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RESEARCH ARTICLE

Quantification of Intact and Truncated Stromal Cell-Derived Factor-1α in Circulation by Immunoaffinity Enrichment and Tandem Mass Spectrometry

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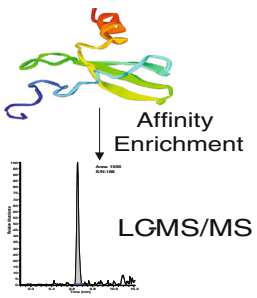

Abstract. Stromal cell-derived factor 1α (SDF-1α) or CXCL12 is a small pro-inflammatory chemoattractant cytokine and a substrate of dipeptidyl peptidase IV (DPP-IV). Proteolytic cleavage by DPP-IV inactivates SDF-1α and attenuates its interaction with CXCR4, its cell surface receptor. To enable investigation of suppression of such inactivation with pharmacologic inhibition of DPP-IV, we developed quantitative mass spectrometric methods that differentiate intact SDF-1α from its inactive form. Using top-down strategy in quantification, we demonstrated the unique advantage of keeping SDF-1α's two disulfide bridges intact in the analysis. To achieve the optimal sensitivity required for quantification of intact and truncated SDF-1α at endogenous levels in blood, we coupled nano-flow tandem mass spectrometry with antibody-based affinity enrichment. The assay has a quantitative range of 20 pmol/L to 20 nmol/L in human plasma as well as in rhesus monkey plasma. With only slight modification, the same assay can be used to quantify SDF-1α in mice. Using two in vivo animal studies as examples, we demonstrated that it was critical to differentiate intact SDF-1α from its truncated form in the analysis of biomarkers for pharmacologic inhibition of DPP-IV activity. These novel methods enable translational research on suppression of SDF-1 inactivation with DPP-IV inhibition and can be applied to relevant clinical samples in the future to yield new insights on change of SDF-1α levels in disease settings and in response to therapeutic interventions.

Key words: SDF-1, DPP-IV, Top-down MS

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Introduction

Stromal cell-derived factor (SDF-1), a pro-inflammatory chemoattractant cytokine, regulates stem/progenitor cell trafficking through binding to CXCR4, a G-protein-coupled seven-span transmembrane receptor on plasma membranes of targeted cells [1]. The SDF-1-CXCR4 axis plays critical roles in homing and retention of hematopoietic stem/progenitor cells in hemato/lymphopoietic organs [2], in trafficking other tissue/organ specific stem/progenitor cells during embryo/organogenesis and tissue/organ regeneration [3], and in regulating processes essential to tumor metastasis such as locomotion and adhesion of malignant cells [4]. The SDF-1–CXCR4 axis is positively and negatively modulated by several external factors. Degradation of the binding partners by proteolytic cleavage attenuates the responsiveness of CXCR4 positive cells to an SDF-1 gradient. The amino terminus of CXCR4 is subjected to proteolytic cleavage by leucocyte-derived protease [5] and SDF-1 is a known substrate for dipeptidylpeptidase IV (DPP-IV), a cell surface-expressed protease [6, 7]. We are particularly interested in the physiological processing of SDF-1 by DPP-IV and sought to investigate the effect of in vivo DPP-IV inhibition on the level of intact SDF-1 in circulation.

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SDF-1 exists in many isoforms that are differentially distributed in various tissues [8]. The highest expressed isoforms α and β differ only at the carboxy terminus, with the β isoform being longer by four amino acid residues (72 versus 68 residues). Both isoforms are found in circulation, but are differentially processed at the carboxy terminus by an unknown peptidase in serum. In vitro studies have shown that the terminal lysine of recombinant α isoform (K68) is immediately removed upon incubation in serum, whereas the recombinant β isoform is resistant to similar degradation [9]. The amino termini of both isoforms, however, are vulnerable to cleavage by DPP-IV with cleavage efficiencies (k_{cat}/K_m) higher than that of GLP-1 [10–12]. Similar to GLP-1, the removal of the two amino terminal residues inactivates SDF-1 by disrupting its binding with CXCR4 and subsequent activation [7]. Recently, there has been increasing interest in the investigation of suppression of SDF-1 inactivation with DPP-IV inhibition. To date, however, most efforts have been limited to in vitro studies or in vivo studies with infused SDF-1 at superphysiological concentrations [13]. These studies, though insightful, failed to provide direct evidence on suppression of endogenous SDF-1 inactivation by in vivo DPP-IV inhibition with therapeutic agents. The hindrance to such efforts is not the lack of specific assays that differentiate SDF-1 isoforms from their DPP-IV cleaved forms. Many previous in vitro kinetics studies successfully employed mass spectrometry (MS)-based methods to generate sequence specific measurements [9]. The sensitivity of these MS methods, however, was not suitable for measurement of SDF-1 at endogenous levels (often sub-nanomolar) in complex biological matrices such as plasma and serum. Instead, the endogenous SDF-1 isoforms in circulation were often quantified with antibody-based immunoassays, which lacked the specificity to differentiate intact SDF-1 isoforms from DPP-IV cleaved ones and, thus, were inadequate for in vivo DPP-IV inhibition studies.

In the present study, we report the development of a tandem MS-based assay with sufficient sensitivity to quantify the α isoform of SDF-1 at the endogenous level while retaining sequence specificity that is required to distinguish intact SDF-1α from its NH_2-terminal truncated inactive form. We chose to quantify SDF-1α using "top-down" approach without converting it to peptide fragments through enzymatic digestion. To achieve optimal sensitivity and absolute quantification, we coupled immunoaffinity enrichment of SDF-1α with stable isotope dilution LC-MS quantification. Leveraging specificity by tandem mass spectrometry and simplification of the background by affinity purification of peptide analytes, the hybrid assay format has been shown to be highly effective in quantifying low abundance proteins/peptides that are not amenable to immunoassays [14–20]. In our method, intact and NH_2 terminus cleaved SDF-1α isoform were enriched from plasma through binding to immobilized antibodies prior to analysis on a triple quadrupole (QQQ) mass spectrometer. The assay exhibited a broad linear range (10^3) with a lower limit of quantification (LLOQ) at low pM level, which is sufficiently lower than the endogenous concentration. Measuring endogenous SDF-1α in two in vivo studies with mice and non-human primates, we demonstrated the shift in the balance between the intact and NH_2-terminal truncated SDF-1α forms at the physiological level upon in vivo DPP-IV inhibition with an inhibitor.

Materials and Methods

Stable Isotope Labeled Peptide Standards

Stable isotope-labeled internal standard peptides representing the sequences of SDF-1α 1–67 and 3–67 were synthesized and analyzed for purity and amino acid content (CPC Scientific, Sunnyvale, CA, USA). The purity was assessed to be 95 % for both internal standards. Subsequent calculations of concentration were based on the purity assessment and weight measurement.

Formation of Intramolecular Disulfide Bonds with a Glutathione Redox Buffer System

Reduced and oxidized glutathione were dissolved in 0.1 M Tris·HCl buffer at pH 8.5 at final concentration 10.0 and 1.0 mM, respectively. Heavy isotope labeled SDF-1α standards were reconstituted in the glutathione redox buffer at 125 μg/mL and stored at room temperature for 24 h for disulfide bond formation and refolding. The complete conversion to refolded SDF-1α standards was confirmed with the disappearance of unfolded SDF-1α standards using a LC-MS/MS method with SRM transitions tracking both unfolded and refolded SDF-1α standards.

High Resolution LC/MS Characterization of Unfolded and Refolded SDF-1α Isoforms

Unfolded and refolded heavy labeled standards of SDF-1α were analyzed by a reversed-phase nano-HPLC coupled to a LTQ-FTICR hybrid mass spectrometer (LTQ-FT Ultra; Thermo Fisher, San Jose, CA, USA). Approximately 1 pmol of SDF-1α standards were injected with an auto-sampler (Series 1100; Agilent, Santa Clara, CA, USA) onto a nano-LC column packed with BioBasic C^8 media (5 cm × 75 μm; New Objective, Woburn, MA, USA). A micro-flow HPLC pump (Agilent Series 1100) delivered a binary gradient increasing from 0 % hydrophobic phase (0.1 M acetic acid in acetonitrile) to 50 % hydrophobic phase at a rate of 1 %/min while maintaining the flow rate at 1 μL/min. Peptides eluting from the nano-LC column were introduced into the mass spectrometer by electrospray ionization using a 3-kV needle voltage, heated metal capillary temperature of 270 °C, and tube lens voltage at 120 V. Ion injection times into linear ion trap were adjusted by the instrument automatic gain control (1×10^7 arbitrary unit setting) with a maximum accumulation time not to exceed 1 s. Ions were passed to the FTICR cell, and full scan spectra with mass-to-

charge ratio (m/z) from 300 to 2000 were acquired approximately every 3 s. While the full scan MS spectra were acquired in the FTICR cell at an instrument-resolving power of 50,000, data-dependent MS/MS scans of the four most intense precursor ions in the preceding full MS scan were collected in the linear ion trap.

Affinity Purification of SDF-1α Isoforms

Polyclonal anti-SDF-1α antibody (RnD Systems) was covalently bound to tosyl-activated Dynal M-280 beads (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In each analysis, 100 μL of plasma was first diluted with 400 μL of Dulbecco's PBS buffer with 0.1 % Tween 20 (vol/vol), 200 fmol of heavy isotope labeled SDF-1α standards were then spiked in and mixed thoroughly with the diluted plasma through vortex. Twenty-five microliter of antibody-conjugated beads was added to each sample for immunoaffinity enrichment. Following incubation for 16 h at 4 °C with rotation, the beads were washed twice with 500 μL 50 mmol/L $(NH_4)_2CO_3$, pH 8.3 and twice with 500 μL water. The bound SDF-1α isoforms were eluted with 50 μL 2 % acetic acid (2 h, room temperature, with rotation). We determined the efficiency of immunoaffinity enrichment by spiking SDF-1α standards prior to and after the immunoaffinity enrichment step and calculating the ratios of the integrated peak areas of the standards between the samples. The efficiency was 72 % for SDF-1α 1–67 and 74 % for SDF-1α 3–67.

Liquid Chromatography Tandem Mass Spectrometry

Eluted peptides were analyzed in SRM mode using a Thermo Vantage triple quadrupole instrument with four m/z transitions with dwell time of 50 ms each. The transitions tracking human and murine SDF-1α isoforms and their corresponding isotope-labeled standards are listed in the table of Figure 3. Peptides were loaded directly onto an analytical nano-spray column (BioBasic C^8, 50 mm by 75 μm i.d., New Objective, Woburn, MA, USA) with 0.1 % formic acid in water at 2 μL/min. After five minutes of loading, the peptides were eluted off the column with a linear gradient to 50 % acetonitrile, 0.1 % formic acid over 5 min at 1 μL/min. We integrated extracted ion chromatograms (XICs) of both SRM transitions and calculated peak area ratios between endogenous SDF-1α isoforms and their heavy standards using LC-Quan (Thermo Fisher, San Jose, CA, USA) ver. 2.0.7. The equation (peak area ratio) (2 nmol/L) was used to calculate the concentrations of endogenous SDF-1α isoforms in the original sample, in nmol/L.

In Vivo DPP-IV Inhibition in Mice

Lean C57BL/6 mice were dosed orally with either vehicle (DMSO) or MK-0626 at 3 mpk (n = 8 in each group). Animals in both groups were sacrificed by cardiocentisis at 1 h post-dosing. Blood was collected in tubes with a cocktail of protease inhibitors (Roche, Mannheim, Germany); plasma was immediately isolated for measurement of SDF-1α and 100 μL was consumed in each analysis.

In Vivo DPP-IV Inhibition in Rhesus Monkeys

On both d 1 and d 15, rhesus monkeys were orally dosed with either vehicle or MK-0626 (n = 6 in each group). Blood was collected at 16 h post-dosing in tubes with protease inhibitors; 100 μL of plasma was used in each analysis.

Results

Design of Staple Isotope Labeled Internal Standards; Formation of Disulfide Bonds with Redox Buffer; Comparison of the Standards Between Before and After Disulfide Bond Formation

Because the lysine residue at the carboxy terminus of SDF-1α is removed instantly by an unknown carboxypeptidase in plasma, SDF-1α sequences without the terminal lysine, namely 1–67 and 3–67, were selected for quantification (Figure 1). Stable isotope-labeled peptides were synthesized for both SDF-1α 1–67 and 3–67 with five heavy isotope-labeled leucine ($^{13}C_6{}^{15}N_1$) residues distributed along the sequences (at positions 36, 42, 55, 62, and 66). The stable isotope-labeled standards, though chemically identical, are heavier by 35 Da than the endogenous SDF-1α forms. The cysteine residues were synthesized in sulfhydryl forms (non-reduced and without being alkylated) so that two pairs of long range disulfide bonds, Cys^9-Cys^{34} and Cys^{11}-Cys^{50}, could be formed under mildly oxidative environment. A glutathione-based redox system was optimized to slowly oxidize the cysteine residues to minimize short range and intermolecular disulfide bond formation so that the peptide standards were refolded to native and globular structures. The refolded peptide standards were characterized with a hybrid LTQ-FTMS platform with a nano-flow LC system providing front end separation. In alternating MS scans, high resolution full MS spectra were acquired with the FTICR analyzer and CID MS/MS spectra were collected with the linear ion trap. Accurate mass measurement was used to confirm the formation of the disulfide bonds (e.g., for refolded stable isotope labeled SDF-1α 1–67 standard) the difference between measured mass (7861.260) and calculated mass (7861.254) is ~1 ppm. Corresponding CID fragment ion spectra verified the correct configuration of the disulfide bonds. In the MS/MS spectrum of refolded SDF-1α 1–67, all three fragment ions in the CID MS/MS spectrum were assigned with delta mass less than 0.5 Da. All three fragment ions ($b_{52}{}^{5+}$, $b_{52}{}^{6+}$, and $y_{15}{}^{2+}$) were derived from the dissociation of the amide bond between Asp52 and Pro51 (Figure 2). The scarcity of fragment ions and backbone

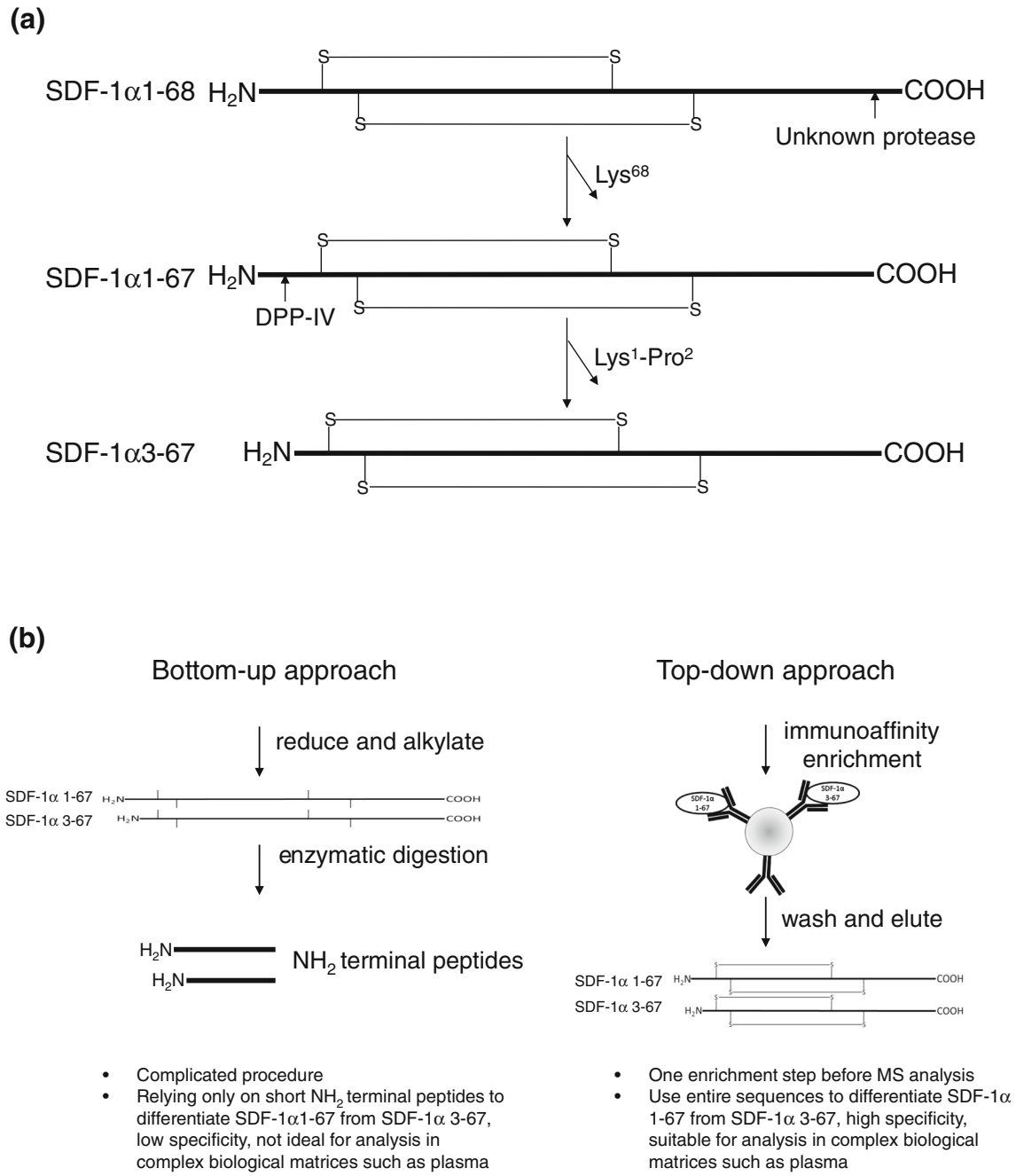

Figure 1. Enzymatic processing of SDF-1α and targeted proteomics methods to quantify intact SDF-1α and its DPP-IV cleaved inactive forms. **(a)** In blood, the full length SDF-1α (1–68) degrades immediately at the COOH terminal when an unknown peptidase removes the last lysine residue (K68). DPP-IV then removes the first two amino acid residues (KP) from the NH_2 terminus with slower kinetics. The relevant analytes in the present study are SDF-1α 1–67 and SDF-1α 3–67, the substrate and product of DPP-IV reaction. **(b)** Comparison between bottom-up and top-down quantification of intact and NH_2-terminal truncated SDF-1α. Targeted proteomics method based on top-down methodology has superior specificity and more elegant workflow

amide bond breakages is obvious when the MS spectrum of refolded SDF-1α 1–67 standard is compared with the MS/MS spectrum of SDF-1α 1–67 standard prior to being refolded. This observation was not unexpected because both the covalent links between cysteine residues and amide backbone need to be dissociated to produce fragment ions, which is not common in CID [21]. The dominance of fragment ions resulted from the breakage of amide bond between Asp52 and Pro53 was also expected because the dissociation of the amide bond NH_2 terminal to proline residue is often favored in CID [22]. The refolded SDF-1α 3–67 had a similar fragmentation pattern with dominant

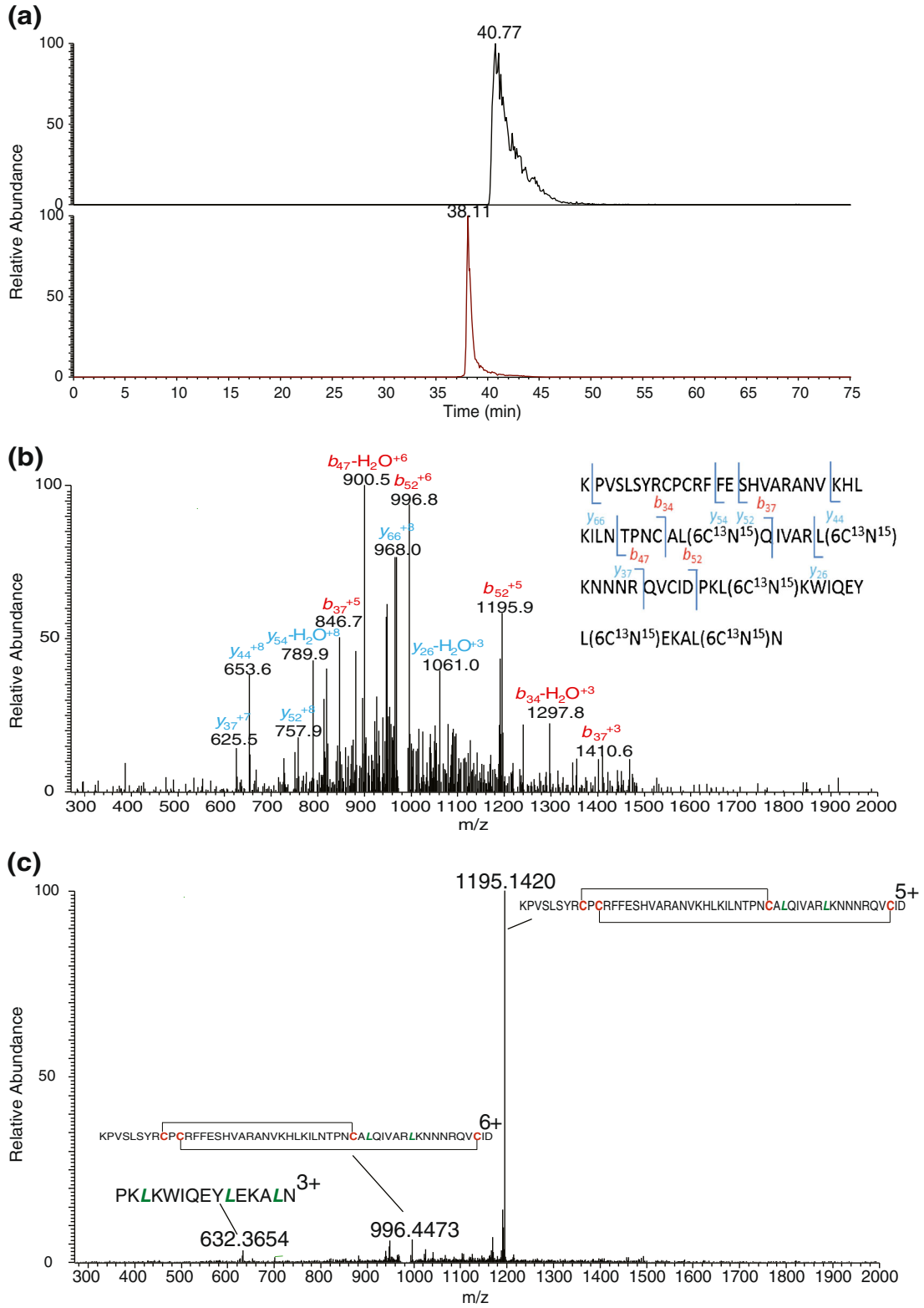

Figure 2. Characterization of synthetic SDF-1α standard with and without long range disulfide bond formation. **(a)** Top panel: extracted ion chromatogram (XIC) of SDF-1α 1–67 without disulfide bond formation; bottom panel: XIC of SDF-1α 1–67 after long range disulfide bonds are formed by oxidation with a redox buffer. **(b)** Collision induced dissociation (CID) spectrum of SDF-1α 1–67 without disulfide bonds. The precursor ion is 984.1681 $[M + 8H]^{8+}$. **(c)** CID spectrum of SDF-1α with long range disulfide bonds in correct configuration. The precursor ion is 983.6645 $[M + 8H]^{8+}$. Because of the restraint by disulfide bonds, CID only yielded very limited number of fragment ions, b_{52}^{5+}, b_{52}^{6+}, and y_{15}^{2+}, with all being products from the fragmentation of the amide bond between Asp52 and Pro53

(a)

SRM Transition	Parent	Q1 m/z		Product	Q3 m/z
#1	**Endogenous SDF-1α 3-67**	951.6 (8+)	→	Light b_{50}	1147.9 (5+)
#2	**Heavy SDF-1α 3-67**	956 (8+)	→	Heavy b_{50}	1150.7 (5+)
#3	**Endogenous SDF-1α 1-67**	979.83 (8+)	→	Light b_{52}	1193.2 (5+)
#4	**Heavy SDF-1α 1-67**	984.2 (8+)	→	Heavy b_{52}	1196 (5+)

(b)

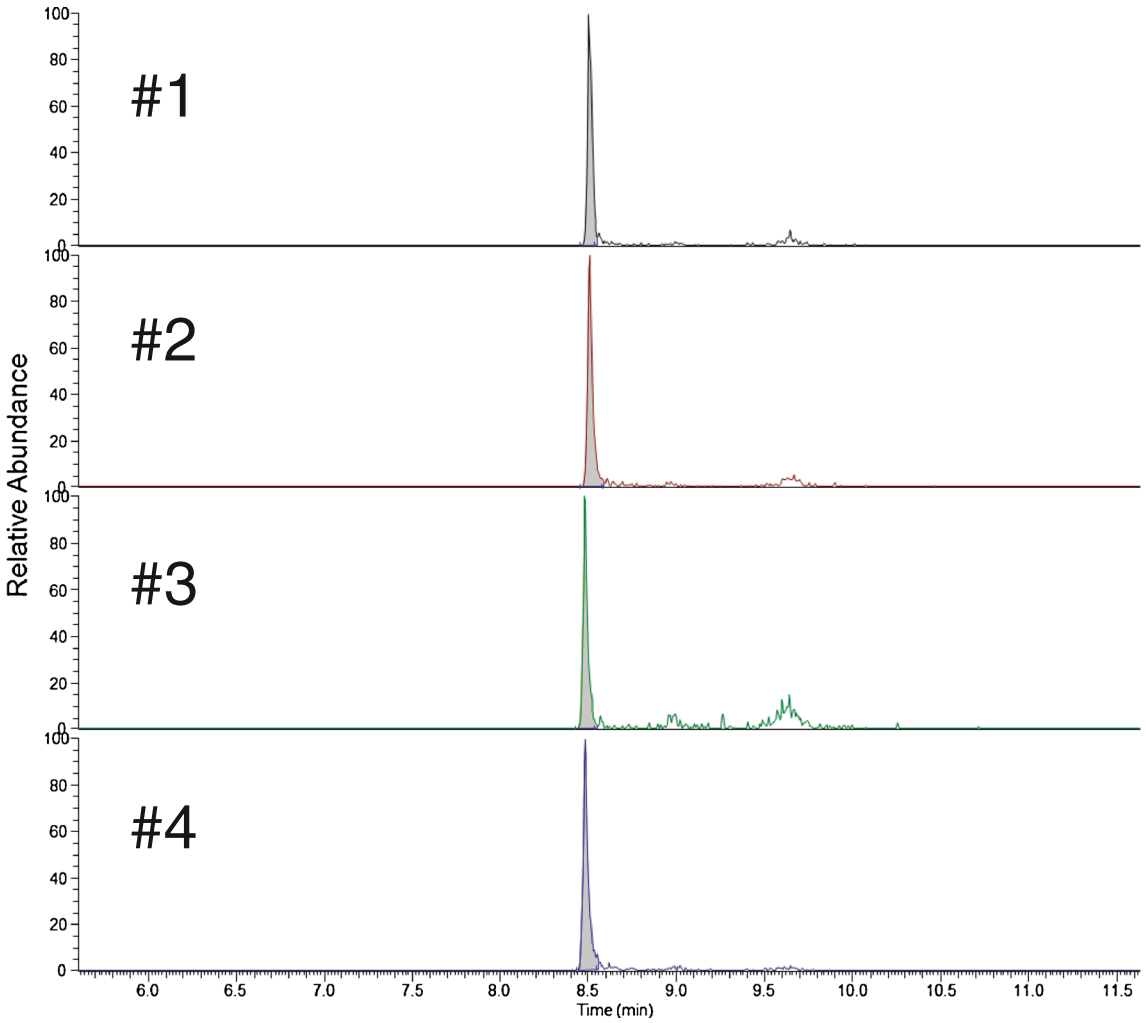

Figure 3. Selected ion monitoring (SRM) coupled with isotope dilution for the quantification of intact and DPP-IV processed SDF-1α. **(a)** SRM transitions for endogenous SDF-1α 1–67 and SDF-1α 3–67 and their respective stable isotope labeled internal standards. **(b)** Representative XICs for endogenous SDF-1α 1–67 and SDF-1α 3–67 and their stable isotope labeled internal standards

fragment ions being b_{50}^{5+} and y_{15}^{2+}, resulting from the dissociation of the amide bond between Asp50 and Pro51. In peptide sequencing, the lack of fragment ions in CID MS/MS spectra due to disulfide bond formation poses as a challenge; in peptide quantification with MRM, however, it provides a unique opportunity in reducing signal loss because the signal of the parent ion is spread over a very limited number of product ions.

Besides fragmentation pattern, the chromatographic behaviors of SDF-1α with and without disulfide bonds were also significantly different. The extracted ion chromatogram (XIC) of the SDF-1α sequences without refolding showed a broad peak with significant rear end tailing, whereas the XIC of the refolded SDF-1α sequences exhibited a sharp, symmetrical peak (Figure 2a). The difference in LC elution profile could be attributed to the difference in protein conformation: the structure of the protein was probably random coil (a collection of many random structures without a specific shape) before being properly folded with the help of disulfide bond formation, after being refolded with proper disulfide bond configuration the protein adopted a globular structure (a unique shape). The singularity in tertiary structure leads to the singularity in hydrophobicity and thus the narrow and symmetrical LC elution profile that resembles those of short peptides and small molecules.

LC-MS Isotope Dilution Quantification of SDF-1α Isoforms in Human, Rhesus, and Murine Plasma

After characterizing unfolded and folded SDF-1α sequences, we chose the folded SDF-1α sequences as analytes in our analysis because of their favorable fragmentation pattern for developing SRM transitions and ideal LC elution profile. A SRM method was established on a QQQ instrument with four alternating Q1 → Q3 transitions tracking fragmentations of SDF-1α 1–67 and 3–67 and their stable isotope labeled standards (Figure 3a). To achieve optimal sensitivity for quantification of endogenous SDF-1α forms, we employed an immunoaffinity enrichment step with an anti-SDF-1α antibody prior to LC-MS/MS analysis. Specifically, the plasma was first spiked with a fixed amount of stable isotope labeled SDF-1α standards (for both intact and NH_2-

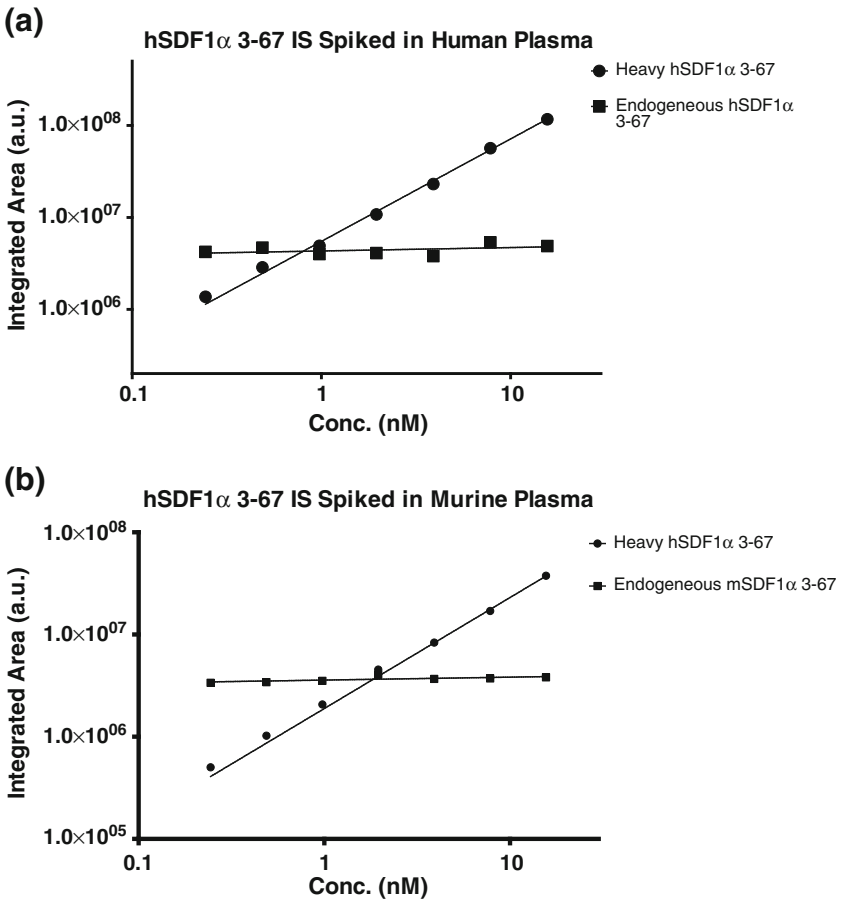

Figure 4. LC-MS/MS response of stable isotope labeled internal standard of SDF-1α 3–67 versus spiked concentration. Heavy isotope labeled internal standard was spiked in plasma at different concentrations prior to immunoaffinity enrichment and LC-MS/MS analysis. **(a)** Concentration–response relationship in human plasma. **(b)** Concentration–response relationship in mouse plasma. In both figures, the horizontal line represents the endogenous concentration of SDF-1α 3–67, which is well above the limit of quantification and within the linear range of the curve

terminal truncated forms, at plasma concentration of 2 nmol/L); an anti-SDF-1α antibody conjugated to paramagnetic beads was then used to selectively bind and enrich SDF-1α from the background and the eluent was analyzed by LC-MS/MS analysis. An example of the total ion current chromatogram of SRM transitions measuring the endogenous SDF-1α forms and their respective isotope labeled standards is illustrated in Figure 3b. The linearity and limit of quantification (LOQ) of the LC-MS/MS assay were evaluated with a "reverse standard curve," where refolded heavy SDF-1α standards were spiked in human plasma at concentrations ranging from 1 pmol/L to 50 nmol/L. An example of the LC-MS response, integrated peak area (AUC) against spiked concentrations for SDF-1α 3–67 heavy isotope-labeled standard is plotted in Figure 4a. The linear range of the assay spanned three orders of magnitude, from 20 pmol/L to 20 nmol/L. The level of endogenous SDF-1α 3–67 in plasma, represented by the horizontal line in the figure, was determined as 0.96 nmol/L (%CV = 5.0 %, n = 12), which is within the quantitative range of the analysis. The lower LOQ of the assay, 20 pmol/L (%CV = 12.2 %, n = 3), was determined according to the method described by Currie [23]. Because of the complete overlap between human and rhesus SDF-1α sequences, the assay can be used for quantification of SDF-1α forms in rhesus plasma without modification. A reverse standard curve using rhesus plasma as background matrix yielded the same quantitative range and LOQ as the standard curve with human plasma as background, with the endogenous SDF-1α 3–67 level in pooled rhesus plasma determined at 0.65 nmol/L (%CV = 4.9 %, n = 12).

A separate SRM method for murine SDF-1α quantification was also developed. Similar to the SRM method quantifying human SDF- 1α forms, four alternating Q1→Q3 transitions were involved with two tracking fragmentation of endogenous murine SDF-1α forms and the other two tracking fragmentation of the corresponding heavy isotope labeled human SDF-1α standards. The human heavy standards were used to in lieu of murine heavy standards because of the nearly complete overlap between human and murine SDF-1α sequences with the only difference at position 18 (Val in human, Ile in mouse). The same enrichment antibody was also used in the murine SDF-1α assay. A similar reverse standard curve was also constructed for murine SDF-1α assay, the linear range and LOQ of which were determined to be the same as the human SDF-1α assay (Figure 4b). The level of endogenous SDF-1α 3–67 of murine SDF-1α in pooled plasma was determined as 1.89 nmol/L (%CV = 5.2 %, n = 12), which is within the linear range of the analysis.

Suppression of Inactive SDF-1α by DPP-IV Inhibition in an In Vivo Study with Mice

Although in vitro studies showed that SDF-1 isoforms (α and β) are substrates for DPP-IV and *ex vivo* studies with sera from DPP-IV-deficient mice confirmed the finding [13], no in vivo studies have been performed to explore the effect of DPP-IV inhibition on the balance between endogenous SDF-1α active and inactive forms. The main obstacle to such study was the lack of sensitive analytical methods that differentiate the intact SDF-1α from the DPP-IV cleaved inactive form. Since our LC-MS/MS method offered a LLOQ much lower than the endogenous level of SDF-1α in pooled murine plasma, we took the opportunity to investigate the suppression of inactive SDF-1α (3–67) by DPP-IV inhibition in an in vivo study using mice. Plasma samples were collected from lean C57BL/6 mice treated with a DPP-IV inhibitor, MK-0626, or vehicle. Although the exposure of DPP-IV inhibition in this study was limited to 1 h post-oral dosing and the animals were sacrificed shortly after MK-0626 reached maximum blood concentration (T_{max} ~20 min), there was a statistically significant difference (P = 0.0062) in the levels of intact SDF-1α between treatment and vehicle groups with means being 1.54 and 0.76 nM, respectively (Figure 5). The total SDF-1α level (the sum of SDF-1α 1–67 and SDF-1α 3–67), however, did not exhibit a statistically significant difference between the treatment and vehicle groups with means being 2.53 and 2.72 nM respectively. This observation highlighted the very importance of the development of a specific assay that differentiates the two SDF-1α forms; no effects of DPP-IV inhibition on SDF-1α would have been detected if the assay could not differentiate the two forms and only measure total concentration.

Suppression of Inactive SDF-1α by DPP-IV Inhibition in an In Vivo Study with Non-Human Primates

We further investigated the effect of DPP-IV suppression on the balance of SDF-1α forms in higher species and with longer exposure of DPP-IV inhibition. Plasma from rhesus monkeys dosed with vehicle or MK-0626 in acute and chronic fashions were collected 16 h post-oral dosing (overnight) for measurements of intact SDF-1α and the truncated form. In both acute (d 1) and chronic dosing (d 15), similar to the murine study, there were statistically significant differences in the levels of intact SDF-1α between the treatment and vehicle groups (Figure 6). The total SDF-1α level, however, exhibited a statistically significant decrease from the vehicle groups to the treatment groups in both acute dosing and chronic dosing. Apparently, the longer exposure of DPP-IV inhibition shifted the balance between intact and truncated SDF-1α in rhesus monkeys so drastically that it almost completely "stripped" the truncated form from circulation. There was, therefore, less SDF-1α in circulation in the monkeys treated with the DPP-IV inhibitor; however, the circulating SDF-1α was mostly intact and active in these monkeys.

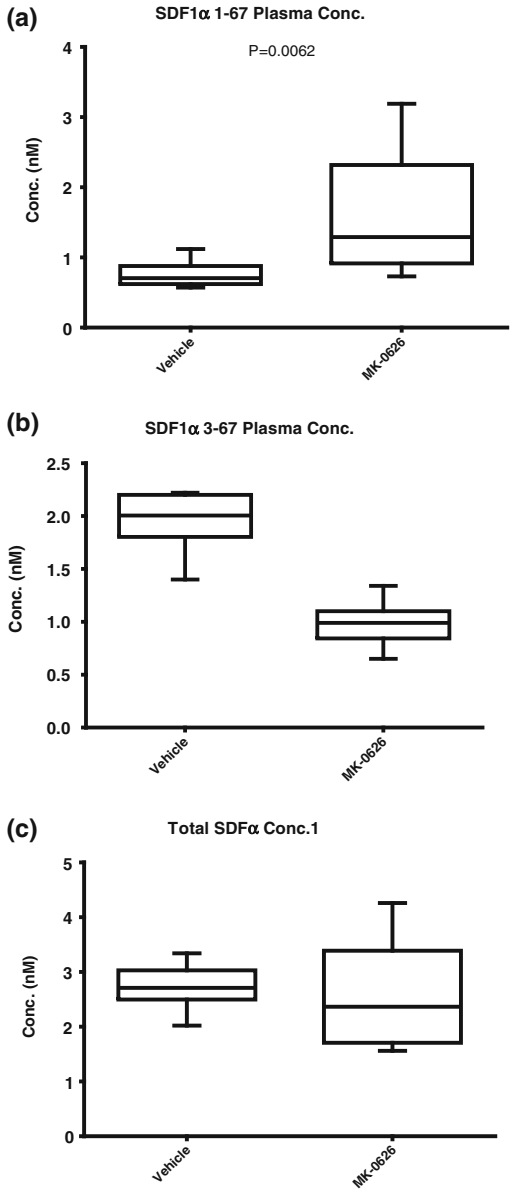

Figure 5. Box and whisker plots of levels of intact and total SDF-1α in mice treated with vehicle or a DPP-IV inhibitor. **(a)** Levels of intact SDF-1α in vehicle group compared with the treatment group, with mean levels being 0.76 nM and 1.54 nM, respectively (n = 8 in both groups, P = 0.0062). **(b)** Levels of SDF-1α 3–37, the truncated form, in vehicle group and the treatment group. **(c)** Levels of total SDF-1α, the sum of SDF-1α 1–67 and 3–67, in vehicle group and the treatment group

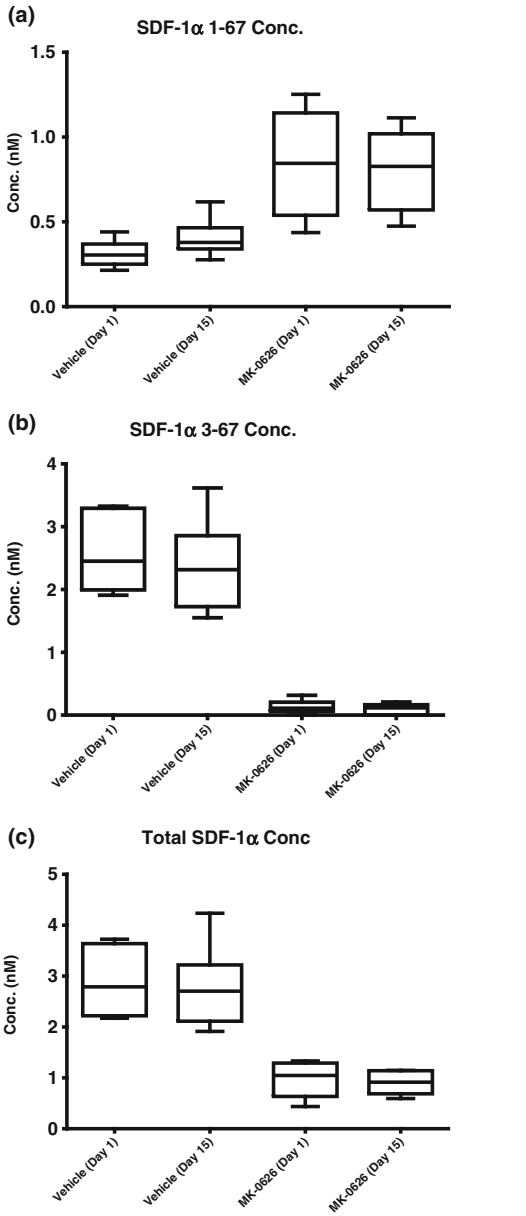

Figure 6. Box and whisker plots of levels of intact and total SDF-1α in rhesus monkeys treated with vehicle or a DPP-IV inhibitor. **(a)** Levels of intact SDF-1α in vehicle groups compared with treatment groups (n=6 in all groups), dosed either acutely (d 1) or chronically (d 15). From left to right, mean values for each group are 0.31, 0.41, 0.84, and 0.81 nM. In both dosing regimens, rises in the level of intact SDF-1α in the treatment groups are statistically significant, whereas there is no statistically significant difference in comparisons between dosing regimens within the vehicle or the treatment groups. **(b)** Levels of SDF-1α 3–37, the truncated form, in vehicle groups and treatment groups. **(c)** Levels of total SDF-1α in vehicle groups and treatment groups. From left to right, mean values for each group are 2.89, 2.77, 0.97, and 0.90 nM. The decreases in the level of total SDF-1α in the treatment groups compared with the vehicle group are statistically significant; within the vehicle or the treatment groups, there is no statistically significant difference in comparisons between dosing regimens

Discussion

To investigate the suppression of SDF-1 inactivation with in vivo DPP-IV inhibition, we developed a highly specific and sensitive assay for quantification of endogenous SDF-1 in plasma. To achieve optimal performance, we coupled immunoaffinity enrichment with stable

isotope dilution quantification by tandem mass spectrometry. The measurements of intact and DPP-IV cleaved SDF-1 were multiplexed in a single method on a QQQ mass spectrometer. Through isotope dilution with stable isotope-labeled internal standards, the assay generates absolute quantification of the analytes. Using α isoform as an example, we demonstrated that the assay has a linear range for quantification relevant to the physiological level of SDF-1 analytes. Exploiting the performance of the assay, we studied the effects of in vivo DPP-IV inhibition on the levels of intact and NH_2 terminal truncated SDF-1α and found marked increase of intact SDF-1α in animals dosed with a DPP-IV inhibitor. In both studies using mice and rhesus monkeys, the balance between intact and truncated SDF-1α was tilted toward the intact form while the total amount of SDF-1α remained unchanged in mice and significantly decreased in rhesus monkeys.

Two strategies are generally involved in protein quantification with mass spectrometry: "bottom-up" and "top-down." In the bottom-up approach, proteins are first enzymatically digested and then quantified through signals of representative peptides. In the top-down approach, intact proteins are directly quantified without being enzymatically digested first. Obviously, the top-down approach is more straightforward in operation with fewer steps involved than bottom-up approach, but its sensitivity diminishes when molecular mass of analytes increases. For proteins with higher masses, bottom-up approach is the ideal method because of more favorable chromatographic behavior and ionization efficiency of ESI MS for peptides than for proteins; however, because it only relies on a handful of selected peptides for quantification, it rarely distinguishes protein isoforms or truncated forms of the same protein. In developing LC-MS assays that are specific to SDF-1 isoforms as well as to their NH_2 terminal truncated forms, we were bound to choose top-down approach while being aware of the limit on assay sensitivity. To achieve the optimal assay sensitivity, we decided to leave the disulfide bonds intact and quantify relevant SDF-1 sequences in their native and non-reduced states. Besides simplicity in sample preparation, the decision not to reduce and alkylate the samples so that SDF-1 isoforms are folded globularly was also based on the native structure's ideal LC elution profile as well as its favorable CID pattern for SRM quantification. In addition, the native structure is probably favored over the disulfide bond-reduced random coil in binding to enrichment antibodies, which determines the efficiency of the affinity enrichment step. In the end, we were able to achieve a lower limit of quantification of 20 pM (with 100 µL plasma as starting material) from a streamlined method with just essential binding and elution steps. The performance of the assay is on par with similar hybrid affinity enrichment LC-MS assays for smaller peptide hormones [24–28], and we believe keeping SDF-1α in its native structure throughout the process was critical to achieving the optimal sensitivity. The specificity of the assay, reliant on peptide sequence readout by tandem mass spectrometry rather than on the enrichment antibody, can be exploited to study physiological processing of SDF-1 by other proteases [29] as well as polymorphism of SDF-1 isoforms. Furthermore, because chemokines are structurally similar, the general strategy of the assay—measuring folded proteins in native state—can be extended to quantify other chemokines with similar arrangements of disulfide bridges such as CC, CXC, and CX_3C.

The CXCR4-SDF-1 chemotaxis is negatively modulated by DPP-IV, which is expressed on many hematopoietic cell populations and is present in a catalytically active form in circulation [30]. DPP-IV removes the NH_2-terminal dipeptide from SDF-1, producing a truncated form that is devoid of chemotactic activity and instead blocks chemotaxis of the full length SDF-1. The suppression of such inactivation has been investigated with pharmacologic inhibition of DPP-IV by small molecules, peptides, and engineered DPP-IV resistant SDF-1 [9, 31, 32]. In many in vivo studies, immunoassays were used to quantify SDF-1 level in peripheral blood. Such measurements did not differentiate intact SDF-1 from NH_2-terminal truncated form and only yielded total SDF-1 levels. Total SDF-1 level is not the ideal biomarker for target engagement (i.e., inhibition of DPP-IV) in these studies. In our mouse study with relatively short exposure of DPP-IV inhibition, there was no statistically significant difference in the level of total SDF-1α in blood between vehicles and mice dosed with a DPP-IV inhibitor; however, there was a significant change in the balance between intact SDF-1α and NH_2-truncated form with the portion of intact SDF-1α increasing from 27.7 % to 57.9 % upon DPP-IV inhibition. The level of intact SDF-1α clearly indicated efficacy for the DPP-IV inhibitor, whereas the level of total SDF-1α was misleading. Total SDF-1α level was even more misleading in the rhesus study, where it was a third in the monkeys treated with a DPP-IV inhibitor compared with the monkeys in the vehicle group. Meanwhile, the level of intact SDF-1α more than doubled in the DPP-IV inhibitor treatment group compared with the vehicle group. The level of NH_2-terminal truncated SDF-1α, which represents SDF-1α inactivation, almost disappeared completely upon inhibition of DPP-IV (> 85 % of total SDF-1α composition in the vehicle group versus <15 % in the treatment group). Given that truncated SDF-1α is at least inactive and could even potentially act as an antagonist to the SDF-1-CXCR4 axis, our results indicate that there is a feedback mechanism in which the level of *intact* SDF-1α in peripheral blood is used as signal to regulate the systemic production of SDF-1α. Without DPP-IV inhibition, a high level of SDF-1α was produced to counter the pressure of inactivation by DPP-IV; when such suppression is alleviated with DPP-IV inhibition, much less SDF-1α was needed to

maintain the SDF-1 gradient between tissues and peripheral blood. This feedback apparently requires a certain exposure level of the DPP-IV inhibitor; in the study with mice, although the intact SDF-1α was preserved immediately upon DPP-IV inhibition, we did not observe an immediate drop in the level of total SDF-1α. Further studies with longer exposure of DPP-IV inhibition in rodents are needed to confirm this mechanistic feedback in lower species.

In summary, we developed a novel method for quantifying SDF-1α and its NH_2-terminal truncated form in circulation via LC-MS/MS coupled with immunoaffinity enrichment of the analytes. Our results demonstrated that the method offers high sensitivity and specificity. Our work thus illustrated the analytical strength and unique advantage of top-down mass spectrometric quantification of small proteins in their native and folded state. The SDF-1α assay for human could be applied to relevant clinical samples in the future and may yield new insights on change of SDF-1α levels in disease settings and in response to therapeutic interventions. The methods for preclinical animal models were developed to enable translational research on suppression of SDF-1 inactivation with DPP-IV inhibition. Using two in vivo animal studies as examples, we demonstrated that it was critical to differentiate intact SDF-1α from its truncated form in the analysis of biomarkers for pharmacologic inhibition of DPP-IV activity.

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