed by applying a constant voltage of 50 V for 30 min for the stacking gel followed by applying a voltage of 100 V for approximately 2 h at room temperature to separate the plasma components. At the end of electrophoresis, the gel was stained with soaking by Bio-Safe Coomassie stain solution (Bio-Rad Laboratories) for 30 min with shaking. The protein proteins in the gel were detected by UV absorption. Radioactive bands were obtained by exposing the dried gel to a phosphor imaging plate (BAS-ccsr c020; Fuji Photo Film Co., Tokyo, Japan) for 4 days, followed by scanning the plate with a Fuji FLA-5000 scanner.

Tryptic Digestion. The radioactive band was cut from the SDS-PAGE gel and subjected to trypsin digestion using the reagents included in a ProteoExtract All-in-One Trypsin Digestion kit (Calbiochem) as follows. The gel pieces were transferred to a siliconized microfuge tube and washed three times with 80 μl of wash buffer at 37°C and then dried in a Savant SpeedVac (Thermo Fisher Scientific) for 20 min. The gel pieces were then transferred to a clean tube; 20 μl of digestion buffer and 2 μl of reducing agent were added; and the sample was mixed and incubated at 37°C for 10 min. After 10 min, the sample was removed from the heater and cooled to room temperature; then 2 μl of blocking agent was added, and the sample was incubated at room temperature for 10 min. The tryptic digestion was then initiated by adding 0.5 μl of trypsin and incubating the sample at 37°C for 2 h with shaking. After 2 h, the sample was centrifuged for 10 to 15 min at 10,000 g, and the supernatant was transferred to a vial for LC/MS analysis.

Hydrolysis by HCl. An aliquot of 200 μl of the human plasma incubated with [14C]HKI-272 for 6 h was transferred into 1 ml of HCl solution (2 N) followed by incubation at 90°C for 2 h with stirring. At the end of the incubation, the mixture was neutralized by adding 1 ml of 2 N NaOH and then adjusted to pH 3 to 4 by using acetic acid or NH4OH solution. An aliquot of 200 μl of the solution was mixed with 800 μl of 10% (v/v) formic acid before LC/MS analysis.

LC/MS Analysis. HPLC was performed on a Thermo Accela HPLC system (Thermo Fisher Scientific) equipped with an autosampler and a diode array detector. Separation was accomplished on a Jupiter column (4 μm, 2 × 250 mm, 300 A; Phenomenex, Torrance, CA) equipped with a guard column. During sample analysis, the HPLC column was at ambient temperature (~25°C), and the autosampler was maintained at 4°C. Mass spectrometric analysis was performed on a Thermo-Finnigan LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). Positive electrospray ionization was performed for all the analyses. The first 4 min of the HPLC flow was diverted to waste. From 4 min until 1 min before ending the HPLC run, the eluent was directed to the mass spectrometer. Settings used for the ionization source were capillary
temperature, 300°C; electrospray voltage, 4 kV; nebulizer gas flow, 80 (arbitrary units); and auxiliary gas flow, 25 (arbitrary units). Mass scan range was from 500 to 2000. Full-scan and product ion mass spectra (30% of collision energy) were both acquired on an Orbitrap with resolution at 30,000, and precursor and product ions were accurately measured with mass accuracy of <5 ppm. UltraTrak (Lancaster Synthesis, Windham, NH) was used as an external calibrant to calibrate Orbitrap before LC/MS analysis.

Radioactivity Profiles. β-RAM radioactivity detector (300 μl of liquid cell; INUS Systems, Tampa, FL) was configured on-line with HPLC to obtain radioactivity profiles. Ultima-Flo (PerkinElmer Life and Analytical Sciences, Wallham, MA) was delivered into the cell at 600 μl/min (3 times the HPLC flow rate). Luna 3.3 software (LabLogic Systems Ltd., Broomhill, UK) was used to control the operation of the β-RAM and record the radioactive chromatograms.

Synthesis and Purification of HKI-272-Lysine Adduct. One milliliter of HKI-272 (20 mg/ml in dimethylformamide) was added to 2 ml of Boc-Lys-OHMe (100 mg/ml in CH2CN) solution followed by the addition of 100 mg of K2CO3 powder to the solution. The mixture was heated at 90°C for 2 h with stirring. The formation of HKI-272 Boc-Lys-OHMe was followed by monitoring the MH+ at m/z 817 by LC/MS.

One milliliter of LiOH (1 M) solution and 5 ml of tetrahydrofuran were added to the reaction mixture. The mixture was stirred at 60°C for 1 h. The formation of the free carboxylic acid of Boc-Lys-OH HKI-272 adduct was monitored by LC/MS at MH+ at m/z 803.

Ten milliliters of 2 N HCl was added to the reaction mixtures to remove the Boc protecting group. The hydrolysis was performed at room temperature overnight. At the end of the reaction, the organic solvent was removed, and the aqueous mixture was subjected to purification on a semipreparative HPLC column (Phenomenex C18, 250 mm × 10 mm, 5 μm; Phenomenex, Torrance, CA) using an Agilent Technologies (Santa Clara, CA) 1200 HPLC system equipped with a fraction collector. Mobile phase A comprised 5 mM ammonium acetate in water, and mobile phase B was acetonitrile. The mobile phase flow rate was 4 ml/min (3 times the HPLC flow rate). Laura 3.3 software (LabLogic Systems Ltd., Broomhill, UK) was used to control the operation of the β-RAM and record the radioactive chromatograms.

Results

Molecular Mass of HKI-272 Adduct of Albumin. Human plasma incubated with [14C]HKI-272 was directly injected onto LC/MS to determine the intact molecular ions of HKI-272 protein adducts. The radioactivity profiles from 0-h (top) and 6-h (bottom) incubations are shown in Fig. 2A. A radioactive peak corresponding to free [14C]HKI-272 was observed in the 0-h incubation mixture at a retention time of 30.1 min, whereas two radioactive peaks were obtained in the 6-h incubation mixture. The [14C]HKI-272-related radioactive peak observed at a retention time of 47.2 min was postulated to be the HKI-272 adduct of albumin because its retention time was consistent with that of HSA based on the UV chromatogram (data not shown).

Besides unchanged HKI-272, the single HKI-272-related radioactive peak observed in the 6-h incubation mixture suggested the likelihood of only one major human plasma protein involved in the covalent binding of HKI-272.

When the radioactive eluent from the HPLC was introduced to the LTQ-Orbitrap, two multiply charged molecular ion envelopes were recorded and labeled with * and **, respectively, as shown in Fig. 2B. The m/z values of each ion and the lowest and highest charge states, M + 34H+ (m/z 1955.2 for albumin and 1971.6 for HKI-272 albumin adduct) and M + 55H+ (m/z 1209.1 for albumin and 1219.1 for HKI-272 albumin adduct), are labeled and shown in the figure. The deconvoluted molecular masses—66,443 Da for the dominant envelope * and 66,499 Da for the minor envelope **—are shown in the inset. The difference in molecular mass between two envelopes, 56 Da, corresponds to the molecular mass of HKI-272, suggesting that the multiply charged ions of the major envelope * correspond to HSA and the minor one to HKI-272 HSA adduct. Based on the AA sequence of HSA shown in the protein knowledgebase of Swiss-Prot (see Table 1; http://www.expasy.org/prot) and taking into account 17 intramolecular disulfide bonds, the molecular mass of HSA should be 66,438 Da, which is 5 Da less than the measured value. The molecular mass of the adduct also suggests that HKI-272 was attached to an HSA molecule.

SDS-PAGE Separation of HKI-272 Human Plasma. The results of SDS-PAGE separations of human plasma with the Coomassie-stained gel (left) and the radioactive image obtained by exposing the dried gel to a phosphor imaging plate, followed by scanning (right), are illustrated in Fig. 3. The radioactive protein band migrated the same distance as albumin, whereas radioactivity was nearly absent from the sample after HSA depletion. The single radioactive band also implies that albumin was the only plasma protein associated with HKI-272, which is consistent with that observed in Fig. 2A. To accurately identify the binding site of albumin with HKI-272, the radioactive band was cut for on-gel tryptic digestion and subsequently analyzed by LC/MS for peptide mapping.

Pepidite Mapping to Identify HKI-272 Binding Site by LC/MS. The tryptic digested mixture was injected on HPLC for separation and detected by mass spectrometer, UV, and β-RAM. Representative profiles of LC/MS [total ion chromatogram (TIC), base peak], UV (λ = 355 nm), and radiometric detector of the tryptic digestion products are shown in Fig. 4, where the UV absorption of HKI-272 was inserted showing one of the major absorption peak at 355 nm, which is similar to the HKI-272 peptide adduct (peak A). The figure shows that only one radioactive peak (A) was observed, which corresponds to the major peak in the UV chromatogram and a peak in TIC. The two other HKI-272-associated peaks are shown in the UV chromatograms, which correspond to an HKI-272 adduct of AA 185 to 195 (**) (a further tryptic digested product of peak A) and an incompletely digested HKI-272 HSA adduct (**). It is important to note that all the peaks shown in the TIC correspond solely to HSA but not any other plasma proteins, strongly indicating that the digested

Reversibility of HKI-272 from Protein Pellet. Reversibility of HKI-272 from covalent protein binding was evaluated by measurement of the recovery of HKI-272 from the protein pellet, which was the residue acetonitrile-precipitated human plasma treated with HKI-272. In the study, an aliquot of 50 μl of HKI-272 was added to 1.0 ml of human plasma at a final concentration difference of HKI-272 in the supernatant at 0 and 4 h. The pellet of HKI-272 from the protein pellet, which was the residue acetonitrile-precipitated human plasma treated with HKI-272. In the study, an aliquot of 50 μl of HKI-272 was added to 1.0 ml of human plasma at a final concentration difference of HKI-272 in the supernatant at 0 and 4 h. The pellet was removed at 0, 1.5, 3, and 18 h, followed by exhaustive extraction with acetonitrile, and analyzed by LC/MS as described above.

Insertion: deconvoluted molecular weights of HSA and HKI-272 HSA adduct.
(Mass difference = 66,999 – 66,443 = 556 Da (MW of HKI-272 = 556 Da)).

HPLC conditions:
Column: Jupiter, 4µ, 250 X 2.00 mm, 300 Å;
Mobile phase: A. 0.1 TFA in water, B. 0.1% TAF in acetonitrile;
Mobile phase gradient (B%): 5% (0-5 min), 5-90% (5-90 min), 90% (90-95 min), 90-5% (95-100 min), 5% (100-110 min).

**Fig. 2.** A. radioactivity profiles of human plasma incubated with $^{14}$C-HKI-272. B. mass spectra of albumin and HKI-272 albumin adduct obtained by direct injecting of human plasma incubated with $^{14}$C-HKI-272 on HPLC column.
radioactive band was principally albumin. More importantly, all the tryptic peptides of HSA having molecular masses >500 Da were identified, as indicated by the underlined letters in Table 1, which ensures that any peptides potentially bound to HKI-272 had been identified. Based on mass spectral data, peak A is an adduct of HKI-272 bound to peptide fragments 24 to 26 (AA residues of 182–195), and peak B is the peptide fragment 5 (residues of 21–41) containing Cys34, the only free sulfhydryl residue in HSA, which suggests that Cys34 did not contribute to the covalent binding of HKI-272 to human plasma.

The mass spectra of peak A obtained by full-scan and data-dependent MS2 on LTQ-Orbitrap are shown in Fig. 5, A and B, respectively. The high resolution (30,000) and accurate mass measurement on Orbitrap allowed us to readily determine the charge state and the accurate monoisotopic molecular mass of peak A. Based on $m/z$ difference between the isotopic ions, the charge state of $m/z$ 692.329 of peak A was determined to be 3+ and $m/z$ 1037.991 to be 2+. Therefore, the monoisotopic molecular mass of peak A was calculated to be 2073.967 Da. By searching the AA sequence of HSA displayed in the Swiss-Prot protein database, the molecular mass of peak A uniquely matches the adduct of HKI-272 with peptide LDELRDEGKASSAK (calculated 2073.967 Da).

The product ion spectrum of peak A, which was obtained by selecting the doubly protonated molecular ions at $m/z$ 1038 as the precursor ion, is shown in Fig. 5B. In the figure, the accurate $m/z$ values of product ions and their proposed identities (b, y) are labeled, and the proposed fragmentation pathways are also shown. HKI-272 can be confirmed to bind to the peptide based on the observation of the protonated HKI-272 at $m/z$ 557.207. The observation also suggests that intact HKI-272 was bound to HSA without being previously metabolized after incubation for 6 h. In addition, the AA sequence of the peptide was confirmed by the observation of y and b product ions. More significantly, the product ions labeled with * in the figure could lead us to identify the accurate site of HKI-272 binding, which shows that HKI-272 was bound to GKASSA. Considering the nucleophilicity of the side chain of the AA residues of GKASSA, we believe that Lys190 is the most likely site of binding with HKI-272.

In an effort to confirm the identity of the plasma protein that binds with HKI-272, HSA (40 mg/ml) was incubated with HKI-272 for 6 h in phosphate buffer, pH 8.0, followed by tryptic digestion and LC/MS analysis. Strikingly, the same peptide mentioned above (residue 182–195) was found to bind with HKI-272. This further confirmed that HSA is the plasma protein to which HKI-272 binds.

HCl Digestion of HKI-272 Plasma. The information obtained from the LC/MS analysis of the tryptic digested radioactive band suggests that HKI-272 was bound to Lys190 of HSA. To further confirm the binding site, the [14C]HKI-272-treated human plasma was directly hydrolyzed by HCl at 90°C for 2 h. The product profiles represented by radioactivity, UV ($\lambda = 355$ nm), and extract ion chromatogram (XIC) are shown in Fig. 6. It is interesting to note that the UV chromatogram matches well with the radioactivity profile. These radioactive peaks were identified by mass spectral data to be

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**TABLE 1**

Peptide mapping of HSA by trypsin digestion combined with LC/MS

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<th>$2 \times 10^4$</th>
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<th>$4 \times 10^4$</th>
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Underlined, identified by LC/MS on tryptic digested radioactive band of SDS-PAGE; bold, the tryptic peptide (residue 182–195) bound to HKI-272; *, Lys190, the residue bound to HKI-272.
the incompletely digested HKI-272 albumin adduct (the latest eluted), amide hydrolyzed of HKI-272 (m/z 446), HKI-272 (m/z 557, trace), as well as a group of HKI-272 adducts that include HKI-272-lysine (m/z 703), HKI-272 adducts of peptides GK (m/z 760), KA (m/z 774), GKA (m/z 831), KAS (m/z 861), GKAS (m/z 918), and EGKA (m/z 960). The mass spectral data, such as accurate molecular masses and the diagnostic product ions of these adducts, were summarized in Table 2. It can be noted that protonated HKI-272 (m/z 557) is a product ion for all the peptide adducts listed in the table, which is evidence that HKI-272 was bound to these peptides. Proposed general fragmentation pathways of these HKI-272 peptide/lysine adducts are shown in Fig. 7, including the cleavage of the amide bond of HKI-272 leading to fragment a, cleavage of C-C of the carbonyl group to fragment b, subsequent neutral loss of dimethyl amine from fragment b to fragment c, and C-N bond cleavage of peptide to HKI-272 to either fragment d (protonated peptide/AA) or protonated HKI-272. Strikingly, the lysine residue in the strings of EGKA, GKAS, GKA, and KAS, except for GK and KA, matches uniquely Lys190. This proves that Lys190 is the unique AA residue that binds covalently to HKI-272, which is consistent with the conclusion from tryptic digestion.

NMR of HKI-272-Lysine Adducts. The HKI-272-lysine adduct was purified by HPLC (purity of 98% based on UV response) before the sample was analyzed by NMR. The chemical shifts of 1H and 13C of HKI-272 and HKI-272-lysine adduct are listed in Table 3. The two resonances for the vinylic protons in the -unsaturated amide (C35, C36) are not present in the spectrum of the HKI-272-lysine adduct, suggesting the saturation of the double bond by an addition reaction with lysine. The correlation spectroscopy spectrum of the HKI-272-lysine adduct (data not shown) shows that the new methine proton is coupled to the two adjacent methylene groups. Therefore, the addition site was assigned to position C36 (β to the amide group). The structure of the HKI-272-lysine adduct was shown in Fig. 8.
To further confirm the identity of the HKI-272-lysine adduct present in the HCl hydrolyzed plasma, the synthetic HKI-272-lysine adduct was spiked into the HCl hydrolyzed plasma followed by analysis on LC/MS using two different mobile phase systems. Figure 9 shows the XIC of HKI-272-lysine adduct (m/z 703) and IS and shows that the HKI-272-lysine adducts released from the synthetic standard coeluted with that released from HCl hydrolysis. In addition, the same mass spectra were observed for the synthetic standard and that released from HCl hydrolysis of plasma (data not shown).

FIG. 5. A, full-scan mass spectrum of peak A obtained on LTQ-Orbitrap. B, product ion spectrum of peak A obtained on LTQ-Orbitrap by selecting m/z 1038 as the precursor ion for CID.
Reversibility of HKI-272 from Protein Pellet. The protein pellet obtained from human plasma treated with HKI-272 after exhaustive extraction with acetonitrile was subsequently reconstituted with phosphate buffer, pH 7.4, followed by incubation at 37°C to assess the reversibility of HKI-272 bound to plasma proteins. The time course for the recovery of HKI-272 bound to plasma protein pellet is shown in Fig. 10. After 4-h incubation with human plasma, the amount of HKI-272 extracted by acetonitrile was 49%, and then 51% remained in the protein pellet, which equals 0.82 in MS signal ratio of HKI-272/IS. After resuspension of the pellet in phosphate buffer, pH 7.4, the amount of extractable HKI-272 from pellet was almost undetectable at time 0 and gradually increased with incubation time and eventually reached approximately 43% of the total trapped in the pellet at 18 h, which was determined based on the total of 0.82 in MS peak area ratio of HKI-272/IS.

Discussion

Species-Specific Binding of HKI-272 to Plasma Protein. The recovery of HKI-272 from plasma, after exhaustive extraction with organic solvent, exhibited species-specific, significant binding to monkey and human plasma proteins but minimal or no binding to plasma protein obtained from dogs, rabbits, and rodents (see Table 4). Comparing the AA sequence displayed in the Swiss-Prot protein database, the AA sequence of human albumin is similar to monkey but very different from that of other species tested despite the similarity in molecular size of the albumin, ~66 kDa, from all the species. The peptide LDELRDEGKASSAK, which was identified to covalently bind to HKI-272 in the tryptic digested mixture, is unique in human and monkey albumin, as seen in Table 5, where the AA residues of 182 to 195 of albumin of mice, rats, rabbits, dogs, monkeys, and

<table>
<thead>
<tr>
<th>HKI-272 Adducts</th>
<th>Matching Residue</th>
<th>Precursor MH</th>
<th>Product Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>K*</td>
<td>190**</td>
<td>703,311</td>
<td>557, 658, 488, 147(d)</td>
</tr>
<tr>
<td>GK*</td>
<td>189–190**</td>
<td>760,333</td>
<td>557, 315(a), 273(b), 228(c), 204(d)</td>
</tr>
<tr>
<td>K*A</td>
<td>190–191**</td>
<td>774,349</td>
<td>557, 329(a), 287(b), 242(c), 218(d)</td>
</tr>
<tr>
<td>GK*A</td>
<td>189–191</td>
<td>831,370</td>
<td>557, 386(a), 344(b), 298(c), 275(d)</td>
</tr>
<tr>
<td>K*AS</td>
<td>189–192</td>
<td>861,382</td>
<td>557, 416(a), 374(b), 329(c), 305(d)</td>
</tr>
<tr>
<td>GK*AS</td>
<td>189–192</td>
<td>918,403</td>
<td>557, 473(a), 431(b), 386(c), 362(d)</td>
</tr>
<tr>
<td>EGK*A</td>
<td>188–191</td>
<td>960,413</td>
<td>557, 515(a), 473(b), 428(c), 404(d)</td>
</tr>
</tbody>
</table>

*, the AA residue to which HKI-272 is bound; **, more than one matching; (), fragmentation pathway of product ion formation (see Fig. 1).
Therefore, the recovery of HKI-272 from plasma, which occurred in a species-specific manner, is believed to be a consequence of the difference in AA sequence between human and monkey to other species. In addition, the same radioactivity profiles were observed for in vitro and in vivo plasma samples from monkeys administered \([^{14}C]\)HKI-272 at 100 mg/kg. This suggests that the covalent binding properties of HKI-272 to HSA characterized in vitro can present very much what happens to HKI-272 in vivo human plasma.

**Lys190 of HSA Covalent Binding to HKI-272.** In this study, Lys190 was identified as the sole AA residue in HSA to covalently bind to HKI-272. Lys190 is located at the junction of domains I (1–188) and II (189–385). This is a high-affinity region for substrate protein binding (Sudlow et al., 1975) and has been shown to have a pocket consisting mostly of hydrophobic and positively charged residues in which a very wide range of compounds may be accommodated. There are many examples of nonspecific binding of exogenous ligands (drugs) to this region. For example, Lys140, Lys195, and Lys199 have been observed to bind to ligands in a nonspecific manner (Yvon et al., 1989). In addition, many covalent binding reports of exogenous ligands (drugs) to HSA occurred around this region or the same residue. For example, Lys190 was found to covalent bind to pyridoxal 5' phosphate via Schiff base condensation (Bohney et al., 1992). Methylglyoxal, used as a covalent binding probe to interact with arginine residues of HSA, has been found to react with arginine 186 and 218, leading to the covalent binding (Ahmed et al., 2005). Lys195 was found to interact with acyl glucuronides of tolmetin and benoxaprofen, likely via Schiff base condensation when the acyl glucuronides of tolmetin and benoxaprofen were incubated with human plasma (Qiu et al., 1998). Alkylation of Lys199 with acetylsalicylic acid via acid-based neutralization was reported (Walker, 1976) as well.

Structural characterization of the synthetic HKI-272-lysine adduct by NMR showed that the covalent binding took place via Michael addition of e-amine of lysine to the \(\beta\)-carbon of the amide functional group of HKI-272. However, it is generally considered that Michael reactivity is not efficient for the amino group of lysine reacting with \(\alpha,\beta\)-unsaturated amide because the amino group is a hard nucleophile, whereas the \(\alpha,\beta\)-unsaturated amide is a soft electrophile. Bolton et al. (1997) used AA and peptide models to study the influence of paraquinone methide reactivity on the alkylation of thiol and amino groups in protein. Their results indicated that peptide alkylation should occur...
IS 20
0.35

Fig. 9. XIC of HKI-272-lysine adduct released by HCl hydrolysis of human plasma (A) and spiked with the standard reference (B).

FIG. 10. Time course of HKI-272 recovery from human plasma pellet after reconstitution with phosphate buffer, pH 7.4.

TABLE 4
Recovery of HKI-272 after incubation with plasma from various species at 37°C for 6 h

<table>
<thead>
<tr>
<th>Species</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>100</td>
</tr>
<tr>
<td>Rat</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit</td>
<td>100</td>
</tr>
<tr>
<td>Dog</td>
<td>100</td>
</tr>
<tr>
<td>Monkey</td>
<td>100</td>
</tr>
<tr>
<td>Human</td>
<td>100</td>
</tr>
</tbody>
</table>

in the order of cysteine thiol > ω-terminal amino > ω-lysine based on chemical reactivity (Bolton et al., 1997). Sayre et al. (2006) also indicated that the rank order of Michael addition reactivity for 4-hydroxy-2-nonenal is Cys >> His > Lys >> Arg. In fact, para-quinone methide from 2-tert-butyl-4,6-dimethylphenol was at least 10-fold more reactive to form adducts with cysteine than with the ω-amino group of lysine (McCracken et al., 1997). To evaluate the reactivity of different nucleophilic groups of AAs to HKI-272, we carried out a study in which HKI-272 was incubated with Phe-Lys, ω-acetyl lysine, ω-acetyl lysine, and GSH at 37°C for 24 h in buffers of pH 4.0, 7.4, and 10.0, respectively. It was found that only the GSH adduct of HKI-272 was formed in all the conditions. However, there are reports that Michael addition can take place between the ω-amine of lysine and the functional group of α,ω-unsaturated keto under physiologic pH and temperature if some electrophilic groups are introduced. For example, Barshteyn and Elfarra (2009) recently observed that a lysine adduct with hydroxymethylvinyl ketone was formed when ω-ω-acetyl lysine was incubated with the compound at 37°C, pH 7.4. We can propose that the intramolecular hydrogen bond between the hydroxyl oxygen and the hydrogen of the α- or β-carbon of the carbonyl group could contribute to enhancement of electrophilicity of the β-carbon, leading to Michael addition at physiologic pH and temperature. In addition, on incubation of hydroxymethylvinyl ketone with hemoglobin followed by tryptic digestion, the compound was found to be covalently bound to several nucleophilic AA residues, including cysteine and lysine residues in hemoglobin (Barshteyn and Elfarra, 2009). Zhang et al. (2003) reported direct binding evidence of the ω-amine of lysine residue of albumin to exogenous ligands via Michael addition. They identified a conjugate of lysine to desmethyl-7-defluorinated [14C](5S)-3-(5-chloro-2-methoxyphenyl)-3-fluoro-6-(trifluoromethyl)-1,3-dihydro-2H-indol-2-one (BMS-204352) in the

TABLE 5
AA sequence of residues 182 to 195 of albumin from various species

<table>
<thead>
<tr>
<th>AA Residues of 182 to 195 in Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
</tr>
<tr>
<td>Monkey</td>
</tr>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>Rabbit</td>
</tr>
<tr>
<td>Dog</td>
</tr>
<tr>
<td>Monkey</td>
</tr>
<tr>
<td>Human</td>
</tr>
</tbody>
</table>
HCl digestion mixture of the plasma from rats that were orally dosed with the compound. They proposed that the mechanism of adduct formation would be via Michael addition between an ε-amine of lysine and an ortho-quinone methide, a reactive intermediate formed as a result of CYP3A activation of BMS-204352 (Zhang et al., 2003).

Hence, we propose that to favor the Michael addition between HKI-272 and the lysine residue of HSA, the electrophilicity of α,β-unsaturated amide of HKI-272 needs to be enhanced. To illustrate our proposal, the following pathways are postulated. On initiation of the incubation with plasma, HKI-272 falls in the binding pocket of HSA via a nonspecific protein binding manner. In the binding site, the Michael reactivity of α,β-unaturated amide of HKI-272 could be enhanced after interaction with the AA residues with electro-withdrawing property in the vicinity. For example, the carbonyl oxygen of HKI-272 could form a hydrogen bond with the carboxylic acid or the hydroxyl group of the respective AA residues, which could result in the increase of electrophilicity of the function group. Ultimately, the Michael reactivity of HKI-272 could be high enough to form a covalent bond with Lys190. In addition, it is possible that the reverse interaction between HKI-272 and AAs in the active site could occur at a different rate, leading to the release of intact HKI-272.

Intact Cys34. In this study, it was shown that Cys34 did not contribute to the covalent binding of HKI-272, although Cys34 is the only known free sulfhydryl among 35 cysteine residues, given the fact that a thiol group is 10-fold more reactive than the ε-amino group of lysine. This is supported by the accurate measurement of the molecular mass of HSA (<5 Da) and the tryptic peptide fragment of 5 kDa of HSA, as well as the consistent AA sequence of fragment 5. In addition, we conducted an experiment to assess the involvement of Cys34 in covalent binding, in which HKI-272 was incubated in human plasma with/without coexistence of iodoacetamide (5 mM), and the recovery was similar in both conditions. Fasco et al. (2007) observed the formation of CN-Cys34 adduct on treatment of HSA with CN. They proposed that Cys34 sulfhydryl moiety should exist as a disulfide bond before reacting with CN because CN could not react with a free sulfhydryl group (Fasco et al., 2007). However, Bertucci et al. (1998) reported the derivatization of the Cys34 in human albumin by ethacrynic acid using mass spectrometry and circular dichroism, which suggests that Cys34 is a free sulfhydryl. Furthermore, Cys34 possesses a free sulfhydryl according to the results of x-ray crystal structural characterization of HSA, which indicated that Cys34 is located at the surface of the HSA molecule with its 5y atom oriented toward the interior and surrounded by side chains of Pro35, His39, Val77, and Tyr64 (Sugio et al., 1999). One explanation for not observing an HKI-272 adduct with Cys34 of HSA is that noncovalent protein binding proceeds so rapidly that the sulfhydryl group of Cys34 has little time to interact with HKI-272.

In conclusion, results from this study showed that low recovery of HKI-272 from human plasma was caused by the covalent protein binding that occurred via Michael addition, and Lys190 was determined to be the sole AA residue participating in covalent binding. In addition, the covalent binding of HKI-272 appeared to be reversible in nature, although further investigation is needed to obtain an in-depth understanding of the mechanism and its impact on the pharmacological activity of this drug. In future studies, it would be interesting to evaluate the potential involvement of any transporters and enzymes in

covalent binding of HKI-272 to human plasma. A crystallographic study may provide insight about such covalent binding.

Acknowledgments. This article is dedicated to the memory of Dr. Joseph McDevitt.

References


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