Mechanism for Covalent Binding of MLN3126, an Oral Chemokine C-C Motif Receptor 9 Antagonist, to Serum Albumins[§]

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ABSTRACT

N-[4-Chloro-2-[(1-oxidopyridin-4-yl)carbonyl]phenyl]-4-(propan-2-yloxy)benzenesulfonamide (MLN3126) is an orally available chemokine C-C motif receptor 9 selective antagonist. In nonclinical pharmacokinetic studies of MLN3126, nonextractable radioactivity was observed in plasma after oral administration of ¹⁴C-labeled MLN3126 (¹⁴C-MLN3126) to Sprague-Dawley (SD) rats. In this study, the nonextractable radioactive component was digested with trypsin or a nonspecific protease, pronase, after chemical reduction to obtain drug-peptide adducts or drug-amino acid adducts. The chemical structure of these adducts was characterized by liquid chromatography/mass spectrometry. The results demonstrated that the major part of the nonextractable

radioactivity was accounted for by covalent binding via the Schiff base formed specifically between the ε-amino group of lysine residue 199 in rat serum albumin and the carbonyl group of MLN3126. The half-life (*t*_{1/2}) of the total radioactivity in plasma during and after 21 daily multiple oral administrations of ¹⁴C-MLN3126 to SD rats was approximately 5-fold shorter than the reported *t*_{1/2} of albumin in rats. The data indicated that the covalent binding was reversible under physiologic conditions. The formation of the covalent binding was also confirmed in *in vitro* incubations with serum albumins from rats, humans, and dogs in the same manner, indicating that there are no qualitative interspecies differences in the formation of the Schiff base.

Introduction

N-[4-Chloro-2-[(1-oxidopyridin-4-yl)carbonyl]phenyl]-4-(propan-2-yloxy)benzenesulfonamide (MLN3126) is an orally available chemokine C-C motif receptor 9 (CCR9) selective antagonist that was discovered at Millennium Pharmaceutical Inc. CCR9 and its only known ligand, chemokine C-C motif ligand 25, play a critical role in the selective homing of lymphocytes to the intestine under inflammatory conditions. The migration of leukocytes to inflammatory sites is essential for host response to chronic inflammatory disease, with chemokines and their receptors serving as key factors in this process (Moser and Loetscher, 2001; Charo and Ransohoff, 2006). Additionally, it is reported that CCR9⁺ T cells in mucosal lymphoid tissue in patients with small bowel Crohn's disease have an activated phenotype in mesenteric lymph nodes and exhibit a T helper 1 and T helper 17 cells cytokine profile in small bowel lamina propria lymphocytes. Thus, interaction of CCR9 and chemokine C-C motif ligand 25 is considered

to contribute to the pathophysiology of inflammatory bowel disease (Hosoe et al., 2004; Johansson-Lindbom and Agace, 2007; Saruta et al., 2007; Andoh et al., 2008).

In nonclinical pharmacokinetic studies, ¹⁴C-labeled MLN3126 (¹⁴C-MLN3126) was administered orally to Sprague-Dawley (SD) rats. The extraction recovery of the radioactivity by organic solvents was not quantitative in rat plasma after oral dosing of ¹⁴C-MLN3126. In comparison, the proportion of the nonextractable radioactive fraction significantly increased with time after dosing. The formation of the nonextractable fraction suggested the involvement of a covalent binding of MLN3126 and/or its metabolites to plasma proteins. Although some drug candidates that are intentionally designed to covalently bind to a target biomolecule for the enhancement and prolongation of pharmacological effects can potentially form a covalent bond with off-target proteins (Kalgutkar and Dalvie, 2012), MLN3126 was not designed as such a covalent inhibitor. Furthermore, in some cases it is believed that covalent binding of drugs or their metabolites to endogenous macromolecules leads to toxicity or idiosyncratic adverse drug reactions (Tang and Lu, 2010). Therefore, understanding the cause of the nonextractable fraction is essential for assessment of not only the pharmacokinetic properties but also the subsequent impact on potential toxicological safety concerns for MLN3126.

Mass spectrometry is a powerful tool for the characterization of drug-protein adducts (Tailor et al., 2016). In particular, mass spectrometry-

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ABBREVIATIONS: AGP, α₁-acid glycoprotein; AUC, area under the plasma concentration-time curve; CCR9, chemokine C-C motif receptor 9; DSA, dog serum albumin; HPLC, high-performance liquid chromatography; HSA, human serum albumin; LC/MS, liquid chromatography/mass spectrometry; LSC, liquid scintillation counter; M-I, *N*-[4-chloro-2-[hydroxy(oxidopyridin-4-yl)methyl]phenyl]-4-(propan-2-yloxy)benzenesulfonamide; MLN3126, *N*-[4-chloro-2-[(1-oxidopyridin-4-yl)carbonyl]phenyl]-4-(propan-2-yloxy)benzenesulfonamide; MLN3126-lysine, *N*′-[5-chloro-2-[[4-(1-methylethoxy)phenyl]sulfonyl]amino]-phenyl[(1-oxidopyridin-4-yl)methyl]-L-lysine; MP, mobile phase; PBS, phosphate-buffered saline; RSA, rat serum albumin; SD, Sprague-Dawley; TFA, trifluoroacetic acid; *t*_{1/2}, half-life.

based proteomics can identify precise amino acid modifications in the protein structure. In this study, we characterized the nonextractable radioactivity observed in plasma collected after dosing of [^{14}C] MLN3126 to SD rats by liquid chromatography/mass spectrometry (LC/MS) after chemical and enzymatic derivatization. Additionally, we investigated the qualitative interspecies differences in the *in vitro* formation of the nonextractable fraction using plasma proteins from humans and other animals.

Materials and Methods

Materials

MLN3126 was synthesized by Albany Molecular Research, Inc. (Albany, NY). [^{14}C]MLN3126 with a specific radioactivity of 4.68 MBq/mg was synthesized by Sekisui Medical Co., Ltd. (Tokyo, Japan). The positions of the ^{14}C -labels are shown in Fig. 1. The specific activity was approximately 15% of the theoretical maximum specific activity (30.19 MBq/mg). The radiochemical purity (99.0%) and chemical identity of the labeled compound were verified by high-performance liquid chromatography (HPLC). *N*-[4-Chloro-2-[hydroxy(oxidopyridin-4-yl)methyl]phenyl]-4-(propan-2-yloxy)benzenesulfonamide (M-I) and *N*⁶-[5-chloro-2-([4-(1-methylethoxy)phenyl]sulfonyl)amino-phenyl]-(1-oxidopyridin-4-yl)methyl]-L-lysine (MLN3126-lysine) dihydrochloride were prepared by Takeda Pharmaceutical Company Ltd. (Osaka, Japan). Chemical structures of MLN3126, M-I, and MLN3126-lysine are shown in Fig. 1. Rat serum albumin (RSA), human serum albumin (HSA), dog serum albumin (DSA), human α_1 -acid glycoprotein (AGP), and human globulin Cohn fractions II and III were purchased from Sigma-Aldrich Co. (St. Louis, MO). Pronase (protease from *Streptomyces griseus*) was purchased from EMD Biosciences, Inc. (San Diego, CA). Sequencing grade modified trypsin was purchased from Promega Corporation (Madison, WI). Pooled plasma from rats and dogs was purchased from Charles River Laboratories Japan, Inc. (Ibaraki, Japan). Pooled plasma from humans was purchased from Nossan Corporation (Yokohama, Japan). All other chemicals and reagents were obtained from commercial sources.

Animals

Male Crl:CD(SD) rats (weight, 270.5–322.2 g; Charles River Laboratories Japan, Inc.) were used. They were fed laboratory chow (CR-LPF; Oriental Yeast Co., Ltd., Tokyo, Japan), had free access to water, and were housed for more than a week prior to use in temperature- and humidity-controlled rooms (20–26°C and 40%–70%, respectively) with 12-hour light/dark cycles. All experiments were performed in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee at the Takeda Pharmaceutical Company, Ltd.

Dosing and Plasma Sample Collection

[^{14}C]MLN3126 and unlabeled MLN3126 were dissolved in 10% (w/v) 2-hydroxypropyl- β -cyclodextrin solution in 0.1 M sodium hydrogen carbonate. In the single administration study, the solution of [^{14}C]MLN3126 was administered orally to SD rats ($n = 3$) at a dose of 10 mg at 9.26 MBq/10 ml/kg. Blood was taken from the abdominal aorta 8 hours after administration under anesthesia with diethyl ether. Blood samples were centrifuged at 1500g and 4°C for 10 minutes and the obtained plasma specimens were pooled. In the multiple administration study, the solution of [^{14}C]MLN3126 was administered orally to rats ($n = 3$) once daily for 21 days at a dose of 10 mg at 4.71 MBq/10 ml/kg. Blood was collected from the tail vein at 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 hours after the first, fourth, seventh, 11th, 14th, and 18th administrations. Collection of blood after the 21st administration was conducted at 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 hours and every 24 hours until 168 hours. Collection of blood after every other administration was performed 24 hours after dosing. Blood samples were centrifuged at 1500g and 4°C for 10 minutes to obtain plasma specimens. Radioactivity in the plasma specimens was measured using a liquid scintillation counter (LSC) (Hitachi, Ltd., Tokyo, Japan).

In Vitro Plasma Protein Binding

[^{14}C]MLN3126 dissolved in dimethyl sulfoxide/acetonitrile (1:9, v/v) solution (30 μl) was added to 3 ml of the plasma of rats, dogs, and humans, 0.05% (w/v)

[^{14}C]MLN3126 (*: ^{14}C -labeled position)

M-I

MLN3126-lysine

Fig. 1. Chemical structures of the compounds used in this study.

human AGP solution, 4% (w/v) HSA solution, and 0.05% human AGP/4% HSA solution in phosphate-buffered saline (PBS) at final concentrations of 0.1, 1, and 10 $\mu\text{g}/\text{ml}$, respectively. The samples (2.9 ml) spiked with [^{14}C]MLN3126 were centrifuged at approximately 230,000–270,000g and 4°C for 14 hours. The concentrations of the radioactivity in the spiked samples and supernatants after ultracentrifugation were determined. The percentage of protein binding was calculated using the following equation:

$$R = (1 - C_f / C_p) \times 100$$

where R = percentage of protein binding of the radioactivity (%); C_f = concentration of the radioactivity in the supernatant after ultracentrifugation (disintegrations per minute per milliliter); and C_p = concentration of the radioactivity in the spiked sample (disintegrations per minute per milliliter).

In Vitro Incubation of [^{14}C]MLN3126 with Plasma Proteins

RSA, HSA, DSA, and AGP from humans and globulins from humans were dissolved in Dulbecco's PBS at a concentration of 1% (w/v). RSA and HSA were also dissolved in PBS at a concentration of 0.1% (w/v). An aliquot of 10 μl of [^{14}C] MLN3126 solution (500 $\mu\text{g}/\text{ml}$ in acetonitrile) was added into 990 μl of the protein solutions and plasma from rats, humans, and dogs at a final concentration of 5 $\mu\text{g}/\text{ml}$, and then incubated for 16 hours at 37°C.

Extraction of the Radioactivity from In Vitro Incubations of [^{14}C]MLN3126 in the Presence of Hydride Reagent

An aliquot of 200 μl of rat plasma and solutions of RSA, HSA, and DSA (1% in PBS, w/v) spiked with [^{14}C]MLN3126 (5 $\mu\text{g}/\text{ml}$) was treated with sodium borohydride (final concentrations of 25 to 26 mg/ml) at room temperature for 2 hours. Trifluoroacetic acid (TFA) (20 μl) was added to the mixtures and left at room temperature for 2 hours. Then, 1 ml of acetonitrile was added to the samples and centrifuged at 1500g and 10°C for 10 minutes. Radioactivity in the supernatants was measured by LSC.

Extraction of the Radioactivity from Rat Plasma and In Vitro Incubations

An aliquot of each sample (200 μl) of pooled rat plasma collected 8 hours after the single dosing of [^{14}C]MLN3126 and of the in vitro incubations of [^{14}C]MLN3126 in protein solutions and plasma was mixed with 1 ml of methanol. Acetonitrile (1 ml) was added to another aliquot of each sample (200 μl) after treatment with 20 μl of TFA for 2 hours at room temperature. The radioactivity in the supernatants after centrifugation at 1500g and 10°C for 10 minutes was measured by LSC. Extracts (800 μl) of rat plasma collected after dosing of [^{14}C]MLN3126 with and without treatment with TFA were evaporated to dryness under a stream of nitrogen gas at room temperature and the residues were reconstituted in 400 μl of 4:1 (v/v) mixture of HPLC mobile phase (MP)-2A and MP-2B. The supernatants obtained by centrifugation at 1500g and 10°C for 10 minutes were subjected to HPLC and LC/MS analyses. In the multiple administrations study, an aliquot of 100 μl of rat plasma collected 2, 8, and 24 hours after the first, seventh, 14th, and 21st administrations was added with 500 μl of methanol. The radioactivity in the supernatants after centrifugation at 1500g and 10°C for 10 minutes was measured by LSC.

Chemical Reduction of Rat Plasma and In Vitro Incubations

An aliquot of 200 μl of rat plasma collected 8 hours after single dosing and from in vitro incubations of [^{14}C]MLN3126 with albumins (RSA, HSA, and DSA) was treated with sodium borohydride (final concentrations of 25 to 26 mg/ml) at room temperature. After 2 hours, 20 μl of TFA was added and the mixtures were left at room temperature for 2 hours. Then, 2 ml of acetonitrile was added to the reactions and the supernatants were removed after centrifugation at 1500g and 10°C for 10 minutes. The precipitates were rinsed with 1 ml of acetonitrile three times and then treated with proteases.

Trypsin Digestion

The precipitates from 200 μl of rat plasma collected after dosing of [^{14}C]MLN3126 and the in vitro incubations of [^{14}C]MLN3126 with RSA (0.1% in PBS), HSA (0.1% in PBS), and DSA (1% in PBS) after chemical reduction were used. These samples were treated with 250 μl of 10 mM dithiothreitol in 50 mM ammonium hydrogen carbonate buffer at 37°C for 2 hours. After 2 hours, 250 μl of 54 mM iodoacetamide in 50 mM ammonium hydrogen carbonate buffer was added and incubated in the dark at room temperature for 2 hours. Acetonitrile (2 ml) was added to the reaction mixtures and the supernatants were removed after centrifugation at 1500g and 10°C for 10 minutes. The precipitates were rinsed with 1 ml of acetonitrile and dried under a stream of nitrogen gas at room temperature. The precipitates were reconstituted in 200 μl of 50 mM ammonium hydrogen carbonate buffer. The reconstituted proteins were treated with 200 μl of 100 $\mu\text{g}/\text{ml}$ trypsin solution in 50 mM ammonium hydrogen carbonate buffer at 37°C overnight. Acetonitrile (150 μl) was added to an aliquot of 300 μl of the digested peptides and centrifuged at 1500g and 10°C for 10 minutes. After dilution with an equal volume of 50 mM ammonium hydrogen carbonate buffer, the supernatants were subjected to LC/MS and on-line flow scintillation analyses.

Pronase Digestion

The precipitates from 200 μl of rat plasma collected after dosing of [^{14}C]MLN3126 and the in vitro incubations of [^{14}C]MLN3126 with RSA (0.1% in PBS), HSA (0.1% in PBS), and DSA (1% in PBS) after chemical reduction were dissolved in 200 μl of 50 mM ammonium hydrogen carbonate buffer. The reconstituted proteins were treated with 200 μl of pronase solution (1365 proteolytic units/ml in 50 mM ammonium hydrogen carbonate buffer) for 16 hours at 37°C. Acetonitrile (250 μl) was added to an aliquot of 250 μl of the reaction solutions and centrifuged at 1500g and 10°C for 10 minutes. After dilution with an

equal volume of 10 mM aqueous solution of ammonium acetate, the supernatants were subjected to HPLC and LC/MS analyses.

HPLC and LC/MS.

Analysis of trypsin digests. HPLC was performed on a Prominence system (Shimadzu Corporation, Kyoto, Japan) equipped with a Jupiter 5 μm C18 column (250 $\times$ 4.6 mm i.d., 300 Å; Phenomenex, Inc., Torrance, CA) at 40°C. Mixtures of TFA, water, and acetonitrile (1:900:100 and 1:100:900, by volume) were used as the MP-1A and MP-1B, respectively. The total flow rate was 1.0 ml/min and the time program for the gradient elution was as follows: the concentration of MP-1B (v/v) was linearly increased from 10% to 40% over a period of 50 minutes, linearly increased from 40% to 100% over a period of 0.1 minutes, and then held at 100% for the following 9.9 minutes. Mass spectrometry was performed using a hybrid ion trap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific, Inc., Waltham, MA) equipped with an electrospray ionization interface. The spectrometer was calibrated with Tyrosin-1,3,6 (CS Bio Co., Menlo Park, CA) as an external reference prior to the experiments. The spectra were obtained in the positive ion mode at the resolving power of 30,000 (full width at half-maximum) for full scan and 7500 for product ion scan. The interface and mass spectrometer were operated under the following conditions: capillary temperature, 300°C; sheath gas flow rate (arbitrary units), 80; auxiliary gas flow rate (arbitrary units), 20; source voltage, 4.5 kV; capillary voltage, 28 V; and tube lens voltage, 75 V. The precursor ions selected with an isolation width of mass-to-charge ratio (m/z) 2.0 underwent collision-induced dissociation in the ion trap by collision with helium to give the product ion spectra. The value of the collision-induced dissociation energy was 40%. The radioactive components in the eluate from the HPLC were separately monitored by a flow scintillation analyzer (Radiomatic 610TR; PerkinElmer, Inc., Waltham, MA) with a cell volume of 0.5 ml. Ultima Flo-AP scintillation cocktail (PerkinElmer, Inc.) was used at a flow rate of 3.0 ml/min and the detector update time was set at 12 seconds.

Analysis of pronase digests and rat plasma extracts. HPLC was performed on a Prominence system equipped with a Luna 3- μm Phenyl-Hexyl column (75 $\times$ 4.6 mm i.d.; Phenomenex, Inc.) at 40°C. Mixtures of 10 mM ammonium acetate and acetonitrile (9:1 and 1:9, by volume) were used as the MP-2A and MP-2B, respectively. The total flow rate was 0.7 ml/min and the time program for the gradient elution was as follows: the concentration of MP-2B (v/v) was linearly increased from 20% to 60% over a period of 20 minutes, linearly increased from 60% to 100% over the following 5 minutes, and then held at 100% for 10 minutes. To acquire HPLC radiochromatograms, the radioactivity in the HPLC eluate collected in fractions every 0.5 minutes was measured by LSC. Mass spectrometry was separately performed using an LTQ Orbitrap XL equipped with an electrospray ionization interface. The spectra were obtained in the positive ion mode using the linear ion trap as an analyzer. The interface and mass spectrometer were operated under the same conditions as described previously.

Measurement of Radioactivity

The samples (50–500 μl) for measurement of the radioactivity were mixed with 10 ml of Liquid scintillator A (Wako Pure Chemical Industries, Ltd., Shiga, Japan) and counted by LSC for 1 or 5 minutes. The counting efficiency was determined by the external standard radiation source method.

Calculation of the Extraction Recovery

The extraction recovery of radioactivity (%) from samples was calculated using the following equation:

$$E = \frac{C_{\text{ex}} \times V_{\text{org}} \times (V_{\text{spl}} + V_{\text{soln}})}{C_{\text{org}} \times V_{\text{ex}} \times V_{\text{spl}}} \times 100$$

where E is the extraction recovery of radioactivity (%); C_{ex} is the ^{14}C count in the extract (disintegrations per minute); C_{org} is the ^{14}C count in the original sample (disintegrations per minute); V_{ex} is the volume of the extract applied for the ^{14}C count (microliters); V_{org} is the volume of the original sample applied for the ^{14}C count (microliters); V_{spl} is the volume of the sample applied for extraction (microliters); and V_{soln} is the volume of the organic solvent and acid added to the sample (microliters).

Pharmacokinetic Analysis of Total Radioactivity in Rat Plasma

The concentrations of total radioactivity were expressed as the MLN3126 equivalent value. The C_{max} , the concentration 24 hours after administration, and

Fig. 2. Concentration of the radioactivity in the plasma of rats during and after 21 daily multiple oral administrations of [^{14}C]MLN3126 at a daily dose of 10 mg at 4.71 MBq/kg. Each point represents the mean for three animals.

the time to reach $C_{\max}$ after the first, fourth, seventh, 11th, 14th, 18th, and 21st administrations were taken from the actual values. The half-life ($t_{1/2}$) and area under the plasma concentration-time curve (AUC) after the first, fourth, seventh, 11th, 14th, 18th, and 21st administrations were calculated using the non-compartmental model in WinNonlin version 4.1 (Pharsight Corporation, Inc., Sunnyvale, CA). The $t_{1/2}$ value was calculated from the actual values by the least-squares method. The AUC was calculated by the trapezoidal method until the sampling point 24 hours after dosing was reached. Each value was expressed as the mean $\pm$ S.D. of three animals.

Results

Concentrations and Pharmacokinetic Parameters of Radioactivity in the Plasma during and after Multiple Oral Administrations of [^{14}C]MLN3126 to Rats. After the first administration, the plasma concentrations of the radioactivity reached a $C_{\max}$ value of 4.321 $\mu\text{g Eq/ml}$ at 1.0 hour (Fig. 2; Table 1). The concentrations decreased to 0.845 $\mu\text{g Eq/ml}$ at 24 hours after administration. The $t_{1/2}$ and $\text{AUC}_{0-24 \text{ hour}}$ values after the first administration were 11.5 hours and 48.587 $\mu\text{g Eq-h/ml}$, respectively. The $C_{\max}$, the concentration 24 hours after administration, and the $\text{AUC}_{0-24 \text{ hour}}$ values slightly increased by the fourth administration compared with those after the first administration, and they attained almost steady state within the first 4 days. The $C_{\max}$, the time to reach $C_{\max}$, the concentration 24 hours after administration, $t_{1/2}$, and $\text{AUC}_{0-24 \text{ hour}}$ values after the fourth administration were

1.0 hour, 5.392, 1.214 $\mu\text{g Eq/ml}$, 13.1 hours, and 62.364 $\mu\text{g Eq-h/ml}$, respectively, and those after the 21st administration were 1.0 hour, 5.936, 1.237 $\mu\text{g Eq/ml}$, 13.9 hours, and 63.425 $\mu\text{g Eq-h/ml}$, respectively. The plasma concentrations of the radioactivity after the 21st administration gradually decreased to an undetectable level after 168 hours. The extraction recovery percentages of the radioactivity from rat plasma collected 8 hours after the first, seventh, 14th, and 21st administrations after dosing with five volumes of methanol were 38.3%, 25.5%, 28.6%, and 28.5%, respectively. The proportion of the nonextractable radioactive fraction increased to approximately 95% of total radioactivity 24 hours after each dosing.

Nonextractable Radioactive Fraction of Rat Plasma Collected after Dosing of [^{14}C]MLN3126. The extraction recovery percentage of the radioactivity from pooled rat plasma collected 8 hours after single oral dosing of [^{14}C]MLN3126 with methanol was 47.1%. In the HPLC radiochromatogram of the methanol extract, unchanged MLN3126 accounted for more than 80% of the total radioactive component. In contrast, the radioactivity in the rat plasma after treatment with TFA was quantitatively recovered in the extract. The radiochromatogram of the extract after treatment with TFA showed that approximately 90% of the total radioactivity was comprised of unchanged MLN3126.

In Vitro Formation of the Nonextractable Fraction. The extraction recovery percentages of radioactivity from in vitro incubations of [^{14}C]MLN3126 with rat plasma, human plasma, dog plasma, RSA,

TABLE 1

Pharmacokinetic parameters of the radioactivity in the plasma of rats during and after 21 daily multiple oral administrations of [^{14}C]MLN3126 at a daily dose of 10 mg at 4.71 MBq/kg

Each value represents the mean $\pm$ S.D. value for three animals.

Day	Pharmacokinetic Parameter				
	$T_{\max}$	$C_{\max}^a$	$C_{\min}^{a,b}$	$t_{1/2}$	$\text{AUC}_{0-24 \text{ h}}^a$
	h	$\mu\text{g Eq/ml}$	$\mu\text{g Eq/ml}$	h	$\mu\text{g Eq-h/ml}$
1	1.0 $\pm$ 0.0	4.321 $\pm$ 1.351	0.845 $\pm$ 0.280	11.5 $\pm$ 0.8	48.587 $\pm$ 15.443
4	1.0 $\pm$ 0.0	5.392 $\pm$ 1.041	1.214 $\pm$ 0.337	13.1 $\pm$ 0.3	62.364 $\pm$ 16.729
7	0.7 $\pm$ 0.3	5.717 $\pm$ 0.507	1.062 $\pm$ 0.171	12.5 $\pm$ 0.4	57.117 $\pm$ 7.340
11	0.8 $\pm$ 0.3	6.857 $\pm$ 1.963	1.129 $\pm$ 0.294	12.5 $\pm$ 0.1	63.574 $\pm$ 15.484
14	0.7 $\pm$ 0.3	7.502 $\pm$ 1.195	1.349 $\pm$ 0.298	13.6 $\pm$ 0.5	70.882 $\pm$ 13.612
18	0.7 $\pm$ 0.3	5.331 $\pm$ 1.092	1.184 $\pm$ 0.302	14.1 $\pm$ 0.6	58.867 $\pm$ 15.921
21	1.0 $\pm$ 0.0	5.936 $\pm$ 1.527	1.237 $\pm$ 0.445	13.9 $\pm$ 1.1	63.425 $\pm$ 23.450

$C_{\min}$, concentration 24 hours after administration; $T_{\max}$, time to reach $C_{\max}$.

^aMLN3126 equivalent.

^bConcentration 24 hours after administration.

TABLE 2

Extraction recovery of the radioactivity from in vitro incubations of [^{14}C]MLN3126 at a final concentration of 5 $\mu\text{g/ml}$ in blank plasma and protein solutions for 16 h at 37°C

NaBH₄ (+) TFA(+) indicates extracted with 5 volumes of acetonitrile after treatment with NaBH₄ followed by treatment with TFA; *TFA (-)* indicates extracted with 5 volumes of methanol; and *TFA (+)* indicates extracted with 5 volumes of acetonitrile after treatment with TFA. Mean values: n = 3.

Sample	Extraction Recovery (%)		
	TFA (-)	TFA (+)	NaBH ₄ (+) TFA (+)
Rat plasma	54.9	104.9	N.D.
Human plasma	47.3	104.7	N.D.
Dog plasma	74.8	104.6	N.D.
1% RSA	30.2	103.8	31.9
1% HSA	41.0	103.0	39.1
1% DSA	81.7	105.0	78.0
1% AGP	105.7	103.6	N.D.
1% Globulins	100.4	100.8	N.D.

N.D., not determined.

HSA, and DSA were 54.9%, 47.3%, 74.8%, 30.2%, 41.0%, and 81.7%, respectively, whereas the radioactivity from incubations with AGP and globulins from humans was quantitatively recovered (Table 2). After treatment of the in vitro incubations with TFA, the radioactivity was quantitatively recovered from all samples.

Hydride Reduction of Rat Plasma and In Vitro Incubations. Rat plasma collected 8 hours after single oral dosing of [^{14}C]MLN3126 and from in vitro incubations of [^{14}C]MLN3126 with RSA, HSA, and DSA was treated with an excess of sodium borohydride. The extraction recovery percentages of the radioactivity from rat plasma collected after dosing of [^{14}C]MLN3126, RSA, HSA, and DSA were 46.4%, 31.9%, 39.1%, and 78.0%, respectively. The predominant radioactive component in the extract after hydride and TFA treatments consisted of M-I, derived by reduction of the carbonyl group of MLN3126 to the secondary alcohol, whereas unchanged MLN3126 was not detected.

Identification of Radioactive Components in the Pronase Digests. Rat plasma collected after dosing of [^{14}C]MLN3126 and from in vitro incubation samples after hydride reduction was treated with a nonspecific protease, pronase, which hydrolyzes proteins into individual amino acids. In each sample after pronase treatment, the extraction recovery of radioactivity with acetonitrile was quantitative. A predominant radioactive peak was detected after approximately 9 minutes in the HPLC radiochromatograms of the extracts (Fig. 3). The full ion mass spectrum of the predominant peak in the rat plasma extract gave the protonated molecule at m/z 577 (Fig. 4A). The product ion mass spectrum of m/z 577 showed a dehydrated ion at m/z 559 and a fragment ion at m/z 431, which corresponded to MLN3126 lacking an oxygen derived by the loss of the lysine moiety from the molecule (Fig. 4B). Other fragments observed at m/z 413, 389, and 231 were assumed to be formed by additional dehydration and losses of isopropyl and 4-(propan-2-yloxy)benzenesulfonyl moieties, respectively. The predominant peak detected in the extracts of the RSA, HSA, and DSA samples gave identical mass spectra as those detected in rat plasma. The mass spectra and the HPLC retention time of the predominant peak were in good agreement with those of the synthetic MLN3126-lysine (Fig. 4, C and D).

Identification of Radioactive Components in the Trypsin Digests. Rat plasma collected after dosing of [^{14}C]MLN3126 and from in vitro incubation samples after hydride reduction and alkylation was treated with trypsin. The radioactivity was quantitatively extracted from the samples. The extracts showed predominant radioactive peaks in the HPLC radiochromatograms (Fig. 5). The full ion mass spectrum of

Fig. 3. ^{14}C -Radiochromatograms of pronase digests of rat plasma collected 8 hours after dosing of [^{14}C]MLN3126 (A) and from in vitro incubations of [^{14}C]MLN3126 with RSA (B), HSA (C), and DSA (D) after hydride reduction. Min, minutes.

peak-A in RSA gave singly and doubly protonated ions at m/z 1457.5211 and 729.2668, respectively (Fig. 6A). The product ion mass spectrum of m/z 729.3 showed characteristic ions at m/z 1027.4471 and 431.0822 (Fig. 6B). The fragment ion at m/z 431.0822 corresponded to protonated MLN3126 lacking an oxygen atom (calculated 431.0827 Da) and the ions at m/z 389 and 231 were identical with the product ions of MLN3126-lysine. The product ion at m/z 1027.4471 was derived by loss of the MLN3126 moiety (calculated 430.0754 Da). In the Swiss-Prot protein database, the ion observed at m/z 1027.4471 matched the peptide MKCSSMQR of RSA with carbamidomethylation of the cysteine residue (calculated 1027.4482 Da). The product ion mass spectrum of m/z 1027.4 showed y- and b-type fragment ions, confirming the amino acid sequence of the peptide (Fig. 6C).

In the same way, the predominant radioactive peaks of the rat plasma collected after dosing of [^{14}C]MLN3126 (peak-A) and from in vitro

Fig. 4. Mass spectra of the predominant radioactive peak detected in the pronase digest of the rat plasma after hydride reduction and the synthetic MLN3126-lysine. Full ion mass spectra for rat plasma (A) and the synthetic MLN3126-lysine (C), and MS² spectra of the precursor ion at *m/z* 577 for rat plasma (B) and the synthetic MLN3126-lysine (D).

incubations with HSA (peak-B) and DSA (peak-C) showed the characteristic product ions at *m/z* 1027.4478, 947.5332, and 981.5178, respectively (Supplemental Figs. 1–3). These ions were assigned as the peptides MKCSSMQR (calculated 1027.4482 Da), LKCASLQK (calculated 947.5342 Da), and FKCASLQK (calculated 981.5186 Da) of RSA, HSA, and DSA, respectively. The peptide MKCSSMQR observed in rat plasma collected after dosing of [¹⁴C]MLN3126 was identical to that in RSA. Each peptide was assigned to a specific amino acid sequence from 198 to 205 in serum albumins across the species (Table 3). In the radiochromatogram of DSA, another radioactive peak (peak-D) was observed (Fig. 5D). The full ion mass spectrum of peak-D gave singly and doubly protonated ions at *m/z* 875.3911 and 438.1991, respectively (Fig. 7A). The product ion at *m/z* 445.3131 derived from the precursor ion at *m/z* 875.4 (Fig. 7B) matched the peptide KLGK (calculated 445.3133 Da) in the Swiss-Prot protein database. The amino acid sequence of the peptide was confirmed by the fragment ions of *m/z*

445.3 (Fig. 7C). The peptide KLGK corresponded to the amino acid residues from 429 to 432 in DSA.

In Vitro Plasma Protein Binding. The percentages of the in vitro plasma protein binding of [¹⁴C]MLN3126 at concentrations of 0.1, 1, and 10 μ g/ml were 99.0%, 98.8%, and 98.7%, respectively, in rats; 97.3%, 97.3%, and 97.0%, respectively, in dogs; and >99.8%, 99.8%, and 99.8% in humans, respectively. The percentages of protein binding of [¹⁴C]MLN3126 at the concentrations of 0.1, 1, and 10 μ g/ml were 99.0%, 99.0%, and 98.9%, respectively, in 4% HSA; 71.9%, 64.6%, and 39.5%, respectively, in 0.05% AGP; and 99.1%, 99.1%, and 98.8%, respectively, in the 4% HSA/0.05% AGP mixture, respectively.

Discussion

The extraction recovery of the radioactivity from the rat plasma collected 8 hours after single oral dosing of [¹⁴C]MLN3126 with

Fig. 5. ^{14}C -Radiochromatograms of trypsin digests of rat plasma collected 8 hours after dosing of ^{14}C MLN3126 (A) and from in vitro incubations of ^{14}C MLN3126 with RSA (B), HSA (C), and DSA (D). Min, minutes.

methanol was 47.1%, and about one-half of the total radioactivity was considered to be the nonextractable fraction. The formation of the nonextractable fraction was also observed in the in vitro incubations of ^{14}C MLN3126 in plasma and in the solutions of RSA, HSA, and DSA (Table 2). After hydride reduction and pronase digestion of the nonextractable fractions of the in vivo and in vitro samples, a predominant radioactive peak was detected in the digests (Fig. 3). When

the nonextractable fraction had not been treated with reducing agent, unchanged MLN3126 was released during pronase digestion. The chemical structure of the peak was identified by LC/MS analysis as MLN3126-lysine, in which lysine and MLN3126 were linked via the secondary amine with loss of the carbonyl oxygen of MLN3126 (Fig. 4). Considering that MLN3126 was bound to the specific amino acid sequences from 198 to 205 of serum albumins (Table 3) and the C-terminal side of Lys-199 was not digested by trypsin, Lys-199 was assumed to be the amino acid residue covalently bound to MLN3126 via the secondary amine. Furthermore, Lys-429 in DSA was considered to be bound to MLN3126 as well because MLN3126 was also bound to the peptide KLGK located from 429 to 432 in DSA. The formation of covalent binding with this residue was presumed to be caused by the unique Lys-429 in DSA since residue 429 is an asparagine in RSA and HSA (Table 3). Some of the lysine residues, including Lys-199, in HSA have been reported to covalently react with drugs or metabolites. For example, Lys-199 and Lys-195 form covalent binding with the acyl glucuronide of tolmetin via Schiff base formation (Ding et al., 1995). Aspirin also transfers an acetyl group to Lys-199 and is hydrolyzed to salicylic acid by HSA (Yang et al., 2007). Furthermore, neratinib (HKI-272) is covalently bound to Lys-190 in HSA via Michael addition (Wang et al., 2010).

The nonextractable fraction of rat plasma released unchanged MLN3126 by TFA treatment. In contrast, the nonextractable fraction was quantitatively converted to an acid-stable form after hydride reduction. These results suggest that the secondary amine linkage originally exists as the acid-labile Schiff base, not as a hemiaminal intermediate, formed between the carbonyl group of MLN3126 and the ϵ -amino group of Lys-199 in intact rat plasma and in vitro incubations. In some cases, treatment of carbonyl compounds and primary amines with hydride reagent could accelerate the formation of secondary amines by reducing an unstable Schiff base. However, although rat plasma collected after dosing of ^{14}C MLN3126 and from in vitro incubation samples was treated with hydride reagent, additional formation of the secondary amine (nonextractable fraction) was not detected under the conditions of this study. The carbonyl group of MLN3126 is considered to be rapidly reduced to the nonreactive secondary alcohol resulting in M-I prior to additional Schiff base formation. The stabilization of the Schiff base by reduction and the extraction recovery of the radioactivity from enzymatic digests were both estimated as being quantitative. Furthermore, the treatment of rat plasma collected after dosing of ^{14}C MLN3126 with pronase generated a predominant radioactive component, MLN3126-lysine. From these results, the major part of the mechanism of the nonextractable binding is considered as being accounted for by the Schiff base formation.

Formation of the covalent binding was not observed in AGP and globulins from humans, unlike in serum albumins (Table 2). The covalent binding of MLN3126 was formed with only the specific lysine residues (Lys-199 and Lys-429) of serum albumins, although serum albumins contain more than 50 lysine residues. From the site-specific covalent binding of MLN3126 with serum albumins, we propose the following binding mechanism. First, MLN3126 binds to the drug binding sites in serum albumins in a nonspecific protein binding manner. Consequently, MLN3126 is held at the favorable position, in terms of steric and/or electronic conditions, to react with the neighboring specific lysine residues. Then, the ϵ -amino group of lysine residues nucleophilically attacks the carbonyl carbon of MLN3126 to form the Schiff base. The amide group of Asn-429 in RSA and HSA could not form a Schiff base with MLN3126 because of its weak nucleophilicity, even if MLN3126 was held close to the asparagine residue in the binding site. Similar reacting pathways initiated by noncovalent protein binding have been proposed (Yang et al., 2007; Wang et al., 2010). From the proposed

Fig. 6. Mass spectrum of the peak-A detected in the trypsin digest of RSA (A), MS² spectrum of the precursor ion at m/z 729.3 (B), and MS³ spectrum of the precursor ion at m/z 1027.4 (C). C*, carbamidomethyl cysteine.

binding mechanism, formation of the covalent binding would not involve reactive metabolites, which could lead to toxicity or idiosyncratic adverse drug reaction.

It has been reported that there are irreversible and reversible types of protein covalent binding (Zhang et al., 2005; Wang et al., 2010). For example, BMS-204352 forms an irreversible covalent binding with

TABLE 3

Amino acid sequences of the binding sites of MLN3126 in albumins

Albumin	Amino Acid Sequence	
	198–205 in Albumin	429–432 in Albumin
RSA	MKCSSMQR	NLGR
HSA	LKCASLQK	NLGK
DSA	FKCASLQK	KL GK

plasma proteins, mostly serum albumins, in humans, dogs, and rats (Zhang et al., 2005). After administration of radiolabeled BMS-204352 to humans, dogs, and rats, the $t_{1/2}$ values of radioactivity (11, 7, and

1 day, respectively) were similar to the reported $t_{1/2}$ values of albumins (19, 6.8, and 2.6 days, respectively) (Schreiber et al., 1971; Morris and Preddy, 1986; Reed et al., 1988). Accordingly, the elimination of radioactivity derived from irreversible covalent binding would depend on degradation of the bound proteins. In comparison, MLN3126 forms a covalent binding via a Schiff base, which is presumed to be reversible to release unchanged MLN3126 under physiologic conditions owing to its chemical nature (Fig. 8). During 21 daily multiple oral administrations of [14 C]MLN3126, the concentration of radioactivity in the rat plasma reached almost steady state within the first 4 days (Fig. 2; Table 1). The persistence of radioactivity in the rat plasma was not observed after the 21st administration. Moreover, the $t_{1/2}$ values of the total radioactivity (approximately 13 hours) in rat plasma during and after multiple oral

Fig. 7. Mass spectrum of the peak-D detected in the trypsin digest of DSA (A), MS² spectrum of the precursor ion at m/z 875.4 (B), and MS³ spectrum of the precursor ion at m/z 445.3 (C). C*, carbamidomethyl cysteine.

Fig. 8. Presumed mechanism of the formation and degradation of the nonextractable fraction via the Schiff base between MLN3126 and the lysine residues in serum albumins.

administrations of [^{14}C]MLN3126 were approximately 5-fold shorter than the reported $t_{1/2}$ value of albumin in rats. These results suggest that the covalent binding of MLN3126 via the Schiff base is reversible under physiologic conditions and has little impact on the pharmacokinetic properties of MLN3126.

In conclusion, the results of chemical and biologic derivatization of the nonextractable radioactive fraction of rat plasma collected 8 hours after oral dosing of [^{14}C]MLN3126 followed by LC/MS analysis suggested that the major part of the nonextractable radioactivity was accounted for by covalent binding via the Schiff base formed specifically between the ϵ -amino group of Lys-199 in RSA and the carbonyl group of MLN3126. Formation of the covalent binding would not involve reactive metabolites, which could lead to toxicity or idiosyncratic adverse drug reaction. The result of multiple administrations of [^{14}C]MLN3126 to rats suggested that the covalent binding of MLN3126 with RSA was reversible under physiologic conditions. It is considered that nonspecific protein binding of MLN3126 to subdomain IIA in albumin is required to hold MLN3126 at a favorable position prior to the reaction. The formation of the covalent binding was also confirmed in the *in vitro* incubations with RSA, HSA, and DSA in the same manner, suggesting that there are no qualitative interspecies differences in formation of the Schiff base with Lys-199.

Authorship Contributions

Participated in research design: Narita, Morohashi, Tohyama, Takeuchi, Tagawa, Kondo, Asahi.

Conducted experiments: Narita, Morohashi, Tohyama, Takeuchi.

Performed data analysis: Narita, Morohashi, Tohyama, Takeuchi, Tagawa, Kondo.

Wrote or contributed to the writing of the manuscript: Narita, Morohashi, Tohyama, Takeuchi, Tagawa, Kondo, Asahi.

References

Andoh A, Yagi Y, Shioya M, Nishida A, Tsujikawa T, and Fujiyama Y (2008) Mucosal cytokine network in inflammatory bowel disease. *World J Gastroenterol* **14**:5154–5161.

- Charo IF and Ransohoff RM (2006) The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* **354**:610–621.
- Ding A, Zia-Amirhosseini P, McDonagh AF, Burlingame AL, and Benet LZ (1995) Reactivity of tolmetin glucuronide with human serum albumin. Identification of binding sites and mechanisms of reaction by tandem mass spectrometry. *Drug Metab Dispos* **23**:369–376.
- Hosoe N, Miura S, Watanabe C, Tsuzuki Y, Hokari R, Oyama T, Fujiyama Y, Nagata H, and Ishii H (2004) Demonstration of functional role of TECK/CCL25 in T lymphocyte-endothelium interaction in inflamed and uninfamed intestinal mucosa. *Am J Physiol Gastrointest Liver Physiol* **286**:G458–G466.
- Johansson-Lindbom B and Agace WW (2007) Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunol Rev* **215**:226–242.
- Kalgutkar AS and Dalvie DK (2012) Drug discovery for a new generation of covalent drugs. *Expert Opin Drug Discov* **7**:561–581.
- Morris MA and Preddy L (1986) Glycosylation accelerates albumin degradation in normal and diabetic dogs. *Biochem Med Metab Biol* **35**:267–270.
- Moser B and Loetscher P (2001) Lymphocyte traffic control by chemokines. *Nat Immunol* **2**:123–128.
- Reed RG, Davidson LK, Burrington CM, and Peters T, Jr (1988) Non-resolving jaundice: bilirubin covalently attached to serum albumin circulates with the same metabolic half-life as albumin. *Clin Chem* **34**:1992–1994.
- Saruta M, Yu QT, Avanesyan A, Fleschner PR, Targan SR, and Papadakis KA (2007) Phenotype and effector function of CC chemokine receptor 9-expressing lymphocytes in small intestinal Crohn's disease. *J Immunol* **178**:3293–3300.
- Schreiber G, Urban J, Zähringer J, Reutter W, and Froesch U (1971) The secretion of serum protein and the synthesis of albumin and total protein in regenerating rat liver. *J Biol Chem* **246**:4531–4538.
- Taylor A, Waddington JC, Meng X, and Park BK (2016) Mass spectrometric and functional aspects of drug-protein conjugation. *Chem Res Toxicol* **29**:1912–1935.
- Tang W and Lu AY (2010) Metabolic bioactivation and drug-related adverse effects: current status and future directions from a pharmaceutical research perspective. *Drug Metab Rev* **42**:225–249.
- Wang J, Li-Chan XX, Atherton J, Deng L, Espina R, Yu L, Horwatt P, Ross S, Lockhead S, Ahmad S, et al. (2010) Characterization of HKI-272 covalent binding to human serum albumin. *Drug Metab Dispos* **38**:1083–1093.
- Yang F, Bian C, Zhu L, Zhao G, Huang Z, and Huang M (2007) Effect of human serum albumin on drug metabolism: structural evidence of esterase activity of human serum albumin. *J Struct Biol* **157**:348–355.
- Zhang D, Krishna R, Wang L, Zeng J, Mitroka J, Dai R, Narasimhan N, Reeves RA, Srinivas NR, and Klunk LJ (2005) Metabolism, pharmacokinetics, and protein covalent binding of radiolabeled MaxiPost (BMS-204352) in humans. *Drug Metab Dispos* **33**:83–93.

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