

Title

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

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Running Title

MLN3126 Covalent Binding to Serum Albumins

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Abbreviations: AGP, α_1 -acid glycoprotein; AUC, area under the plasma concentration-time curve; CCL25, chemokine C-C motif ligand 25; 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; C_{\max} , maximum concentration; C_{\min} , concentration at 24 hours after administration; MLN3126-lysine, *N*⁶-{[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; TFA, trifluoroacetic acid; T_{\max} ,
time to reach C_{\max}

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 non-clinical pharmacokinetic studies of MLN3126, non-extractable radioactivity was observed in plasma after oral administration of ¹⁴C-labeled MLN3126 ([¹⁴C]MLN3126) to Sprague-Dawley (SD) rats. In this study, the non-extractable 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 were characterized by liquid chromatography/mass spectrometry. The results demonstrated that the major part of the non-extractable 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 *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 physiological 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 not 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 (CCL25), 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 (Charo and Ransohoff, 2006; Moser and Loetscher, 2001). 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 Th1 and Th17 cytokine profile in small bowel lamina propria lymphocytes. Thus, interaction of CCR9 and CCL25 is considered to contribute to the pathophysiology of inflammatory bowel disease (Andoh et al., 2008; Hosoe et al., 2004; Johansson-Lindborn and Agace, 2007; Saruta et al., 2007).

In non-clinical 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 non-extractable radioactive fraction significantly increased with time after dosing. The formation of the non-extractable 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 non-extractable 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-based proteomics can identify precise amino acid modifications in the protein structure. In this study, we characterized the non-extractable radioactivity observed in plasma collected after dosing of [¹⁴C]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 non-extractable 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. Serum albumins from rats (RSA), humans (HSA), and dogs (DSA), α_1 -acid glycoprotein (AGP) from humans, and globulins Cohn fraction II, III from humans 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. (Ibariki, Japan). Pooled plasma from humans was purchased from Nosan Corporation (Yokohama, Japan). All other chemicals and reagents were obtained from commercial sources.

Animals. Male Crl:CD(SD) rats (weight, 270.5 to 322.2 g; Charles River 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 to 26°C and 40 to 70%) 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-9.26 MBq/10 ml/kg. Blood was taken from the abdominal aorta at 8 hours after administration under anesthesia with diethyl ether. Blood samples were centrifuged at $1500 \times g$ 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-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 1st, 4th, 7th, 11th, 14th, and 18th administration. 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 at 24 hours after dosing. Blood samples were centrifuged at $1500 \times g$ 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 ($30 \mu\text{l}$) was added to 3 ml of the plasma of rats, dogs, and humans, 0.05% (w/v) 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 $10 \mu\text{g/ml}$, respectively. The samples (2.9 ml) spiked with [^{14}C]MLN3126 were centrifuged at approximately $230,000\text{--}270,000 \times g$ 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 by 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 (dpm/ml); and C_p = concentration of the radioactivity in the spiked sample (dpm/ml).

***In Vitro* Incubation of [¹⁴C]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 μ l of [¹⁴C]MLN3126 solution (500 μ g/ml in acetonitrile) was added into 990 μ l of the protein solutions and plasma from rats, humans, and dogs at a final concentration of 5 μ g/ml and then incubated for 16 hours at 37°C.

Extraction of the Radioactivity from *In Vitro* Incubations of [¹⁴C]MLN3126 in the Presence of Hydride Reagent. An aliquot of 200 μ l of rat plasma and solutions of RSA, HSA, and DSA (1% in PBS, w/v) spiked with [¹⁴C]MLN3126 (5 μ g/ml) was treated with sodium borohydride (final concentrations of 25–26 mg/ml) at room temperature for 2 hours. Trifluoroacetic acid (TFA) (20 μ l) 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 1500 \times g 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 μ l) of pooled rat plasma collected at 8 hours after the single dosing of [¹⁴C]MLN3126 and of the *in vitro* incubations of [¹⁴C]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 μ l) after treatment with 20 μ l of TFA for 2 hours at room temperature. The radioactivity in the supernatants after centrifugation at 1500 \times g and 10°C for 10 minutes was measured by LSC. Extracts (800 μ l) of rat plasma collected after dosing of [¹⁴C]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 $1500 \times g$ and 10°C for 10 minutes were subjected to HPLC and LC/MS analyses. In the multiple administration study, an aliquot of 100 μl of rat plasma collected at 2, 8, and 24 hours after the 1st, 7th, 14th, and 21st administration was added with 500 μl of methanol. The radioactivity in the supernatants after centrifugation at $1500 \times g$ 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 at 8 hours after single dosing and *in vitro* incubations of [^{14}C]MLN3126 with albumins (RSA, HSA, and DSA) was treated with sodium borohydride (final concentrations of 25–26 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 $1500 \times g$ 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 were 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 $1500 \times g$ 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 μ l of 100 μ g/ml trypsin solution in 50 mM ammonium hydrogen carbonate buffer at 37°C overnight. Acetonitrile (150 μ l) was added to an aliquot of 300 μ l of the digested peptides and centrifuged at 1500 \times g 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 μ l of rat plasma collected after dosing of [¹⁴C]MLN3126 and the *in vitro* incubations of [¹⁴C]MLN3126 with RSA (0.1% in PBS), HSA (0.1% in PBS), and DSA (1% in PBS) after chemical reduction were dissolved in 200 μ l of 50 mM ammonium hydrogen carbonate buffer. The reconstituted proteins were treated with 200 μ l of pronase solution (1365 proteolytic units/ml in 50 mM ammonium hydrogen carbonate buffer) for 16 hours at 37°C. Acetonitrile (250 μ l) was added to an aliquot of 250 μ l of the reaction solutions and centrifuged at 1500 \times g 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 μ m 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 vol.) 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 held at 100% for the following 9.9 minutes. MS 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, tube lens voltage; 75 V. The precursor ions selected with an isolation width of m/z 2.0 underwent collisionally-induced dissociation in the ion trap by collision with helium to give the product ion spectra. The value of the collisionally-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- μ m 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 vol.) 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 and from 60 to 100% over the following 5 minutes and 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. MS 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 above.

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 were calculated using the following equation.

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

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

Pharmacokinetic Analysis of Total Radioactivity in Rat Plasma. The concentrations of total radioactivity were expressed as the MLN3126 equivalent value. The maximum concentration (C_{max}), the concentration at 24 hours after administration (C_{min}), and time to reach C_{max} (T_{max}) after the 1st, 4th, 7th, 11th, 14th, 18th, and 21st administration were taken from the actual values. The $t_{1/2}$ and AUC after the 1st, 4th, 7th, 11th, 14th, 18th, and 21st administration were calculated by the non-compartmental model in WinNonlin Ver. 4.1 (Pharsight Corporation, Inc., Sunnyvale, CA). The $t_{1/2}$ was calculated from the actual values by the least-squares method. The AUC was calculated by the trapezoidal method until the sampling point at 24 hours after dosing. 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 Administration of [¹⁴C]MLN3126 to Rats. After the 1st administration the plasma concentrations of the radioactivity reached a C_{\max} of 4.321 $\mu\text{g equiv./ml}$ at 1.0 hour (Table 1 and Fig. 2). The concentrations decreased to 0.845 $\mu\text{g equiv./ml}$ at 24 hours after administration. The $t_{1/2}$ and $\text{AUC}_{0-24\text{h}}$ after the 1st administration were 11.5 hours and 48.587 $\mu\text{g equiv.}\cdot\text{hours/ml}$, respectively. The C_{\max} , C_{\min} , and $\text{AUC}_{0-24\text{h}}$ slightly increased by the 4th administration compared with those after the 1st administration and attained almost steady state within the first 4 days. The T_{\max} , C_{\max} , C_{\min} , $t_{1/2}$, and $\text{AUC}_{0-24\text{h}}$ after the 4th administration were 1.0 hour, 5.392 $\mu\text{g equiv./ml}$, 1.214 $\mu\text{g equiv./ml}$, 13.1 hours and 62.364 $\mu\text{g equiv.}\cdot\text{hours/ml}$, and those after the 21st administration were 1.0 hour, 5.936 $\mu\text{g equiv./ml}$, 1.237 $\mu\text{g equiv./ml}$, 13.9 hours and 63.425 $\mu\text{g equiv.}\cdot\text{hours/ml}$, respectively. The plasma concentrations of the radioactivity after the 21st administration gradually decreased to undetectable level at 168 hours. The extraction recovery of the radioactivity from rat plasma collected at 8 hours after 1st, 7th, 14th, and 21st dosing with 5 volumes of methanol were 38.3%, 25.5%, 28.6%, and 28.5%, respectively. The proportion of the non-extractable radioactive fraction increased to approximately 95% of total radioactivity at 24 hours after each dosing.

Non-Extractable Radioactive Fraction of Rat Plasma Collected after Dosing of [¹⁴C]MLN3126. The extraction recovery of the radioactivity from pooled rat plasma collected at 8 hours after single oral dosing of [¹⁴C]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 Non-Extractable Fraction.** The extraction recovery of radioactivity from *in vitro* incubations of [¹⁴C]MLN3126 with rat plasma, human plasma, dog plasma, RSA, 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 at 8 hours after single oral dosing of [¹⁴C]MLN3126 and *in vitro* incubations of [¹⁴C]MLN3126 with RSA, HSA, and DSA were treated with an excess of sodium borohydride. The extraction recovery of the radioactivity from rat plasma collected after dosing of [¹⁴C]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 [¹⁴C]MLN3126 and *in vitro* incubation samples after hydride reduction were 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 at 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 the 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 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. 4C, D).

Identification of Radioactive Components in the Trypsin Digests. Rat plasma collected after dosing of [^{14}C]MLN3126 and *in vitro* incubation samples after hydride reduction and alkylation were 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 the 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 *in vitro* 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 (Supplementary Fig. 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 with that in RSA. Each peptide was assigned to 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 percentage of the *in vitro* plasma protein binding of [¹⁴C]MLN3126 at the concentrations of 0.1, 1, and 10 $\mu\text{g/ml}$ were 99.0%, 98.8%, and 98.7% in rats, 97.3%, 97.3%, and 97.0% in dogs, and >99.8%, 99.8%, and 99.8% in humans, respectively. The percentage of protein binding of [¹⁴C]MLN3126 at the concentrations of 0.1, 1, and 10 $\mu\text{g/ml}$ were 99.0%, 99.0%, and 98.9% in 4% HSA, 71.9%, 64.6%, and 39.5% in 0.05% AGP, and 99.1%, 99.1%, and 98.8% in 4% HSA/0.05% AGP mixture, respectively.

Discussion

The extraction recovery of the radioactivity from the rat plasma collected at 8 hours after single oral dosing of [¹⁴C]MLN3126 with methanol was 47.1% and about a half of the total radioactivity was considered as the non-extractable fraction. The formation of the non-extractable fraction was also observed in the *in vitro* incubations of [¹⁴C]MLN3126 in plasma and in the solutions of RSA, HSA, and DSA (Table 2). After hydride reduction and pronase digestion of the non-extractable fractions of the *in vivo* and *in vitro* samples, a predominant radioactive peak was detected in the digests (Fig. 3). When the non-extractable 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, as 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 non-extractable fraction of rat plasma released unchanged MLN3126 by TFA treatment. In contrast, the non-extractable 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 *in vitro* incubation samples were treated with hydride reagent, additional formation of the secondary amine (non-extractable fraction) was not detected under the conditions of this study. The carbonyl group of MLN3126 is considered to be rapidly reduced to the non-reactive 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 non-extractable 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 non-specific 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 non-covalent protein binding have been proposed (Yang et al., 2007; Wang et al., 2010). From the proposed 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 (Wang et al., 2010; Zhang et al., 2005). For example, BMS-204352 forms an irreversible covalent binding with plasma proteins, mostly serum albumins, in humans, dogs, and rats (Zhang et al., 2005). After administration of radio-labeled BMS-204352 to humans, dogs, and rats, the $t_{1/2}$ of radioactivity (11, 7, and 1 day, respectively) were similar to the reported $t_{1/2}$ of albumins (19, 6.8, and 2.6 days, respectively) (Schreiber et al., 1970; 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 physiological 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 (Table 1 and Fig. 2). The persistence of radioactivity in the rat plasma was not observed after the 21st administration. Moreover, the $t_{1/2}$ of the total radioactivity (approximately 13 hours) in rat plasma during and after multiple oral administrations of [^{14}C]MLN3126 were approximately 5-fold shorter than the reported $t_{1/2}$ of albumin in rats. These results suggest that the covalent binding of MLN3126 via the Schiff base is reversible under physiological

conditions and has little impact on the pharmacokinetic properties of MLN3126.

In conclusion, the results of chemical and biological derivatization of the non-extractable radioactive fraction of rat plasma collected at 8 hours after oral dosing of [¹⁴C]MLN3126 followed by LC/MS analysis suggested that the major part of the non-extractable 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 administration of [¹⁴C]MLN3126 to rats suggested that the covalent binding of MLN3126 with RSA was reversible under physiological 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 *in vitro* incubations with RSA, HSA, and DSA in the same manner, suggesting that there are not 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

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

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Footnotes

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Figures Legends

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

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-4.71 MBq/kg. Each point represents the mean for three animals.

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

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).

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

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.

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 non-extractable fraction via the Schiff base between MLN3126 and the lysine residues in serum albumins.

Table 1. Pharmacokinetic parameters of the radioactivity in the plasma of rats during and after 21 daily multiple oral administrations of [¹⁴C]MLN3126 at a daily dose of 10 mg-4.71 MBq/kg

Day	Pharmacokinetic parameter				
	T _{max} (h)	C _{max} ^a (μg equiv./ml)	C _{min} ^{a,b} (μg equiv./ml)	t _{1/2} (h)	AUC _{0-24 h} ^a (μg equiv. ·h/ml)
1	1.0 ± 0.0	4.321 ± 1.351	0.845 ± 0.280	11.5 ± 0.8	48.587 ± 15.443
4	1.0 ± 0.0	5.392 ± 1.041	1.214 ± 0.337	13.1 ± 0.3	62.364 ± 16.729
7	0.7 ± 0.3	5.717 ± 0.507	1.062 ± 0.171	12.5 ± 0.4	57.117 ± 7.340
11	0.8 ± 0.3	6.857 ± 1.963	1.129 ± 0.294	12.5 ± 0.1	63.574 ± 15.484
14	0.7 ± 0.3	7.502 ± 1.195	1.349 ± 0.298	13.6 ± 0.5	70.882 ± 13.612
18	0.7 ± 0.3	5.331 ± 1.092	1.184 ± 0.302	14.1 ± 0.6	58.867 ± 15.921
21	1.0 ± 0.0	5.936 ± 1.527	1.237 ± 0.445	13.9 ± 1.1	63.425 ± 23.450

Each value represents the means ± standard deviation (SD) for three animals.

^aMLN3126 equivalent.

^bConcentration at 24 hours after administration.

Table 2. Extraction recovery of the radioactivity from *in vitro* incubations of [¹⁴C]MLN3126 at a final concentration of 5 μg/ml in blank plasma and protein solutions for 16 hours at 37°C

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.

Mean values (*n* = 3)

N.D.: Not determined

TFA: Trifluoroacetic acid

TFA (-): Extracted with 5 vol. of methanol

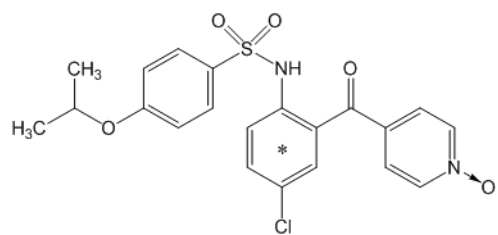
TFA (+): Extracted with 5 vol. of acetonitrile after treatment with TFA

NaBH₄ (+) TFA(+): Extracted with 5 vol. of acetonitrile after treatment with NaBH₄ followed by treatment with TFA

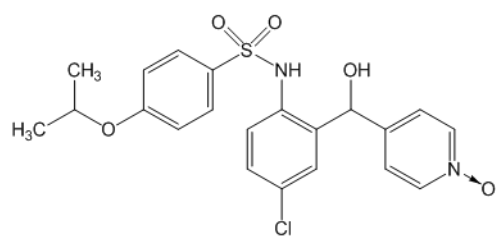
Table 3. Amino acid sequences of the binding sites of MLN3126 in albumins

	Amino acid sequence of 198 to 205 in albumin	Amino acid sequence of 429 to 432 in albumin
RSA	MKCSSMQR	NLGR
HSA	LKCASLQK	NLGK
DSA	FKCASLQK	KL GK

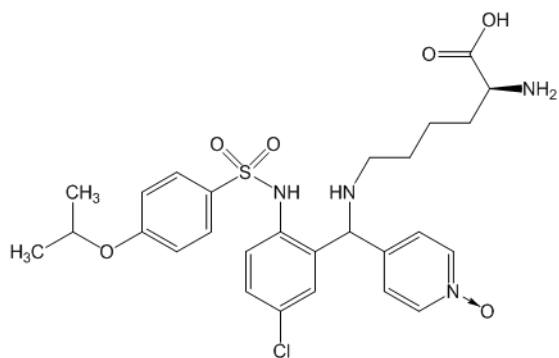
Figure 1



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



M-I



MLN3126-lysine

Figure 2

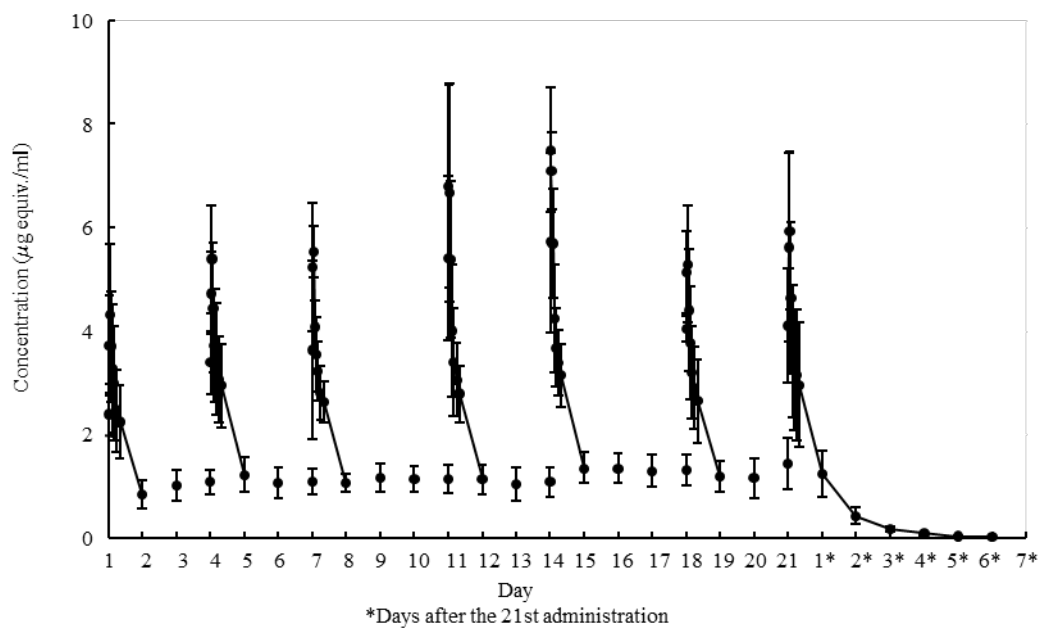


Figure 3

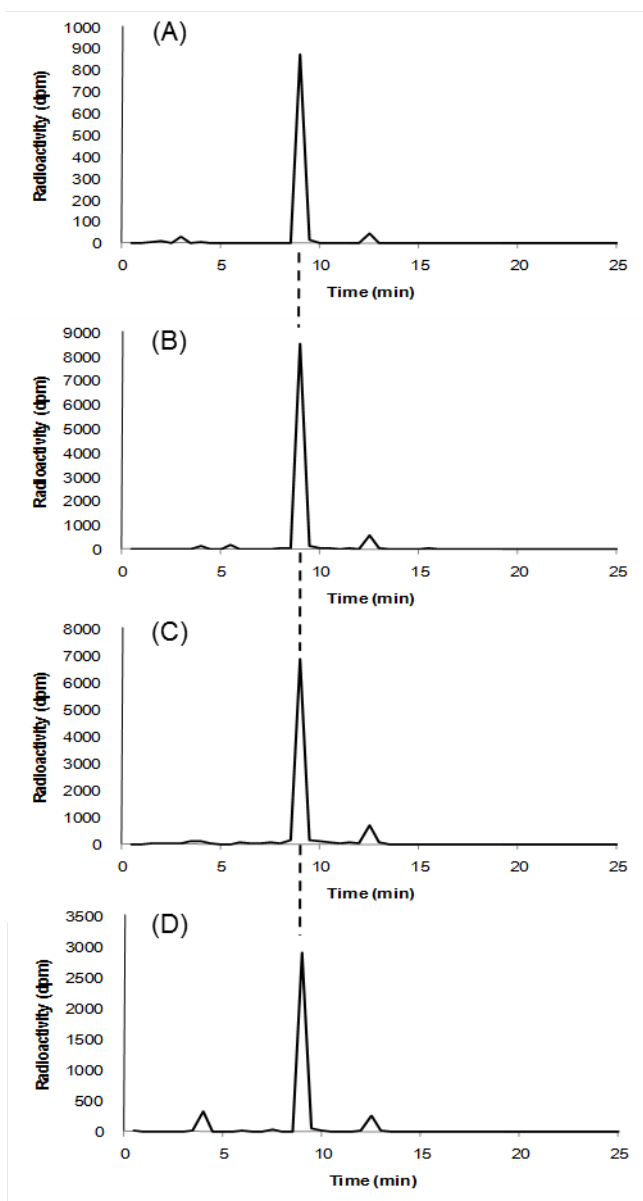


Figure 4

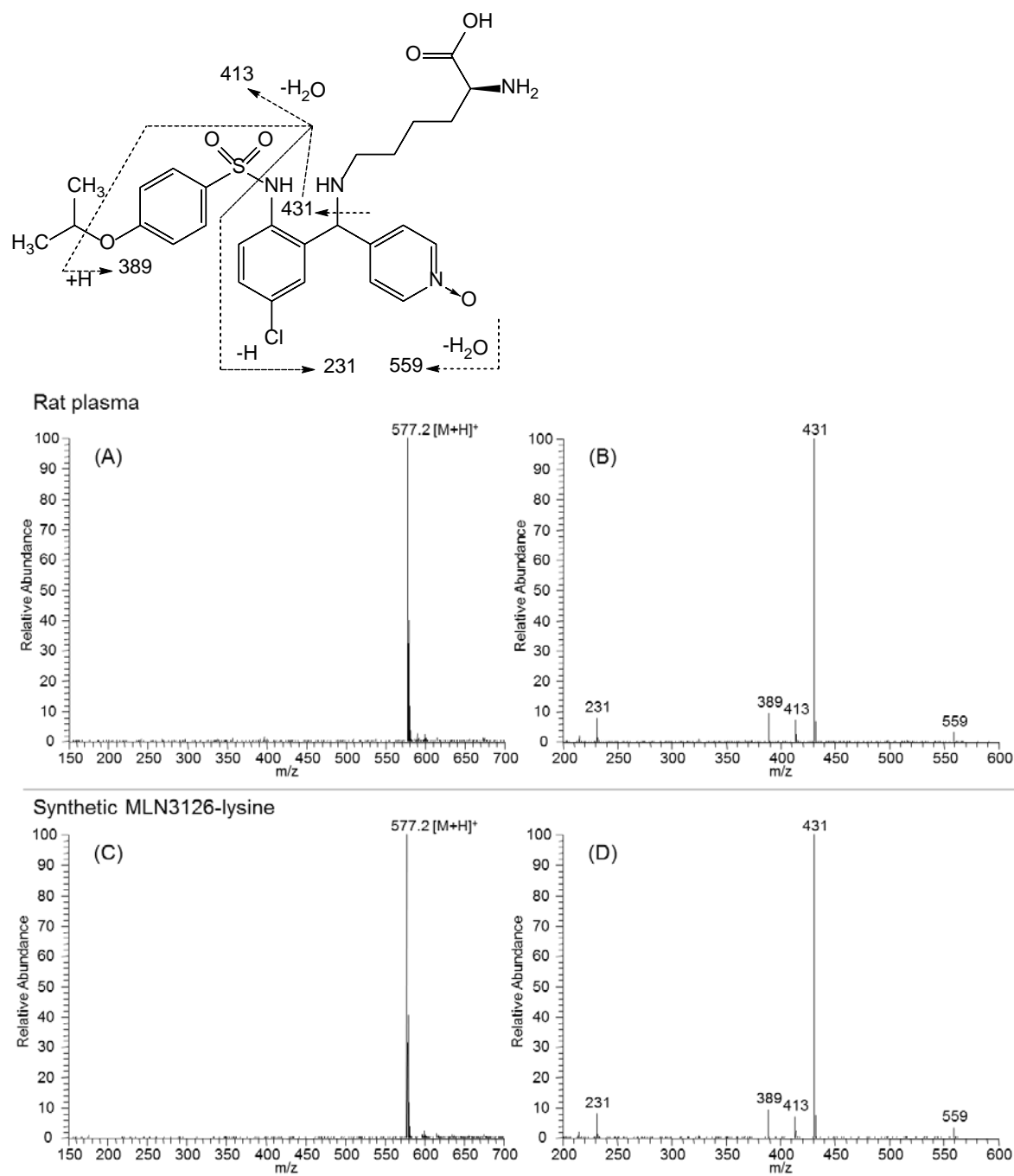


Figure 5

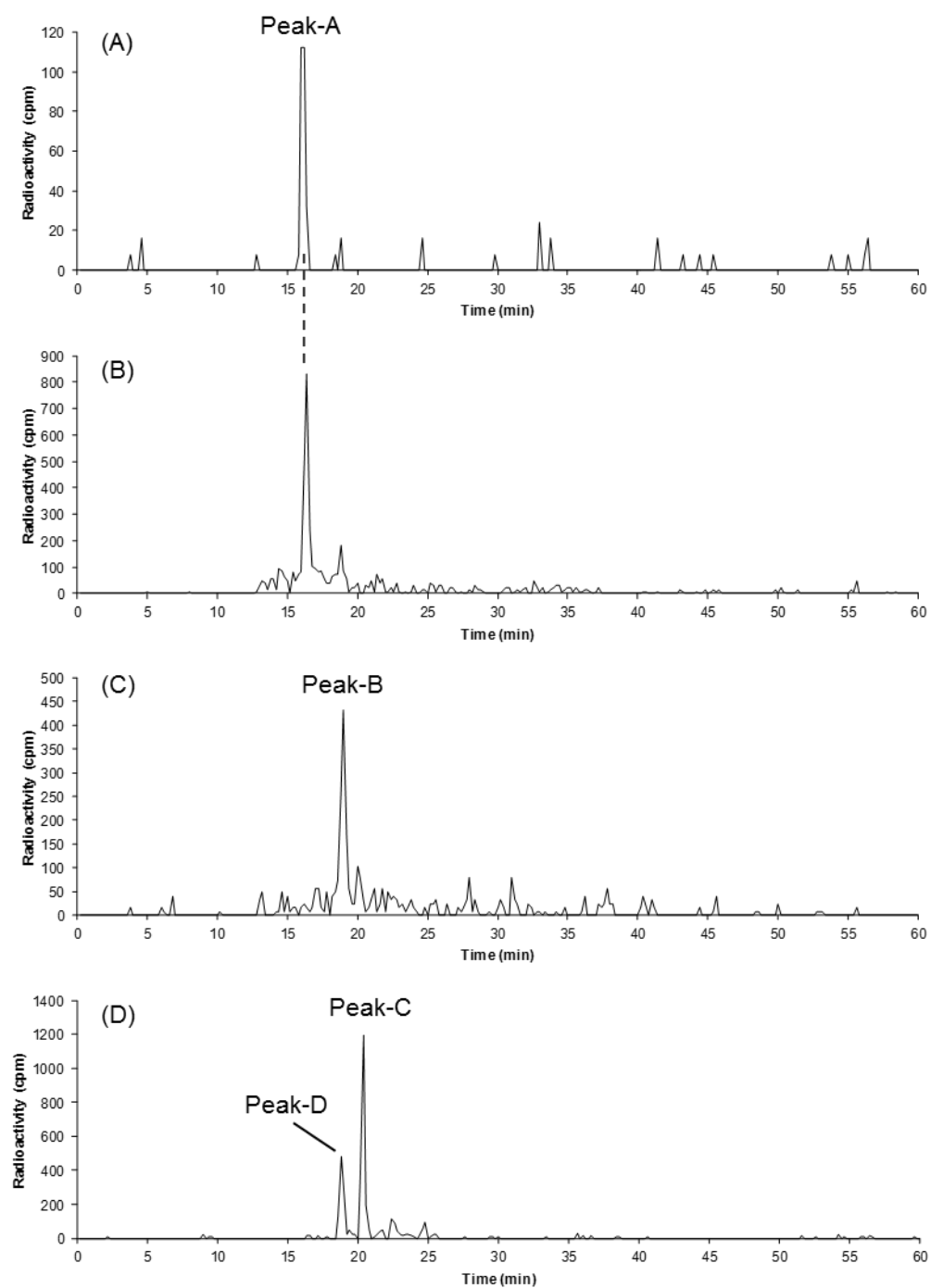


Figure 6

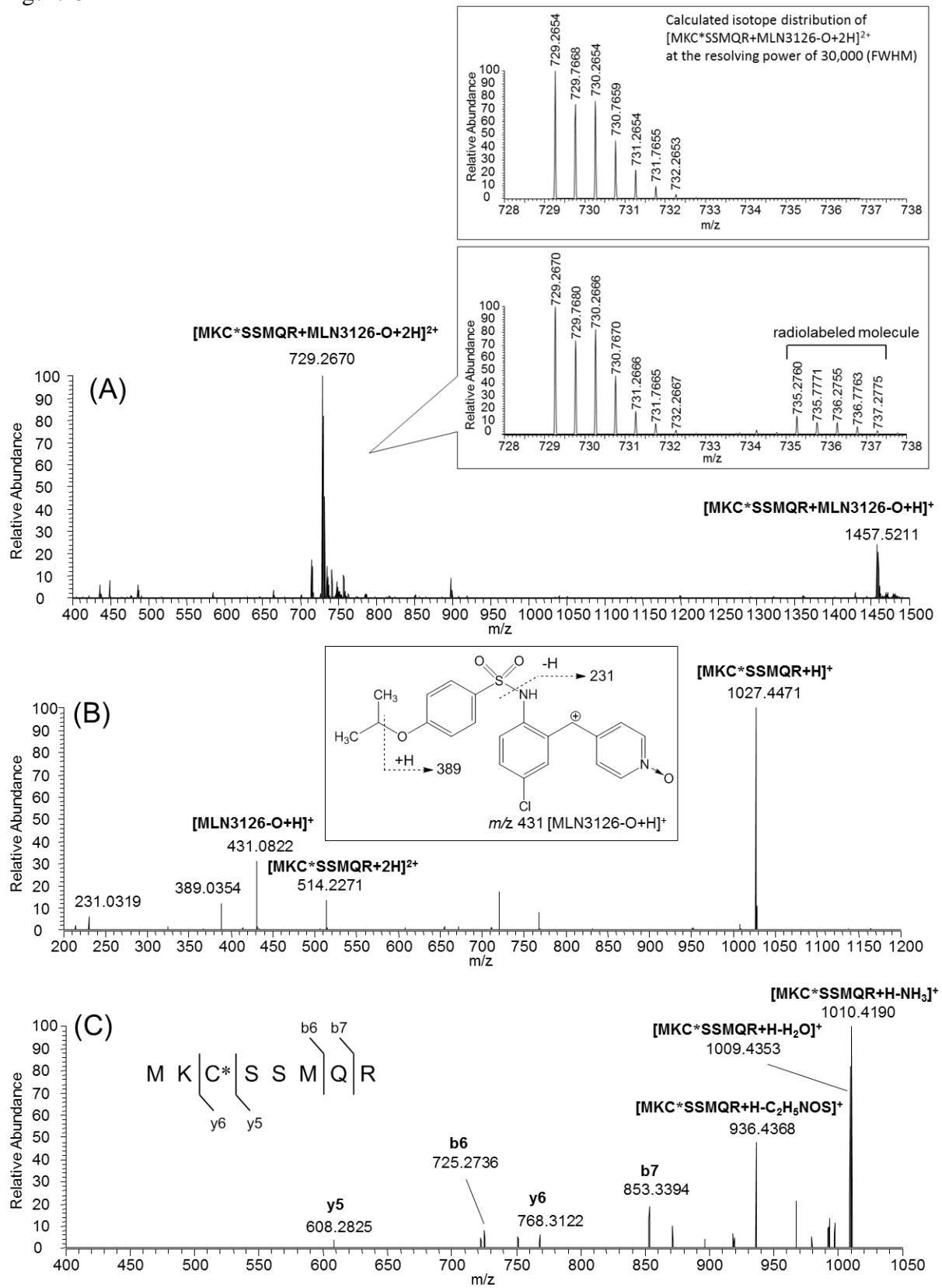


Figure 7

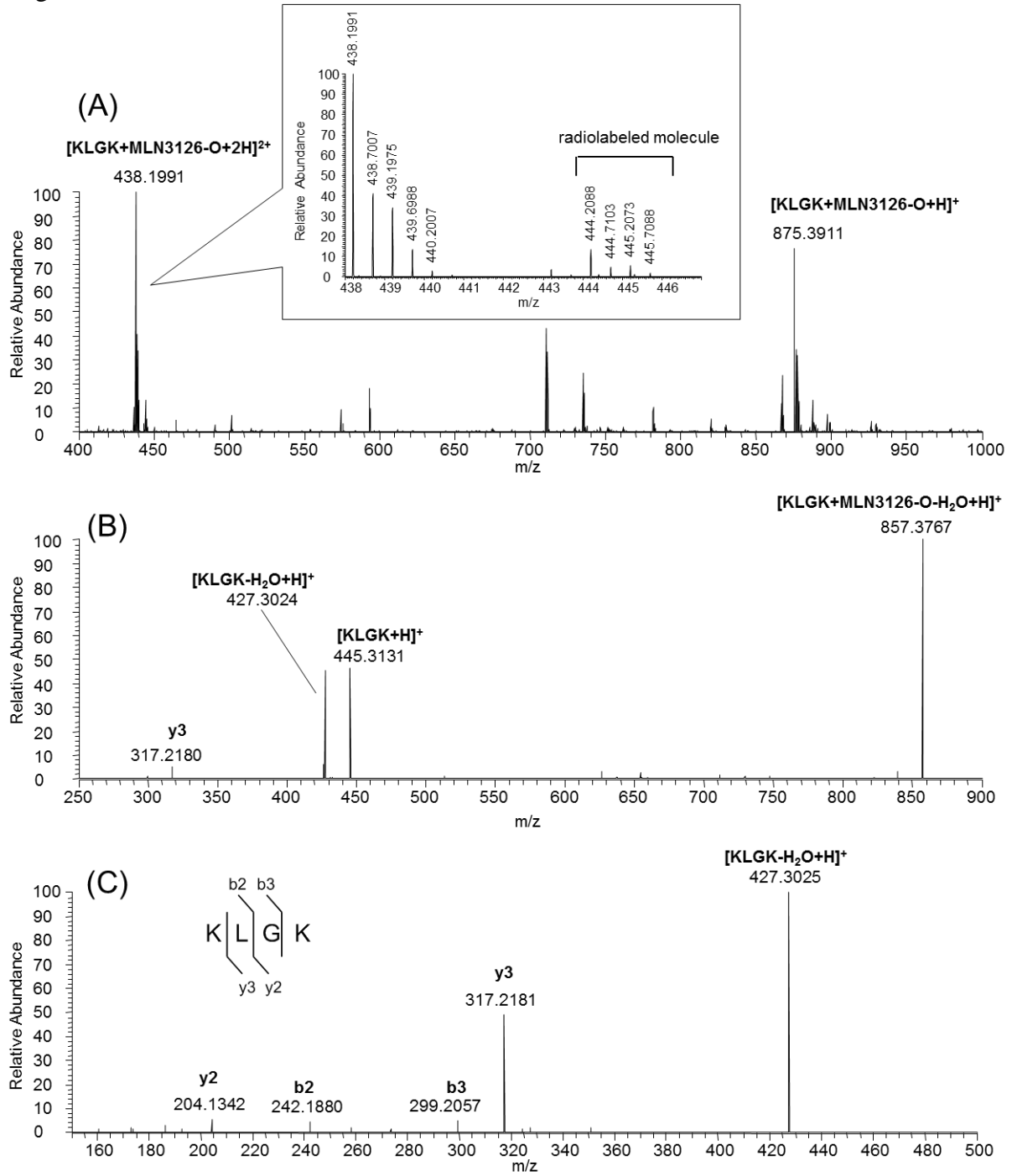


Figure 8

