Biomeasures and mechanistic modeling highlight PK/PD risks for a monoclonal antibody targeting Fn14 in kidney disease

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ABSTRACT

Discovery of the upregulation of fibroblast growth factor-inducible-14 (Fn14) receptor following tissue injury has prompted investigation into biotherapeutic targeting of the Fn14 receptor for the treatment of conditions such as chronic kidney diseases. In the development of monoclonal antibody (mAb) therapeutics, there is an increasing trend to use biomeasures combined with mechanistic pharmacokinetic/pharmacodynamic (PK/PD) modeling to enable decision making in early discovery. With the aim of guiding preclinical efforts on designing an antibody with optimized properties, we developed a mechanistic site-of-action (SoA) PK/PD model for human application. This model incorporates experimental biomeasures, including concentration of soluble Fn14 (sFn14) in human plasma and membrane Fn14 (mFn14) in human kidney tissue, and turnover rate of human sFn14. Pulse-chase studies using stable isotope-labeled amino acids and mass spectrometry indicated the sFn14 half-life to be approximately 5 hours in healthy volunteers. The biomeasures (concentration, turnover) of sFn14 in plasma reveal a significant hurdle in designing an antibody against Fn14 with desired characteristics. The projected dose (>1 mg/kg/wk for 90% target coverage) derived from the human PK/PD model revealed potential high and frequent dosing requirements under certain conditions. The PK/PD model suggested a unique bell-shaped relationship between target coverage and antibody affinity for anti-Fn14 mAb, which could be applied to direct the antibody engineering towards an optimized affinity. This investigation highlighted potential applications, including assessment of PK/PD risks during early target validation, human dose prediction and drug candidate optimization.

Introduction

Successful development of monoclonal antibody (mAb) therapeutics requires thorough characterization and optimization of their properties, not only to achieve the desired pharmacokinetics but also to enable optimal target interaction, which is a prerequisite for enabling a pharmacodynamic effect. Knowledge of relevant characteristics of the therapeutic target can be equally important for designing appropriate drug properties, such as required affinity ranges. Target properties, also referred to as biomeasures, include target concentration and turnover, and can be incorporated into mathematical modelling of the pharmacokinetic/pharmacodynamic (PK/PD) relationship, for example using quantitative site-of-action (SoA) model. The models predictions are frequently sensitive to the accuracy of the biomeasures, which may have to be determined experimentally when no prior information is available. Herein, we describe the application of this strategy to assist with PK/PD risk assessment of an anti-Fn14 mAb therapeutic in early discovery.

The cytokine, tumor necrosis factor-like weak inducer of apoptosis (TWEAK, Apo3L, TNFSF12) and its cognate receptor, fibroblast growth factor-inducible 14 (Fn14, TWEAK receptor, TNFRSF12A, CD266) are members of the TNF/TNFR superfamily. Fn14 is the only characterized TWEAK receptor that can transduce both soluble and membrane TWEAK. The human Fn14 gene encodes a type I transmembrane protein (129 amino acids) that is processed into a mature protein (102 amino acids). The extracellular domain (53 amino acids) contains a cysteine-rich domain necessary for TWEAK binding. The intracellular domain (29 amino acids) of Fn14 lacks a death domain. However, it contains TNFR-associated factor (TRAF)-binding sites that can initiate the signaling events through recruitment of TRAF2/5 and activation of IKK and MAP kinase pathways. While Fn14 is expressed at low levels in healthy tissue, it is upregulated in tissue injury to mediate local tissue responses. Similar to many TNF receptors, Fn14 has only recently been reported to undergo proteolytic cleavage in the extracellular domain. The soluble sFn14 (sFn14) levels are markedly increased in plasma from mouse models of kidney disease and are correlated with disease biomarkers including proteinuria and MCP-1. Additionally sFn14 was detected in human plasma and urine. Moreover, sFn14 levels in urine are significantly increased in diabetic nephropathy (DN) patients and correlated with proteinuria and MCP-1 levels.

Fn14 was originally described as an immediate-early response gene regulated by growth factors in fibroblasts. Accumulating evidence has suggested a role for TWEAK activation of Fn14 receptors in the pathogenesis of acute and
chronic kidney injury, contributing to both glomerular and tubulointerstitial damage in non-immune and immune-mediated kidney diseases. Several studies have supported the pathogenic role of TWEAK/Fn14 pathway by using mouse knock-out or antibody blockade in various mouse models of kidney diseases, including acute kidney disease (AKI) and chronic kidney disease (CKD). In particular, TWEAK antibody and Fn14 KO studies have demonstrated a protective role in various systemic lupus erythematosus (SLE)/lupus nephritis (LN) models, including NTN, MRL/lpr and cGvHD models. In addition to kidney diseases, TWEAK/Fn14 has a potential role in the diseases of different organs, including heart, skeletal muscles, the central nervous system, liver, and gut.

Indeed, several reports have provided data to demonstrate that tissue expression of TWEAK and Fn14, as well as circulating TWEAK levels, are associated with these diseases in both mouse models and human patients. While the neutralizing anti-TWEAK antibody (BIIB023; Biogen) Phase 2 clinical trial exploring the potential for kidney protection in lupus erythematosus/lupus nephritis (LN) has been discontinued, there has been limited exploration of the therapeutic potential of direct antagonistic targeting of Fn14.

The experimental data described above support the rationale for targeting Fn14 for the treatment of renal disease. However, it is possible that the soluble form of the receptor may impact the ability to attain the desired membrane-bound target coverage by occupying mAb binding sites and reducing the number of mAb binding sites available to bind the membrane Fn14. Additionally, efficient neutralization of sFn14 in plasma may not translate to sufficient target coverage in tissue and human efficacy. It is possible to evaluate the feasibility of a target without actually producing the neutralizing antibody by constructing mechanistic models that describe the kinetics of target, antibody, and target-antibody interaction. Since the pharmacokinetics of the antibody can be assumed to be typical IgG mAb kinetics, the sensitive parameters are the target turnover, target abundance and the affinity of the antibody for the target. The interplay between soluble target, membrane-bound target and antibody, as well as the effects of key biomolecules such as target turnover have been integrated using a quantitative site-of-action (SoA) model for an anti-CXCL13 antibody. To aid in the model development, the sFn14 turnover (balance between protein synthesis and protein clearance) was determined for healthy volunteers using pulse-chase administration of a stable isotope labelled (5,5,5-2H3)-leucine (D3-leucine) coupled with immunoaffinity liquid chromatography tandem mass spectrometry (IA-LC-MS/MS) using the method described by Bateman et al. In addition, human sFn14 and human kidney tissue Fn14 concentrations were determined using a quantitative IA-LC-MS/MS method. These experimentally determined biomarkers were subsequently incorporated by the SoA model with a goal of evaluating the feasibility of the target and the optimum Kd for the neutralizing antibody.

**Results**

**Determining levels of kidney Fn14 and serum sFN14 in human**

Quantification of Fn14 in the human kidney has not previously been reported; hence, kidney Fn14 concentrations were determined experimentally. The levels of Fn14 in human kidney were measured by IA-LC-MS/MS, and determined to be 63±10 nM (mean±SD) in tissue samples collected from 5 diabetic nephropathy patients (Figure 2). Soluble Fn14 concentration in human serum, also measured by IA-LC-MS/MS, was determined to be 1.7±0.3 nM (mean±SD) in 16 healthy individuals (Figure 2). This value is similar to the sFn14 concentration range previously reported. In the previous publication, the serum level of sFn14 in 12 healthy individuals was determined to be 2.8±1.4 nM (14±7 ng/ml) (mean±SD) using an ELISA assay.

**Serum turnover rate for sFn14**

The turnover of sFn14 was identified as a critical biomarker for the SoA model through sensitivity analysis as it significantly affected the required clinical dose by model simulation. For instance, a 3-fold higher dose could be required when the half-life of sFn14 was changed from 50 hour to 0.5 hour (simulation result not shown). Therefore, the physiologically relevant turnover of sFn14 was experimentally determined in healthy human volunteers using a pulse-chase study combined with a MS-based measurement of tracer incorporation changes into a sFn14-specific peptide over time (Figure 3a). The resulting data were fitted to first order kinetics equations (Figure 3b) and an average half-life of 5±0.5 hours was determined for sFn14 in the serum of three healthy volunteers. This half-life was considered fairly rapid and could be rationalized based on the small size of sFn14 (molecular weight of approximately 5 kDa), which might be subject to rapid renal filtration.45,46

**Optimal Kd identification for antibody engineering**

During the early development of antibody therapeutics, the optimal binding affinity against the therapeutic target is a critical point to address. It is known that reduction in Kd could help reduce clinical dose in many circumstances. In order to explore the effect of antibody Kd on the target coverage, it requires an understanding of the interplay between anti-Fn14 antibody, mFn14 and sFn14. Hence a mechanistic SoA model was established for human (Figure 1), by incorporating parameters listed in Table 1, which are based on bioanalytical measurements presented earlier in the results and assumptions of typical antibody PK.

The relationship between Kd of anti-Fn14 mAb and the clinical dose required to achieve specific target coverage at trough was explored using SoA model simulations. Due to the uncertainty in mFn14 turnover in the kidney, simulations were conducted for a range of mFn14 half-lives (0.5, 5, and 50 h, shown as 3 panels) (Figure 4). In addition, the effect of sFn14 levels on clinical dose versus Kd relationship was also investigated (shown as the band width of the simulation curves), since sFn14 level was not determined in kidney patients. The sFn14 levels used in the simulation ranges from the measured level in healthy volunteers to a 10-fold higher level, based on the observations that sFn14 levels are markedly increased in plasma from mouse models of kidney disease compared to healthy mouse.

Interestingly, there appears to be a reversed bell-shaped relationship between dose of anti-Fn14 mAb and its Kd. The bell-
The optimal $K_d$ is defined as the $K_d$ at which the lowest anti-Fn14 mAb dose is required to achieve specified target coverage (90% or 50%). The optimal $K_d$ varied depending on the mFn14 turnover. When target turnover for mFn14 is relatively slow ($t_{1/2} \text{Fn14} = 50$ hr), an optimal $K_d$ of about 0.3 nM allows for ≥ 90% target coverage. When target turnover for mFn14 is 5 h, an optimal $K_d$ of 1 nM provides 90% target coverage. When target turnover for mFn14 is 0.5 h, clinical dose versus $K_d$ relationship appears to be monotonic for 90% target coverage, and there is no advantage for having an intermediate $K_d$. In this scenario, $K_d = 5$ nM provides similar target coverage. In summary, an optimal $K_d$ of 0.3 to 1 nM was suggested to the antibody engineers, as it requires the lowest dose for achieving ≥ 90% target coverage in all scenarios (Fn14 half-life varies between 0.5 to 50 hr).
anti-Fn14 antibodies obtain the optimal Kd for target coverage (Kd ≤ 0.3 nM) were investigated during the simulation. It is assumed that coverage. Again, a range of mFn14 turnover half-lives (0.5, 5 and 50 h) was investigated, as expressed in width of bands). Optimal Kd of anti-Fn14 mAb is the Kd at which the required dose is the lowest (illustrated as vertical lines).

**Table 1. Model parameters for mechanistic SoA model.**

| Parameter | Definition | Units | Value | Reference |
|-----------|------------|-------|-------|-----------|
| $S_{cF}$ | sFn14 baseline concentration in plasma | nM | 1.7 | Measured value in healthy volunteers by MS |
| $\gamma_{cF}$ | mFn14 baseline concentration in kidney | nM | 63 | Measured value in diabetic nephropathy patients by MS |
| $k_{int}$ | Internalization rate of mFn14 in kidney | hr⁻¹ | | A range of internalization half-life of 0.5 to 50 hr was investigated |
| $k_{intDT}$ | Internalization rate of mFn14-mAb complex in kidney | hr⁻¹ | 0.014-1.4 | Assumed same internalization rate as mFn14 |
| $k_{el}$ | Elimination rate of sFn14 in plasma | hr⁻¹ | 0.14 | A half-life of 5 hr in healthy subjects by 51Pulse chase study |
| $k_{elD}$ | Elimination rate of drug-sFn14 complex in plasma | hr⁻¹ | 0.0035 | Assumed same elimination rate as mAb for soluble target |
| $K_D$ | Binding affinity of anti-Fn14 mAb | nM | 0.3 | Predicted optimal Kd |
| $k_{on}$ | on rate for anti-Fn14 mAb binding | M⁻¹×hr⁻¹ | 3.6 | Maximum on-rate controlled by diffusion limit of antibody (1) |
| $k_{elD}$ | Elimination rate constant of the anti-Fn14 mAb | hr⁻¹ | 0.0035 | Typical mAb PK parameter |
| $k_{el}$ | Distribution rate constant of anti-Fn14 mAb from plasma to peripheral compartment | hr⁻¹ | 0.0078 | Typical mAb PK parameter |
| $k_{pkD}$ | Distribution rate constant of anti-Fn14 mAb from peripheral compartment to plasma | hr⁻¹ | 0.0079 | Typical mAb PK parameter |
| $k_{pk}$ | Distribution rate constant of anti-Fn14 mAb from plasma to into SoA | hr⁻¹ | 0.043 | Based on the assumption that antibody distribution into tissue is driven by lymphatic flow, which is 0.2% of blood flow (CL,D,DP = 124.6 ml/h × 0.2% = 0.25 ml/h) (2,3) and $k_{pD} = \frac{25}{t_{1/2,Fn14}}$ |
| $k_{pkD}$ | Distribution rate constant of anti-Fn14 mAb from SoA to plasma | hr⁻¹ | 4.21 | Back-calculated based on tissue mAb concentration is about 30% of plasma concentration (4,5) |

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**Human efficacious dose prediction and risk assessment**

Using the SoA model, human efficacious dose was projected for anti-Fn14 antibodies to evaluate developmental risk, in light of the high circulating levels of sFn14 and its potential impact on target coverage. Again, a range of mFn14 turnover half-lives (0.5, 5 and 50 h) were investigated during the simulation. It is assumed that anti-Fn14 antibodies obtain the optimal Kd for target coverage (Kd = 1 nM if $t_{1/2,Fn14} = 0.5$ and 5 hr, Kd = 0.3 nM if $t_{1/2,Fn14} = 50$ hr). The predicted target coverage for mFn14 in kidney and sFn14 in plasma under weekly subcutaneous dosing were presented in Figure 5. The turnover half-life of mFn14 has a large impact on target coverage at site of action. If mFn14 turns over quickly ($t_{1/2,Fn14} = 0.5$ hr), a high dose of 10 mg/kg/week is predicted for achieving >90% target coverage. With a medium to slow turnover ($t_{1/2,Fn14} = 5$ or 50 hr), sufficient target coverage (>90% at trough) can be achieved at 1 mg/kg/week.

**Discussion**

With the maturation of antibody engineering technologies, the development pipeline for mAbs and related modalities is very rich and diverse. PK/PD modeling coupled with critical bioanalytical measures has gained wide applications in all stages of mAb therapeutics development. Although many publications have touched upon the general guiding principles for applying PK/PD modeling.

![Image](image-url)
In this report, a mechanistic SoA model was first established to capture the interplay between anti-Fn14 antibody, the shed Fn14, and membrane Fn14 targets. The model captures crucial elements affecting the target coverage, including the antibody PK and tissue distribution, target levels and kinetics, as well as the binding affinity for the anti-Fn14 antibody. The presented fit-for-purpose SoA model, where sFn14 in the kidney compartment is not considered, was chosen as the final model. The choice was based on the comparison against a “full model”, where sFn14 in kidney is modeled for its potential neutralizing effect on anti-Fn14 antibody. Our evaluations suggest that, due to the limited amount of sFn14 present in kidney (tissue volume of 0.1 L) compared to plasma (tissue volume of 3 L), the effect of sFn14 in kidney on target coverage is negligible (simulations performed; results not presented). Therefore, sFn14 in kidney is not considered in the final model based on the principle of parsimony. Mechanistic SoA models are applied earlier during early drug discovery, for example, toward a quantitative understanding of target pharmacology in preclinical models, or in evaluating the impact of baseline tissue target concentration measurement on target coverage prediction. This report, on the other hand, provides an example for applying the SoA model, in conjunction with critical biomeasures, to aid antibody engineers and early risk assessment for the target.

Bioanalytical experiments were conducted to generate pivotal model parameters. Since moderate level of sFn14 (22.6 ng/ml) was identified in mouse, plasma sFn14 level in human was measured. The sFn14 was present at significant level in healthy subjects (median level at 1.7 nM). The mFn14 level in target tissue (kidney) from diabetic nephropathy patients is high (63 nM) as determined by MS. In addition, considering the potential fast turnover of sFn14 due to its small size (molecular weight of 5 kD), a pulse-chase experiment was conducted to determine the in vivo turnover rate of sFn14 in three healthy human volunteers. Stable isotope labeled amino acids pulse-chase, with minimal system perturbation in conjunction with sensitive MS measurements led to highly reliable physiological turnover data. The biomeasures were integrated into the SoA model, providing more confidence in model prediction.

Our report provides a case study for applying modeling and biomeasure in early drug discovery. The first example is for therapeutic antibody design/optimization, particularly optimal Kd identification. Having guidance on the optimal Kd could greatly facilitate the antibody selection and engineering process, and avoid “over-optimization” situations, where improvement in antibody affinity cannot further reduce the clinical dose but may delay the drug development timeline. Interestingly for anti-Fn14 antibodies, there appears to be a reversed bell-shaped relationship between Kd and required dose (Figure 4). This observation is unique due to the presence of a high amount of soluble target (sFn14) in the circulation considering large plasma volume. In the current case, sFn14 competes against membrane target Fn14 for binding to anti-Fn14 mAb. When the anti-Fn14 binds extremely tightly to targets (both soluble and membrane Fn14), large amounts of antibody are absorbed by the sFn14 “sink” in the circulation. As a result, the required dose for covering the membrane target is high. Conversely, an antibody with weak affinity cannot sufficiently neutralize either soluble or membrane target, leading to a high dose requirement. It is only when Kd is at an intermediate value that the dose for desired target coverage (e.g., 90%) is minimal, since...
antibodies are not trapped to a significant extent by the shed target, but still have the necessary potency to bind to the membrane target. For anti-Fn14 antibody, an optimal affinity of 0.3 to 1 nM was suggested. It would be counter-productive to have developed super potent (e.g., single digit picomolar) antibody without modeling and biomarkers. Considering many membrane-bound targets shed their extracellular domain (e.g., HER2, CD20), the current case study can be applied to these categories of therapeutic targets. It is also worth noting that optimal Kd can be dependent on all the model parameters, including levels and turnover rates of soluble and membrane target. For example, optimal Kd can vary with different turnover half-life of membrane Fn14 (Figure 4). Thus, it is important to determine the relevant bio-measures to have confidence in the prediction of optimal Kd. The early application of modeling may help select the best candidate for entering the clinic.

Coupling critical biomarkers and modeling, potential risk factors for developing anti-Fn14 antibody as a therapeutic agent were investigated during early drug discovery. Biomarkers, including levels for sFn14 and mFn14, and Fn14 turnover rate, were integrated into the SoA model, providing confidence in model prediction. The clinical dose requirement was evaluated, in light of the high level of circulating sFn14. The simulations indicate a relatively high and frequent clinical doses (>$1 mg/kg/week) are likely to be required for anti-Fn14 Ab to achieve over 90% target coverage over the dosing interval in case of fast mFn14 turnover (Figure 5). If close to complete target coverage (>99%) is required for sufficient pharmacology, the predicted dose will likely be 3 mg/kg/week and above (results not shown). It is also worth pointing out that, in the current SoA model, it is assumed that mFn14 (in injured kidney and other healthy tissues) does not have a significant effect on the disposition of anti-Fn14 antibody. As a result, the half-life of the anti-Fn14 mAb is assumed to be similar to a typical mAb. Depending on the amount of mFn14 target present in patients and the binding/internalization property of anti-Fn14 antibody, target-mediated drug disposition (TMDD) may reduce the drug exposure. In case of significant TMDD, the required clinical dose could be even higher than what is currently predicted. This information can be helpful to evaluate the anti-Fn14 mAbs against other antibodies with similar indication, e.g., anti-TWEAK antibodies. In addition, early projection of the clinical dose can influence strategies on molecular properties (e.g., viscosity) and formulations. Modeling also suggests that the clinical dose could be highly dependent on the internalization rate of Fn14 and drug-Fn14 complex in the kidney (Figure 5). Because of the high level of Fn14 in kidney (63 nM), rapid internalization (if $t_{1/2}$ > 0.5 hr) of the drug-Fn14 complex can significantly increase drug clearance, leading to high clinical dose (10 mg/kg/week). Thus, Fn14 and anti-Fn14 antibodies should be carefully evaluated for their internalization properties using in vitro cell assays and in vivo PK experiments. For instance, if Fn14 is rapidly turning over, an antibody with slow internalization rate may be preferable, as it has less target-mediated clearance. On the other hand, if Fn14 is turning over slowly, an antibody with rapid internalization may be better, since it can efficiently deplete Fn14 through internalization, and thus permits higher target coverage. The internalization properties are critical for membrane-targeted antibodies and should be studied in great detail during early drug discovery.

The SoA model could be further validated using experimental approaches, such as preclinical animal experiments. Key model assumptions, such as the neutralizing effect of sFn14, could be tested using a pharmacology study in relevant animal models by measuring the concentration-time profile of total sFn14 (both free and antibody bound). An accumulation of total sFn14 can be supportive of the soluble target binding assumption. Model simulations can be tested preclinically as well. For instance, to test the projection of a bell-shaped relationship between affinity and efficacious dose, a few antibodies with different binding affinities (low, median and high) can be compared side-by-side in animal models. Furthermore, a model parameter that is hard to obtain in human can be estimated by translating the animal parameter determined in preclinical studies.

The changing environment for drug discovery and development (e.g., increased cost, substantial competition when developing drugs against the same target) requires smarter strategies to be more efficient. Although antibody engineering techniques have been matured over decades, rational design and selection of antibody drug is still an evolving field. PK/PD modeling, together with critical biomarker measurement, could provide valuable guidance during early development of mAbs. By integrating directly relevant information, such as the target level and physiological turnover, and disease population, the model simulations can suggest optimal characteristics for the antibody drug, such as modality choices, optimal Kd, and required half-life. This report illustrates an integrated approach that can be applied to many antibody programs.

Materials and methods

Immunoaffinity-LC-MS/MS assay

sFn14 levels were measured in human serum obtained from healthy subjects and Fn14 levels were measured in human kidney tissue from diabetic nephropathy patients. Recombinant human Fn14 (Uniprot ID Q9NP84, amino acids 27–75 corresponding to the predicted extracellular domain of Fn14 fused to a C terminal His tag) was obtained from Pfizer Pharmaceuti
cal. Anti-Fn14 mAb P4A8 was obtained from Pfizer and biotinylated using EZ-link Sulfo-NHS-LC-biotin (Pierce Protein Research Products/Thermo Scientific; Rockford, IL). Dynabeads Streptavidin MyOne T1 was purchased from Invitrogen Life Technologies (Carlsbad, CA). Serum samples were either provided by Pfizer (Cambridge, MA) or purchased from Bioreclamion (Long Island, NY). The reference stable isotope-labeled (SIL) peptide was obtained from Thermo Fisher.

A 20 μL aliquot of serum sample, calibrant or quality control (QC) sample was diluted with 680 μL phosphate-buffered saline (PBS) in a 2 ml 96-deep well plate. One μg of biotinylated anti-Fn14 (P4A8 antibody) was added to each well and samples were incubated overnight at 4°C while shaking at 900 rpm. Twenty μL of MyOne T1 streptavidin magnetic beads were added to each well and the plate was incubated for 45 min at room temperature (RT) on a shaker. Magnetic bead and sample processing was performed on an automated liquid handling system (Microlab Star, Hamilton, Bonaduz, Switzerland). The beads were washed twice with 280 μL CHAPS buffer followed by one wash with 280 μL 10 mM PBS. Captured proteins were eluted with 140 μL of 30 mM HCl and immediately
neutralized with 30 μL of 2 M Tris–HCl, pH 8.0. A 10 μL aliquot of 0.9 fmol/μL stable isotope-labeled reference peptide was added to each sample. Reduction was performed by the addition of 15 μL of 75 mM TCEP and incubation at 56 °C for 45 min. Fifteen μL of 150 mM iodoacetamide was added to each sample and incubated in the dark at RT for 30 min for alkylation. Samples were then digested overnight at 37 °C using 1 μg of trypsin prior to LC-MS/MS analysis.

The calibration consisted of a six point standard curve analyzed in duplicates across a Fn14 quantitation range from 110.2 to 8930 pM. The highest calibrant was prepared by diluting recombinant human Fn14 in 5% bovine serum albumin (BSA). The remaining calibrants were prepared by 3-fold serial dilution of the highest calibrant. QC samples were analyzed in quadruplicates and prepared using human control serum that was either unspiked, spiked with 858.6 pM recombinant human Fn14 or diluted 3 fold using 5% BSA buffer.

A Dionex Ultimate 3000 HPLC system was used for the separation of protein digests. It was configured with a WPS-3000 thermostatted well-plate autosampler, a FLM-3300 flow manager containing a temperature-controlled column compartment and a quaternary micropump, a NCS-3500 module composed of a binary high pressure nanopump, and a tertiary loading pump combined with a temperature-controlled dual 6-port valve column compartment. The system was controlled by Chromelone software (version 6.8).

One hundred ul of the sample was first loaded on a PepMap300 C18 trap column (5 × 0.3 mm, 5 μm, 300 A, Dionex) followed by chromatographic separation on a PepMap C18 RSLC nanocolumn (15 cm × 75 μm, 2 μm, 100 A, Dionex) by gradient elution at a flow rate of 600 nL/min using Solvent A (0.1% formic acid in 2% acetonitrile) and Solvent B (0.1% formic acid in 90% acetonitrile). From 0 to 4 min, the solvent composition was held at 5% B and then ramped up from 5% to 40% B during 4 to 13.5 mins of the cycle, reaching 90% B at 16 mins and then brought back to initial conditions of 5% B for re-equilibration. The total chromatography run time was about 15 min.

A 5500 Qtrap mass spectrometer (AB Sciex) was operated in multiple reaction monitoring (MRM) mode for detection of Fn14 tryptic peptides. The eluate from nonflow chromatography was introduced into a nanospray III source (AB Sciex) using a nanospray stainless steel emitter (50 mm × 30 μm ID, Proxeme, West Palm Beach, FL). The following instrument parameters were employed: Ion spray voltage: 3700 V; nebulizing gas: 5 ps; curtain gas: 10 ps and Interface heater temperature: 180 °C. Q1 and Q3 were operated in unit and low resolution, respectively. The transitions monitored for the target peptide, GSSWSADLDK were: Q1 533.2 to Q3 921.4 (y8+), 834.4 (y7+), 648.3 (y6+) and 561.2 (y5+) representing the doubly charged precursor ion. Q1 533.2 to Q3 648.3 (y6+) was used for quantification of human Fn14.

AB Sciex Analyst software (Version 1.5.2) was used for data acquisition and peak areas integration. For each sample, peak area ratios (PAR) of the Fn14 to the reference SIL peptide were calculated and the PAR’s of calibration samples were used to construct calibration curves by applying a 1/(concentration) weighted non-linear regression model using Labstats (Excel add-on). The Fn14 concentration of calibrants was back-calculated to assess the quality of the curve fit. All sample concentrations were then calculated from their PARs against the calibration curve.

sFn14 serum turnover rate measurement

A pulse-chase study combined with IA-LC-MS/MS was utilized for the measurement of the half-life of sFn14 in three healthy male human volunteers between 21 and 40 years old. Briefly, D3-leucine tracer was administered to the subjects with primed (1.3 mg/kg body weight), continuous (0.022 mg/kg body weight per minute) intravenous infusion for 18 hours. Twenty-four blood samples were collected longitudinally from each subject starting at time 0 before bolus infusion and continued during the 18 hours of infusion, followed by 18 hours sample collection after withdrawal of the infusion. The study was conducted at Clinical Pharmacology of Miami with the consent from the volunteers and approved by the Western Institutional Review Board (Olympia, Washington, USA).

Measurements were made for determination of serum leucine tracer enrichment, as well as incorporation of D3-leucine into the sFn14 -specific peptide (GSSWSADLDK). Gas chromatography-mass spectrometry (GC-MS) was employed to measure the serum leucine isotopologue ratios at each time point. Akin to the method described above for the quantification of sFn14, the incorporation of the leucine tracer into sFn14 was measured using IA-LC-MS/MS analysis. Briefly, 200 μL aliquots of serum samples diluted by addition of 200 μL of PBST were spiked by 1 μg of biotinylated anti-Fn14 antibody (Anti-Human/Mouse CD266 (mouse IgG2b, 12-9018) from ebioscience (San Diego, CA) and incubated overnight at 4 °C. Streptavidin T1 Dynabeads were added to the samples and incubated for 45 min at RT while mixing at 900 rpm. Magnetic bead wash and protein elution were processed on Microbal Star Hamilton as described above. 1 μg trypsin/Lys C mixture (Promega) was added to the immunoffinity enriched samples and incubated for overnight digestion and the resulted peptide mixtures were analyzed on a Dionex Ultimate 3000 (Thermo Scientific) linked to a triple quadrupole mass spectrometer, TSQ-Quantiva (Thermo Scientific, USA) equipped with an EZ-spray ionization source. Three MRM transitions for single charge y3, y6 and y7 fragments of GSSWSADLDK doubly charged peptide for both light and heavy were monitored. Raw data were imported into Skyline v2.5 software (University of Washington) for peak integration and area ratios were exported and converted to H/(H+L).

A tracee-tracer model introduced by Foster et al. for estimating the fractional synthetic rate of plasma proteins was implemented in SAAM II software (The Epsilon Group, University of Washington). The model captures the kinetic processes for the synthesis and degradation of the protein. By fitting the profiles of labeled leucine as well as the labeled protein of interest, the model provides estimations for the synthesis and degradation rates of the target protein.

Mechanistic SoA model

Mechanistic SoA models were established for human (Figure 1) using species-specific parameters. The model describes the PK processes of the anti-Fn14 mAb, its distribution into the target tissue and binding to the tissue target Fn14. Since the shed target, sFn14 was detected in the plasma, the SoA model also incorporates the binding between sFn14 and mAb to account...
for the potential impact of sFn14 on bioactive-Fn14 mAb. The set of ordinary differential equations that describe the mouse SoA model are as follows:

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\begin{align}
\frac{dD_p}{dt} &= -k_\text{eff}D_p + k_{p,D}D_T \frac{V_T}{V_p} - k_{p,D}D_p + k_{p,D}D_p \frac{V_p}{V_T} - k_{p,D}D_p + \frac{V_T}{V_p} - k_{on}D_pS_p - k_{on}D_pS_p \\
\frac{dD_s}{dt} &= k_{p,s}D_s \frac{V_p}{V_T} - k_{p,s}D_s + \frac{V_T}{V_p} - k_{on}D_sT_s \\
\frac{dD_T}{dt} &= k_{p,s}D_s \frac{V_p}{V_T} - k_{p,s}D_s + \frac{V_T}{V_p} - k_{on}D_sT_T \\
\frac{dD_T}{dt} &= k_{eff}D_T - k_{on}D_sT_s + k_{synTi} - k_{intTi}T_s \\
\frac{dD_s}{dt} &= k_{eff}D_S - k_{on}D_sS_p + k_{synSp} - k_{edS}S_p \\
\frac{dD_T}{dt} &= k_{on}D_sT_s - k_{off}D_T - k_{intDT}DT_s \\
\frac{dD_p}{dt} &= k_{on}D_pS_p - k_{off}D_S - k_{edD}D_S
\end{align}
\]

\(D_p, D_T, \) and \(D_s\) represent the drug concentration in plasma, peripheral tissue and site of action (kidney). \(T_s\) and \(S_p\) represent the concentrations of free membrane Fn14 in kidney and free sFn14 in plasma, respectively. \(DT_s\) and \(DS_p\) represent the drug-mFn14 complex in kidney, and drug-sFn14 complex in plasma, respectively. Free drug in the plasma \((V_p)\) distributes into peripheral \((V_T)\) and SoA \((V_S)\) compartments. The binding kinetics between drug and mFn14/sFn14 is characterized by a second-order association \(k_{on}\) and first-order dissociation \(k_{off}\) rate constants. Parameters \(k_{synTi}\) and \(k_{synSp}\) represent the zero-order synthesis rate for the mFn14 and sFn14 respectively, and are calculated based on steady state constrain (Equation 8 and 9). The model parameters are listed in Table 1. Fn14 target coverage by the drug is calculated by equation 10, where \(T_s\) is the predicted free mFn14 concentration and \(T_{SO}\) is the baseline concentration for mFn14 in kidney.

\[
\begin{align}
k_{synTi} &= T_{SO} \times k_{intT} \\
k_{synSp} &= S_{SO} \times k_{edS} \\
\text{% Coverage} &= \left(1 - \frac{T_s}{T_{SO}}\right) \times 100
\end{align}
\]

**Disclosure**

XC, VF, MFO, LLI, HN, and JB are employed by Pfizer, and hold stock in Pfizer. PS and JP were working at Pfizer during their work on this publication and PS held stock in Pfizer.

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