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Utility of LC-MS Surrogate Peptide Methodology in the Development of a Combinectin, a Unique Anti-HIV Biologic Drug

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OBJECTIVES: The anti-HIV biologic drug combinectin is a construct of three independent HIV entry inhibitors and a pharmacokinetic (PK) enhancer, which together exhibit a synergistic increase of potency compared to the individual components. Consequently, the integrity of the macromolecule over time is of concern to maintain high potency.

METHODS: The development of this drug modality was supported by two orthogonal assays. The in vivo samples were analyzed by a custom-developed enzyme-linked immunosorbent assay (ELISA) or were subjected to the universally applicable surrogate peptide methodology followed by a fit-for-purpose liquid chromatography-mass spectrometry (LC-MS) analysis.

RESULTS: The ELISA data allowed for a general differentiation of the stability of the analogues while the LC-MS approach afforded detailed data on the PK profiles of the four distinct subunits.

CONCLUSIONS: A selective proteolytic metabolism of the biologic drug was determined by the specific LC-MS surrogate peptide representative of the free carboxyl-terminus of the analyte.

KEYWORDS: combinectin, anti-HIV biologic drug, LC-MS, surrogate peptide methodology, ELISA.

INTRODUCTION

The chronic condition described as the Acquired Immune Deficiency Syndrome (AIDS) in humans is the result of an infection by the retrovirus termed Human Immunodeficiency Virus (HIV). This virus has two main strains, HIV-1 and HIV-2, with HIV-1 being the more common type. The infection of T-helper (CD4) cells by HIV-1 occurs through a multi-step process depicted in **Figure 1** by which fusion occurs between the cellular membrane and the viral membrane. The generally accepted model of this process is based on the interaction of the viral gp160 trimer glycoprotein complex (composed of gp120 and gp41 subunits) with cell surface receptors on the membranes of the target cells. Following the initial binding of gp120 to a CD4 molecule to the cell membrane, conformational changes take place that allows for the subsequent binding of the gp120 subunit to cellular chemokine co-receptors CCR5 or CXCR4. After binding to the co-receptor, an additional conformational change of the gp120/gp41 complex allows the gp41 subunit to be inserted into the membrane of the target cell, initiating steps required for membrane fusion [1-5].

The standard antiretroviral therapy (ART) to maximally suppress the HIV virus and to stop the progression of the AIDS disease is based on the combination dosing of at least two classes of antiretroviral drugs (ARV, [6]) which are typically orally administered short-acting small-molecule entities. There continue to be an unmet medical need to develop novel drug modalities with improved pharmacological properties. Long-acting agents decrease potential drug-drug interactions and may lead to better treatment compliance. A multi-specific approach could address evolving virus resistance and increase potency along with a refined safety and tolerance profile.

A unique anti-HIV biologic drug, termed combinectin, is shown in **Figure 2**. The combinectin molecule consists of four subunits connected with linkers combining three independent HIV entry inhibitors and human serum albumin (HSA) as an in vivo pharmacokinetic (PK) enhancer. [5,7] The approach to constructing multi-specific drugs to prevent HIV infection is based on the synergistic increase of potency of the macromolecule compared to the individual components. [5,7-9] Similar observations of improved potency were reported when a bispecific HIV-1 neutralizing antibody targeting the gp120 subunit was linked with ibalizumab or a fusion peptide inhibitor was bound to a monoclonal antibody targeting the co-receptor CCR5 [10,11].

The combinectin macromolecule utilizes targeted binding proteins, termed adnectins, as HIV entry inhibitors. Adnectins are derived from the 10th type III domain of the human fibronectin protein and possess modifiable binding loops that can be targeted to any protein epitope, akin to the complementarity determining regions of an antibody [12].

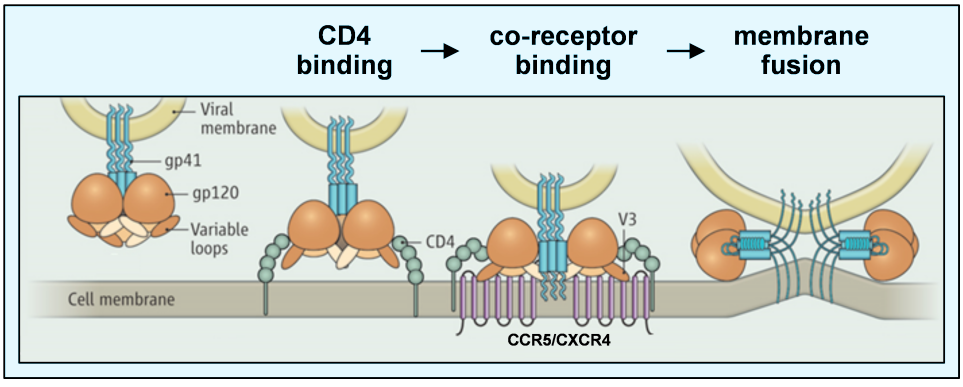

Figure 1. Multi-step process of the infection of human T-helper cells by HIV via fusion of cellular and viral membranes (adapted from [3], credit P. Huey/Science, reprinted with permission from AAAS).

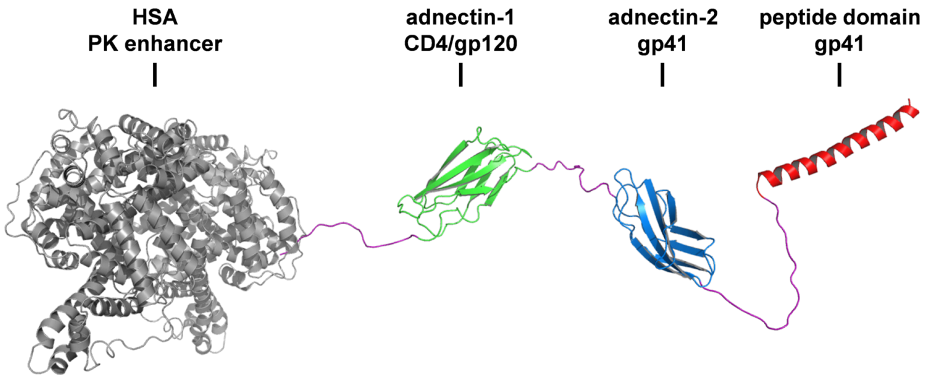

Figure 2. The combinectin molecule, combining three independent HIV entry inhibitors and a PK enhancer. The adnectin-1 component targets the CD4 binding step. The subunits adnectin-2 and peptide domain target the gp41 membrane fusion. An HSA entity improves in vivo PK properties of the construct. [5, 7]

The adnectin-1 component binds to CD4 and inhibits a post CD4-binding step similar to the drug Ibalizumab. [4,13,14] The adnectin-2 and the alpha-helical peptide domain subunits independently target the gp41 membrane fusion similar to the drug Enfuvirtide. [5,9,15,16] Lastly, an HSA unit, in this case, linked to the amino-terminus (N-terminus) of the construct, is employed to improve in vivo PK properties of the construct.

Studies have shown that it is critical for all components of the combinectin molecule to remain intact for optimal biological efficacy [5,8,9]. It was therefore imperative to monitor the in vivo exposure of the intact construct along with any potential degradation to ensure the development of an accurate pharmacokinetic/pharmacodynamics (PK/PD) relationship during the drug discovery and development process. Typically, an enzyme-linked immunosorbent assay (ELISA) would be used to quantify a large protein *in vitro* and *in vivo* samples. However, ELISAs commonly do not provide detailed information on partial clipping, i.e., proteolysis or degradation, of a biologic drug due to the location of the capture and the detection regions, which are normally not at the termini of the proteinaceous analyte. Hence, an orthogonal assay utilizing the universally applicable surrogate peptide approach and a fit-for-purpose liquid chromatography-mass spectrometry (LC-MS) analysis was developed to monitor the state of the peptide domain unit and specifically the free carboxyl-terminus (C-terminus) in support of structure-activity relationship (SAR) efforts including biotransformation studies [17,18]. Presented here is the methodology to apply LC-MS to the development of a unique anti-HIV biologic drug, combinectin, which is exemplified with *in vivo* PK stability data.

EXPERIMENTAL

Anti-HIV Biologic Drugs

The utility of LC-MS in the development of combinectin molecules is demonstrated with two similar in-house generated constructs, PRD-3191, and PRD-3202, which differ only in the identity of the last amino acid residue of the peptide domain; leucine (L) versus serine (S) at the carboxyl-terminus (**Table 1**).

Animal Dosing

The study was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and was approved by the Bristol-Myers Squibb site institutional animal care and use committee.

Table 1. The similar combinectin constructs PRD-3191 and PRD-3202 differ in the last amino acid residue at the carboxyl-terminus of the peptide domain subunit.

Combinectin PRD-3191	[HSA]-[adnectin-1]-[adnectin-2]-[peptide domain=TIAEYAARIEALIRAAQEQQEKNEAALRELYKWAL]
Combinectin PRD-3202	[HSA]-[adnectin-1]-[adnectin-2]-[peptide domain=TIAEYAARIEALIRAAQEQQEKNEAALRELYKWAS]

Male cynomolgus monkeys received a 1 mg/kg dose of drug, PRD-3191 or PRD-3202, in phosphate-buffered saline (PBS) solution by intravenous (IV) infusion. One monkey was dosed per test article. Serial blood samples (~0.3 mL) were collected from a femoral artery pre-dose and at 0.17, 1, 7, 24, 48, 72, 144, 168 hours post-dose. Centrifugation at 4°C yielded serum samples, which were frozen and stored at -80°C until analysis.

Calibration Standards

Calibration standards were prepared by spiking cynomolgus monkey serum with the intact combinectin molecule, PRD-3191 or PRD-3202, and then serially diluting 2-fold to obtain a calibration curve ranging from 0.12 nM to 125 nM.

Enzyme-Linked Immunosorbent Assay (ELISA)

Streptavidin-Coated Gold 96-well plates (Meso Scale Diagnostics, Rockville, MD) were coated with the capture reagent by adding 50 µL of biotinylated PRD-828, an in-house generated peptide derived from the gp41 protein [5], at 1 µg/ml in phosphate-buffered saline with tween (PBST) solution (GE Healthcare Life Sciences, Marlborough, MA) to each well, and shaking for 1 hour at room temperature. The coated plates were washed three times with PBST buffer, incubated with 200 µL blocking buffer (1% of BSA in PBST buffer) for 1 hour, and washed three times with PBST buffer. The serum samples were prepared at three dilution levels (100-, 5000-, and 10000-fold) and added to the assay-ready plates along with the calibration standards. The plates were incubated at room temperature with shaking for 1 hour and washed three times with PBST buffer. The detection reagent was applied by adding 50 µL of Sulfo-Tagged (Meso Scale Diagnostics) anti-HSA antibody (Thermo Scientific, Waltham, MA) at 0.5 µg/mL in 1% BSA in PBST and shaking for 1 hour at room temperature. The plates were washed three times with PBST buffer and 150 µL of MSD Read Buffer (2X) (Meso Scale Diagnostics) was added. Data were obtained on a plate reader (Meso Scale Diagnostics).

Surrogate Peptides for LC-MS Analysis

The LC-MS surrogate peptides of the combinectin molecules were determined via in silico digestion of the proteinaceous subunits using PinPoint software (Thermo Scientific). The amino acid sequence of the protein was the input for a computed proteolytic cleavage by a predefined enzyme. In this case, trypsin was selected which generally cleaves peptides on the carboxyl-terminal side of lysine and arginine amino acid residues except when followed by a proline amino acid residue. The output of the software was a list of potential proteotypic peptides suitable for specific and sensitive tandem mass spectrometric (LC-MS/MS) analysis. The bioanalytical data on these proteolytic peptidic analytes were then a surrogate for concentration levels of the original protein. Initial LC-MS experiments to select peptides for optimal chromatographic performance and detection sensitivity yielded the surrogate analytes listed in **Table 2**.

These surrogate analytes will inform as proxy reporters on the concentration levels of the respective molecular subunits. Specifically, the P4 analyte will inform on the integrity or degradation of the peptide domain unit.

Table 2. Surrogate peptidic analytes of the combinectin subunits monitored by LC-MS analysis.

	HSA	Adnectin-1	Adnectin-2	Peptide Domain			
LC-MS surrogate analytes	**HSA**	**A1**	**A2**	**P1**	**P2**	**P3**	**P4**
Combinectin PRD-3191	DDNPNLPR	YQVFSVPGSK	VHPYR	IEALIR	NEAALR	ELYK	WAL
Combinectin PRD-3202	DDNPNLPR	YQVFSVPGSK	VHPYR	IEALIR	NEAALR	ELYK	WAS

Reagents for Sample Preparation for LC-MS Analysis

Biotinylated goat anti-human serum albumin (anti-HSA) antibody (Rockland Immunochemicals, Limerick, PA) was immobilized on Dynabeads M-280 Streptavidin (Thermo Fisher Scientific, Waltham, MA) as per vendor instructions by mixing a ratio of 10 µg antibody and 1 mg beads for 1.5 hours at room temperature. The final concentration of the antibody-coated beads was 10 mg/mL, and was stored in PBST buffer at 4°C for future use.

Sample Preparation for LC-MS Analysis

Frozen samples were thawed at room temperature for one hour. The serum samples and calibration standards were prepared at three dilution levels (2-, 5-, and 10-fold). Combinectin molecules were isolated from the matrix via immunoaffinity enrichment methodology by mixing 30 µL aliquots of the in vivo study samples and calibration standards with 60 µL goat anti-HSA antibody immobilized on beads in PBST buffer. The mixtures were incubated for 60 minutes at room temperature and the supernatant was separated from the beads using a MC Version Flick & Blot VP 771HH-MC magnetic bead separator (V&P Scientific, San Diego, CA). The beads were washed three times with PBST buffer, and two times with 50 mM ammonium bicarbonate solution (Sigma Aldrich, St. Louis, MO). The beads were reconstituted with 60 µL of 50 mM ammonium bicarbonate solution containing 0.1% RapiGest (Waters, Milford, MA) and 10 µL of 100 mM dithiothreitol (DTT) (Sigma Aldrich), and incubated for one hour at 60°C while mixing at 800 rpm utilizing a thermomixer (Eppendorf, Westbury, NY). After the mixtures were cooled to room temperature, 10 µL of 150 mM of iodoacetamide (Sigma Aldrich) was added and incubated in the dark for 30 minutes at room temperature. To generate the LC-MS surrogate peptides of the combinectin subunits, 10 µL, equating 3 µg of sequence grade modified trypsin (Promega, Madison, WI), was added to the mixtures and incubated overnight at 37°C while mixing at 800 rpm. The proteolysis was quenched with 20 µL of 1% formic acid. The supernatant with the analytes was separated from the beads and analyzed by LC-MS.

Setup of LC-MS Analysis

The LC-MS system consisted of an Acquity I-Class UHPLC system (Waters, Milford, MA) interfaced with an API6500 triple quadrupole MS system (Sciex, Toronto, Canada). The samples were kept at 10°C in the autosampler, and a 10 µL volume was injected onto an Acquity BEH C18 column (2.1 x 50 mm, 1.7 µm, Waters) at a temperature of 60°C with a flow rate of 600 µL/min. The aqueous mobile phase A consisted of water with 0.1% formic acid (LC-MS grade, Thermo Fisher Scientific. Waltham, MA), and the organic mobile phase B consisted of acetonitrile with 0.1% formic acid (LC-MS grade, Thermo Fisher Scientific). Initially, the mobile phase was held at 1% B from 0.0 min to 0.2 min, then a linear gradient was executed to 95% B at 2.0 min and 95% B was held to 2.5 min, followed by a rapid return to 1% B at 2.6 min and 1% B was held for 0.4 min of re-equilibration at initial conditions, for a total run time of 3 minutes.

The following source conditions were used for the mass spectrometer operation in

Table 3. MS conditions of the surrogate peptides of the combinectin molecules.

Combinectin PRD-3191 / Combinectin PRD-3202	Precursor ion [m/z]	Product ion [m/z]	Declustering potential [V]	Collision energy [eV]
HSA (DDNPNLPR)	470.7	596.4	60	20
A1 (YQVFSVPGSK)	556.3	721.4	60	23
A2 (VHPYR)	336.2	435.2	80	25
P1 (IEAUR)	357.7	472.3	100	15
P2 (NEAALIR)	337.2	430.3	100	14
P3 (ELYK)	276.6	310.3	60	16
P4 (WAL)	389.2	258.1	60	16
P4 (WAS)	363.2	258.0	60	16

electrospray positive ion mode: resolution Q1 = unit, resolution Q3 = unit, entrance potential = 10 V, collision cell exit potential = 11 V, curtain gas = 35 AU, collision gas = 9 AU, ion spray voltage = 5000 V, temperature = 600°C, ion source gas 1 = 50 AU, and ion source gas 2 = 60 AU. As shown in **Table 3**, seven precursor-ion to product-ion transitions (MRM scan functions) were simultaneously monitored per combinectin molecule using tandem MS (MS/MS) detection with a dwell time of 25 ms. The data were acquired and analyzed using Analyst Instrument Control and Data Processing software version 1.6.2 (Sciex). Any further data processing was performed using Microsoft Excel computing software and its build-in functions.

Assay Calibration Curves

The dynamic range of the calibration curves for the ELISA of the intact combinectin molecules and the LC-MS analysis of the surrogate peptides of the combinectin molecules are listed in **Table 4** showing the lower limit of quantitation (LLOQ as the signal being 3-times the blank value) and the upper limit of quantitation (ULOQ) in nM units. The data obtained from study samples were plotted against the calibration curves (linear regression fit weighted by reciprocal concentration ($1/x^2$)) to compute concentration levels.

RESULTS AND DISCUSSION

The combinectin constructs are a potent long-acting anti-HIV biologic drug consisting of three distinct HIV entry inhibitors and a PK enhancer (**Figure 2**) [5]. These macromolecules

Table 4. Dynamic range of the calibration curves in nM units for the ELISA and the LC-MS surrogate peptides applied to the analysis of combinectin molecules.

		LC-MS						
	ELISA	HSA	A1	A2	P1	P2	P3	P4
Combinectin PRD-3191	0.002 - 1.0	7.8 - 62.5	0.98 - 62.5	0.49 - 62.5	0.12 - 62.5	0.24 - 62.5	0.98 - 62.5	0.24 - 62.5
Combinectin PRD-3202	0.002 - 1.0	7.8 - 62.5	0.98 - 62.5	0.49 - 62.5	0.12 - 62.5	0.24 - 62.5	0.98 - 62.5	0.24 - 62.5

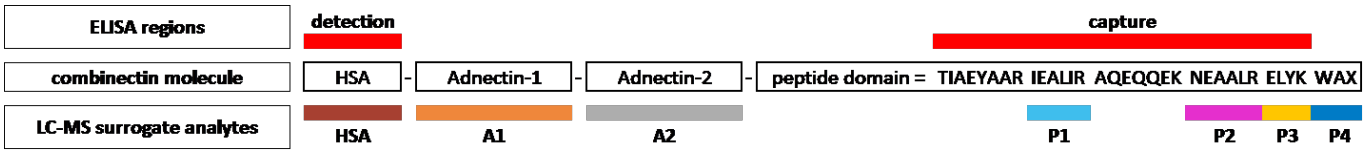

Figure 3. Comparison of the detection coverage of the combinectin molecules (P4 analyte: X = L for PRD-3191 and X = S for PRD-3202) by the ELISA and the LC-MS surrogate peptide approach.

exhibit a unique synergistic increase in potency compared to the individual components along with an improved resistance barrier. However, for these molecules to maintain their potency, the subunits need to remain intact for extended time periods to afford acceptable PK properties such as half-life values [5,8,9]. Thus, any data on the integrity of the linked subunits will inform on SAR efforts to improve pharmacological parameters including potency, report on potential metabolic clipping of amino acid residues at the protein subunits, and contribute to the projection of the human efficacious dose. While the custom ELISA provided bioanalytical PK data on the "assumed" intact analyte, the assay cannot detect partial clipping, i.e., proteolysis or degradation, of the carboxyl-terminus of the peptide domain subunit until enough amino acids in the recognition epitope of the capture reagent are removed, such that the capture reagent no longer binds the combinectin molecule. As conceptualized in **Figure 3**, the capture region (amino acid residue sequence TIAEYAARIEALIRAAQEQQEKNEAALRELYK) of the ELISA covers

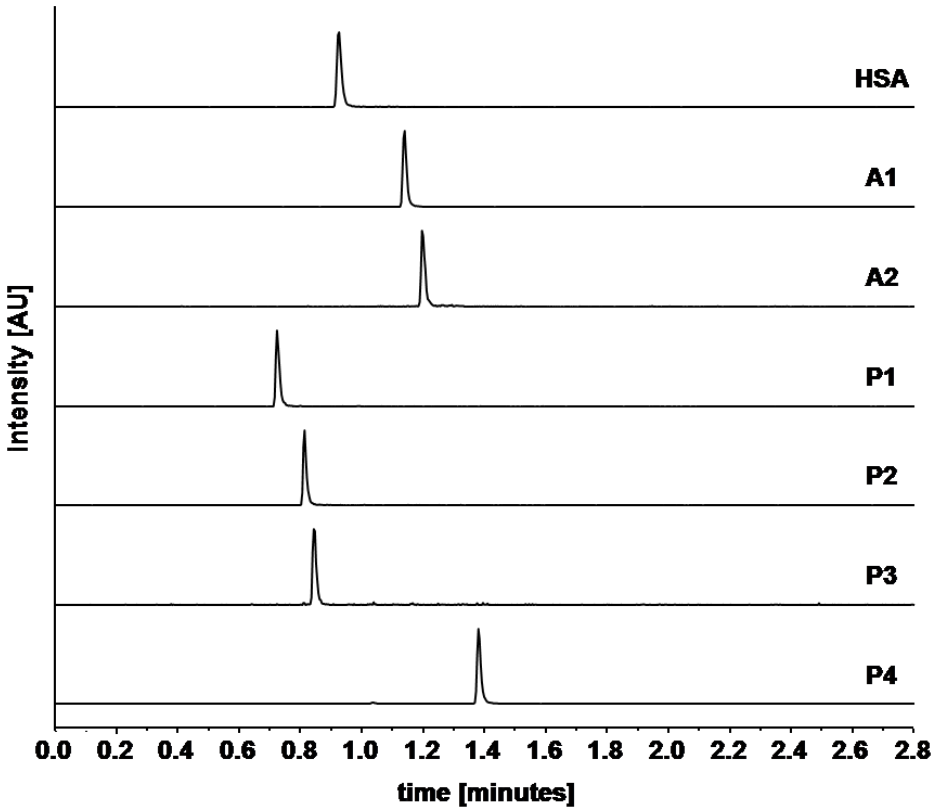

Figure 4. Extracted ion chromatograms of the seven peptide surrogate analytes displaying the selectivity of the LC-MS approach to monitor combinectin molecules in in vivo samples.

Table 5. Half-life data [hours] of the combinectin constructs comparing data derived from ELISA and discrete LC-MS surrogate peptides. Only the data of the LC-MS analyte P4 represents the PK profile of the intact construct, thus correcting the overestimated half-life value of the ELISA data.

		LC-MS						
	ELISA	HSA	A1	A2	P1	P2	P3	P4
Combinectin PRD-3191	13	13	12	12	44	8	6	4
Combinectin PRD-3202	31	41	51	42	40	36	37	23

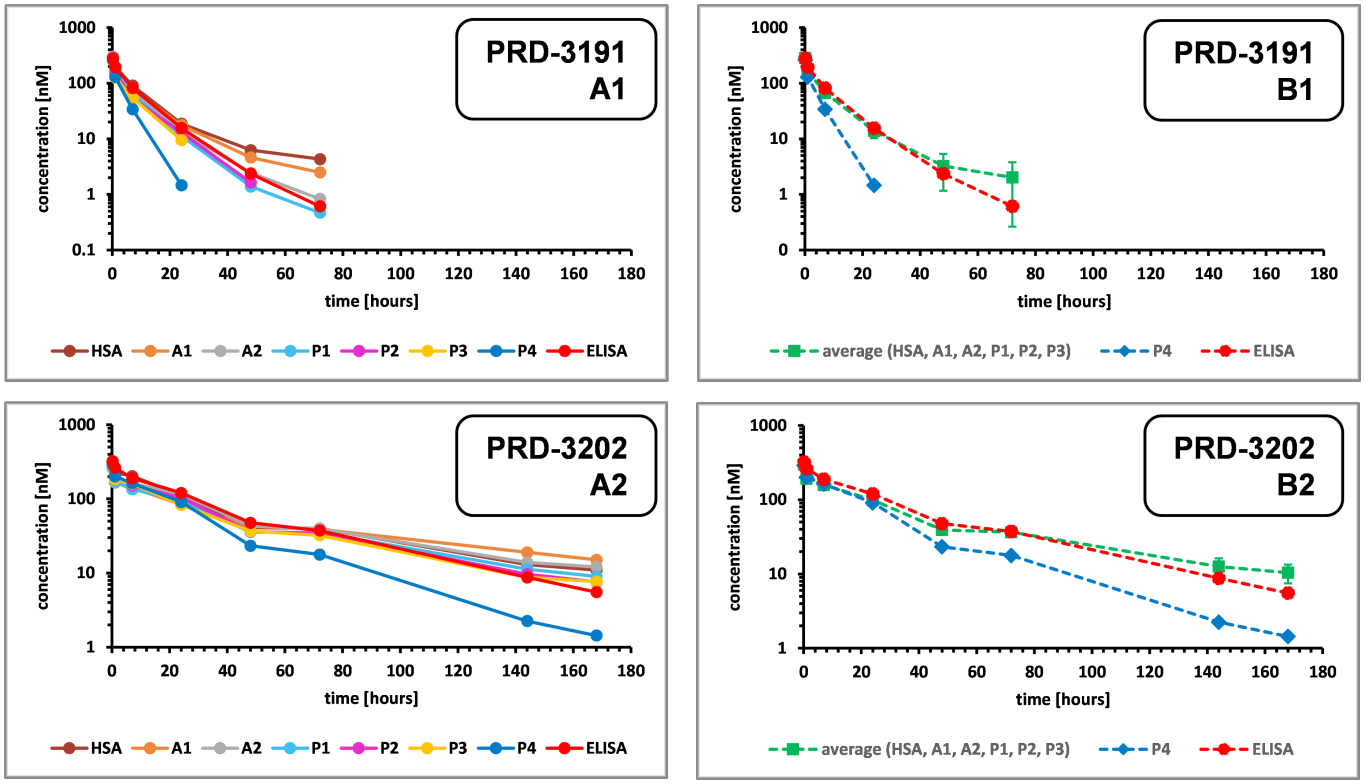

Figure 5. The panels A1 and A2 display PK profiles of the combinectin molecules PRD-3191 and PRD-3202 based on data from the ELISA and the seven discrete LC-MS surrogate peptides HSA, A1, A2, P1, P2, P3, and P4. The panels B1 and B2 are a simplified representation of the data by averaging the analytes HSA, A1, A2, P1, P2, and P3, which are in adequate correlation and reciprocal validation with the ELISA data. However, the data of the P4 analyte indicate a more rapid proteolytic metabolism of the carboxyl-terminus of the peptide domain unit compared to the other components of the macromolecule.

most of the peptide domain subunit and the detection region is within the HSA domain; consequently, the integrity of the amino acid residues of the carboxyl-terminus of the peptide domain is not required for binding and subsequent detection of the combinectin molecule, thus partially confounding the resulting PK data determined using this assay. Conversely, the LC-MS assay informs on the integrity of the peptide domain unit by monitoring the surrogate peptides P1 (IEALIR), P2 (NEAALR), P3 (ELYK), and specifically on the state of its carboxyl-terminus via the analyte P4 (WAL or WAS, respectively) in addition to providing specific data on the PK enhancer HSA and the two HIV entry inhibitors Adnectin-1 (A1) and Adnectin-2 (A2).

An example of the simultaneous analysis and detection of the seven LC-MS surrogate peptides in the one hour time point study sample of PRD-3191 is depicted in **Figure 4** illustrating the selectivity of the LC-MS approach for biologic drugs.

The PK profiles of the combinectin molecules in cynomolgus serum are shown in **Figure 5** and the associated half-life properties are listed in **Table 5**.

The data on the earlier analogue PRD-3191 obtained with the ELISA and the seven discrete LC-MS surrogate peptides are presented in **Figure 5-A1**. The ELISA data and the LC-MS data of the analytes HSA, A1, A2, P1, P2, and P3 are inadequate correlation and reciprocal validation, as expected since the capture and detection regions of the ELISA reagents cover the same domains as these LC-MS analytes of the combinectin molecule (**Figure 3**). The LLOQ of the assays for PRD-3191 was reached at the 72 hour time point. For discussion and simplicity purposes, the data of the LC-MS surrogate peptides HSA, A1, A2, P1, P2, and P3 are displayed in **Figure 5-B1** as the average data (including standard deviation error bar). The data of the LC-MS surrogate peptide P4 does not align with the ELISA data indicating a more rapid proteolytic metabolism of the carboxyl-terminus of the peptide domain unit compared to the other components of the macromolecule; i.e., HSA, Adnectin-1, and Adnectin-2. Consequently, only the data of the LC-MS analyte P4 represent the PK profile of the intact combinectin molecule, which is a crucial contribution to the projection of the human efficacious dose. [5] As listed in **Table 5**, the ELISA data indicate a half-life of 13 hours whereas the data of analyte P4 specify a half-life of 4 hours.

Continued SAR efforts yielded the analogue PRD-3202 featuring a substitute of the carboxyl-terminus amino acid residue from leucine (L) to serine (S) which significantly improved the half-life properties of the combinectin molecule, suggesting a slower metabolic degradation of the molecule (**Figure 5-A2 and B2**). The LLOQ of the assays for PRD-3202 was reached at the 168 hour time point. However, as previously discussed, only the data of LC-MS analyte P4 denote the PK profile of the intact construct thus correcting the overestimated half-life value of the ELISA data from 31 hours to 23 hours (**Table 5**).

CONCLUSIONS

To determine systemic exposure of combinectin analogues in cynomolgus monkeys following intravenous (IV) infusion administration, a custom developed ELISA and the universally applicable surrogate peptide methodology followed by a fit-for-purpose liquid chromatography-mass spectrometry (LC-MS) analysis was utilized for the bioanalysis of serum samples. A combinectin construct consists of four subunits, three distinct HIV entry inhibitors (Adnectin-1, Adnectin-2, peptide domain), and an HSA PK enhancer, which exhibits a synergistic increase in potency compared to the individual subunits, thus the integrity of the macromolecule over time is of concern to maintain potency. While the ELISA data allowed for a general differentiation of the stability of the macromolecules, only the LC-MS surrogate peptide representative of the free carboxyl-terminus of the peptide domain unit informed on the selective proteolytic metabolism of the biologic drug. The specificity of the universally applicable LC-MS surrogate analyte methodology can be applied to SAR efforts including biotransformation studies in the development of complex molecules such as biotherapeutic drugs without the need for custom-made assay reagents.

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