Critical role of bioanalytical strategies in investigation of clinical PK observations, a Phase I case study

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Abbreviations: BSA, bovine serum albumin; CDR, complementarity-determining region; ELISA, enzyme-linked immunosorbent assay; IgG1, immunoglobulin G1; IS, internal standard; HRP, horseradish peroxidase; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDL-c, low density lipoprotein cholesterol; LDLR, low density lipoprotein receptor; mAbs, monoclonal antibodies; MAD, multiple-ascending dose; MQC, minimum quantifiable concentration; MRM, multiple reaction monitoring; NHS, normal human sera; PBS, phosphate buffered saline; PCSK9, proprotein convertase subtilisin/kexin type 9; PD, pharmacodynamics; PK, pharmacokinetics; rhuPCSK9, recombinant human PCSK9; RT, room temperature; SA, streptavidin; SAD, single-ascending dose; SIL, stable isotope-labeled; S/N, signal-to-noise; TMB, 3, 3',5,5'-tetramethylbenzidine; LLOQ, lower limit of quantification

RG7652 is a human immunoglobulin 1 (IgG1) monoclonal antibody (mAb) targeting proprotein convertase subtilisin/kexin type 9 (PCSK9) and is designed for the treatment of hypercholesterolemia. A target-binding enzyme-linked immunosorbent assay (ELISA) was developed to measure RG7652 levels in human serum in a Phase I study. Although target-binding assay formats are generally used to quantify free therapeutic, the actual therapeutic species being measured are affected by assay conditions, such as sample dilution and incubation time, and levels of soluble target in the samples. Therefore, in the presence of high concentrations of circulating target, the choice of reagents and assay conditions can have a significant effect on the observed pharmacokinetic (PK) profile. Phase I RG7652 PK analysis using the ELISA data resulted in a nonlinear dose normalized exposure. An investigation was conducted to characterize the ELISA to determine whether the assay format and reagents may have contributed to the PK observation. In addition, to confirm the ELISA results, a second orthogonal method, liquid chromatography tandem mass spectrometry (LC-MS/MS) using a signature peptide as surrogate, was developed and implemented. A subset of PK samples, randomly selected from half of the subjects in the 6 single ascending dose (SAD) cohorts in the Phase I clinical study, was analyzed with the LC-MS/MS assay, and the data were found to be comparable to the ELISA data. This paper illustrates the importance of reagent characterization, as well as the benefits of using an orthogonal approach to eliminate bioanalytical contributions when encountering unexpected observations.

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) protein regulates plasma low density lipoprotein cholesterol (LDL-c) levels by promoting LDL receptor (LDLR) degradation within hepatocytes and reducing LDLR expression on the hepatocyte surface, resulting in lower plasma LDL-c clearance1–3. RG7652 is a human immunoglobulin G1 (IgG1) antibody that targets PCSK9, thereby inhibiting its binding to LDLR, and it is designed to treat hypercholesterolemia. The Phase I clinical study was a randomized, placebo-controlled, double-blind, single- and multiple-ascending dose (SAD, MAD) study to evaluate the safety, tolerability, pharmacokinetics (PK) and pharmacodynamics (PD) of subcutaneous treatments of RG7652 in healthy subjects. Following RG7652 treatment, free (unbound), partially (one site bound) and fully bound (both sites bound) forms of RG7652 may co-exist in circulation. A target-binding enzyme-linked immunosorbent assay (ELISA)
was developed to measure RG7652 levels in serum samples. This type of assay format is generally utilized for quantifying free therapeutic as well as partially bound therapeutic; however, the actual therapeutic species being measured could be assay reagent and condition dependent. If the goal is to detect free therapeutic, the assay dilution and incubation times are minimized. Nevertheless, in order to achieve the desirable dynamic range in a PK assay, samples are generally prepared at high dilutions prior to analysis to overcome matrix interference, using minimum dilutions is therefore not feasible. In the RG7652 target-binding ELISA, the assay sensitivity is affected by the presence of PCSK9 levels in serum because PCSK9 can bind to RG7652 and prevent it from binding to the plate for detection. This is further complicated in the RG7652 Phase I study, because the baseline endogenous PCSK9 levels, ranging from 132 ng/mL to 593 ng/mL determined using an in-house assay, can increase up to 10-fold after treatment. The challenge was to use a target-binding ELISA, but still achieve the sensitivity requirement of 100 ng/mL. To reach this goal, the assay conditions were changed to favor detection of total RG7652 to avoid target interference in the assay. This was accomplished primarily by extending sample incubation time. Extensive in vitro characterization was performed to understand the therapeutic species being measured. The data indicated that the clinical PK ELISA was capable of measuring total RG7652 (both unbound and PCSK9-bound RG7652) in human serum matrix. Nevertheless, analyses of Phase I PK data revealed a nonlinear dose normalized increase in therapeutic exposure. This observation prompted further characterization of the PK ELISA method to understand whether the ELISA format and reagents had contributed to this observation. In addition, an orthogonal method, an affinity capture liquid chromatography tandem mass spectrometry (LC-MS/MS) assay, was developed to verify the results generated using the ELISA PK assay.

Recently, affinity capture LC-MS/MS methods have been explored as an alternative approach for quantification of protein therapeutics, including monoclonal antibodies (mAbs). In this approach, there is typically an analyte enrichment step using affinity capture followed by enzymatic digestion to generate structurally-related peptides. Subsequently, a signature peptide can be selected as a surrogate to quantify the mAb analyte. The LC-MS/MS approach utilizes the chromatographic separation and characteristic precursor and product ions for multiple reaction monitoring (MRM) analysis, instead of merely relying on the custom reagents in the case of ELISA, to achieve high assay selectivity. Therefore, an orthogonal approach using LC-MS/MS was developed and utilized to verify the performance of the ELISA. The LC-MS/MS assay was capable of quantifying all forms of the mAb therapeutic via a tryptic signature peptide as surrogate.

A subset of PK samples from the RG7652 Phase I clinical study, randomly selected from half of the subjects in the 6 SAD cohorts, was analyzed using affinity capture LC-MS/MS assays, and the results were comparable to those generated by the ELISA. Here, we present the results from the method and reagent characterization, as well as the comparison of RG7652 data generated using the PK ELISA and LC-MS/MS assay formats.

**Results**

**Clinical PK assays developed in ELISA and LC-MS/MS formats**

A target-binding ELISA was developed and validated to quantify total RG7652 levels in human serum (Fig. 1). It uses biotin-labeled recombinant human PCSK9 (rhuPCSK9) for capture and a mouse mAb 1.2A4 raised specifically to the complementarity-determining region (CDR) of RG7652 for detection. This assay is capable of detecting RG7652 over a broad range of concentrations (78.1–5000 ng/mL) in human serum in the presence of PCSK9. The minimum quantifiable concentration (MQC) of the assay was determined to be 100 ng/mL.

An affinity capture LC-MS/MS clinical PK assay was developed as a second orthogonal method to quantify total RG7652 levels in human serum and to confirm the ELISA results. The LC-MS/MS clinical PK assay uses either biotin-labeled mAb1.2A4 or Protein A magnetic beads for affinity capture of RG7652 in human serum, followed with a digestion step using trypsin. A tryptic signature peptide derived from the heavy chain variable region of RG7652 was used as a surrogate for quantification (Fig. 2). The signature peptide and its corresponding stable isotope-labeled (SIL) internal standard (IS) are listed in Table 1. The lower limit of quantification (LLOQ) was determined to be 0.100 μg/mL by the mAb 1.2A4 capture method compared to 3 μg/mL by the Protein A capture method.

**Characterization of the ELISA PK assay**

**Characterization of the detection antibody mAb 1.2A4**

The data demonstrate that mAb 1.2A4 has specific binding to RG7652 and no interaction with generic human IgG and target rhuPCSK9 (Fig. 3A). To verify whether mAb 1.2A4 can detect RG7652 in the presence of PCSK9, 5 in vitro samples of various RG7652 to rhuPCSK9 molar ratios were evaluated. The percent recovery of RG7652 in the 5 samples was determined to range from 82% to 100% (Fig. 3B). This experiment demonstrates that mAb 1.2A4 is capable of detecting RG7652 in the presence of PCSK9. Samples with RG7652 to rhuPCSK9 molar ratios up to 1:8 were recovered well (within 80–120%). The slight signal
reductions in RG7652 recovery observed in the sample with a molar ratio of 1:8 are likely due to enhanced steric hindrance from rhuPCSK9 (molecular weight of ~74 KDa).

Impact of sample incubation time

The effect of sample incubation time on assay performance was evaluated to find out whether RG7652 recovery at low drug concentrations could be improved by extending the incubation time. Two approaches were evaluated. First, 9 normal human serum (NHS) samples, with endogenous PCSK9 levels ranging from 50–454 ng/mL as determined using an in-house assay, were spiked with RG7652 at a low level of 180 ng/mL and a high level of 3000 ng/mL. These spiked samples were then analyzed side-by-side using the same assay conditions, except for sample incubation time at either 2 hours or overnight. Each sample was analyzed in duplicate in 3 separate experiments, the mean percent recovery was summarized and is presented in Figures 4A and 4B, respectively. The overall percent recovery of RG7652 was closer to the expected nominal spike levels with overnight incubation compared to 2 hour incubation. Eight of the 9 samples at low-spike RG7652 met the acceptance criteria (80–120% recovery) after overnight incubation, but none of the samples did so after the 2 hour incubation (Fig. 4A). The RG7652 to endogenous PCSK9 molar ratios in the 9 low-spike samples ranged from 0.2:1 to 1.8:1 with 7 samples having molar ratios less than 1:1, an indication that PCSK9 bound RG7652 was the main form of therapeutic. Overnight incubation allows for an equilibrium shift of fully or partially bound therapeutic to free therapeutic in the serum, which enables analyte binding to the capture reagent on the plate and leads to better recovery.

With the high-spike of RG7652, incubation time didn’t have a significant effect on RG7652 percent recovery due to higher molar ratios of RG7652 to endogenous PCSK9 (3.3:1 to 30.0:1, Fig. 4B). However, we did observe improved assay reproducibility with the overnight incubation condition that showed less standard deviation between the 3 runs (Fig. 4B).

In the second experiment, the in vitro samples were prepared at a fixed level of RG7652 (1 µg/mL) plus biotin-rhuPCSK9 at 0, 0.5, 1, 2, 4 and 8 µg/mL respectively. Each sample was analyzed in parallel using similar protocols except for the sample incubation step, which was either 2 hours or overnight incubation (Fig. 4C). In the absence of rhuPCSK9, similar results were observed between the 2 incubation conditions. In the presence of rhuPCSK9, the same sample tended to recover closer to the expected nominal with overnight incubation. When the RG7652 to biotin-rhuPCSK9 molar ratios were 0.25 (1:4) or higher, the ELISA recovery results indicated that the assay measured total RG7652 after overnight incubation.

Both experiments indicated that overnight incubation enabled the assay to measure total therapeutic species with acceptable recovery, especially in the presence of low level of therapeutic. Consequently, overnight sample incubation was selected as the final assay condition. Our data illustrates that this assay is capable of detecting RG7652 over a broad range of concentrations (78.1–5000 ng/mL) in human serum in the presence of PCSK9. The MQC of the assay was determined to be 100 ng/mL and met the sensitivity requirement. Accuracy was assessed during validation using RG7652 controls prepared in the human serum and were found to be acceptable, with the average recovery ranging from 96 to 112% of the nominal levels. Overall, the mean inter- and intra-assay precision of the assay controls was determined to be in the range of 3% to 10% and 4% to 7%, respectively. Additional assay parameters, such as dilutional linearity, hook effect, interference and stability, were tested during validation and the assay met all the acceptance criteria (data not shown).

Characterization of the 2 affinity capture LC-MS/MS assays

Sensitivity assessment of the affinity capture LC-MS/MS assays

The LC-MS/MS approach, enriching the analyte by affinity capture was assessed using samples containing RG7652 at 0.100 to 100 µg/mL. Immuno capture by mAb 1.2A4 provided specific capture of the analyte RG7652 with less matrix interference and was therefore anticipated to offer higher sensitivity. However, since mAb1.2A4 was utilized for detection in the ELISA, one potential concern was that using the same antibody reagent would not make the LC-MS/MS assay a completely orthogonal method. There was also a question regarding whether the anti-CDR mAb 1.2A4 could efficiently capture all forms of RG7652, including the fully target-bound RG7652. Therefore, a second option of using a generic reagent Protein A for affinity capture was also explored. This alternative method was expected to capture all forms of RG7652 without any bias. The LLOQ was determined to be 0.100 µg/mL by the mAb 1.2A4 method.

Table 1. Multiple reaction monitoring (MRM) transitions for the signature peptide and its corresponding internal standard

| Surrogate Peptide | Sequence                        | Q1 (m/z) | Q3 (m/z) | DP | CE | EXP |
|-------------------|---------------------------------|----------|----------|----|----|-----|
| HC_P4            | FTISADTSK, quantification       | 485.2    | 608.3    | 36 | 23 | 26  |
| HC-P4 Heavy      | FTI[13C6]N7ISADTSK internal standard | 488.67   | 728.1    | 36 | 21 | 24  |

*denotes amino acid containing stable isotope labeling of 13C6 and 15N7.
compared to 3 μg/mL by the Protein A method. The RG7652 recovery achieved was within the acceptance criteria of ± 20.0% of the nominal concentrations (Fig. 5A). For the mAb 1.2A4 immunocapture method, the assay accuracy was found to be acceptable with the average recovery ranging from 86 to 103% of the nominal levels; the mean inter- and intra-assay precision was determined to be in the range of 4% to 9% and 2% to 7%, respectively.

Precision of the LC-MS/MS assays in the presence of PCSK9

To address the concern of whether the assay with the immunocapture using 1.2A4 could potentially under-recover RG7652 in the presence of PCSK9, both capture options were evaluated using the same set of samples prepared by spiking various levels of RG7652 plus rhuPCSK9 at the clinically relevant level of 5 μg/mL, which resulted in samples with molar ratios of 2:1, 1:1, 1:2, 1:4, 1:8 and 1:16 (RG7652: rhuPCSK9), respectively. Control samples with no spiked rhuPCSK9 were also tested. RG7652 percent recovery ranged from 86% to 107% or 74% to 101%, respectively, when using mAb 1.2A4 or Protein A for capture (Fig. 5B). The data demonstrated that the assay with immunocapture using 1.2A4 offered the ability to quantify RG7652 without bias in the presence of PCSK9, even at the excess level of rhuPCSK9-to-RG7652 ratio.

Comparison of the 2 affinity capture LC-MS/MS RG7652 clinical PK assays

The performance of the anti-CDR mAb 1.2A as a capture reagent was further evaluated by testing samples collected at Day 29 post treatment from one of the lower dosing cohorts at 150 mg and one of the higher dosing cohorts at 600 mg, the data was compared with that of the Protein A capture method. Data obtained in all testing samples produced similar results between the 2 LC-MS/MS methods (Fig. 6A and B). This data further justified that using mAb 1.2A4 for the immunocapture step of the LC-MS/MS assay would enable the detection of all forms of RG7652, regardless of its target-binding status (free versus total). Therefore, the LC-MS/MS using mAb1.2A4 for enrichment was used as an orthogonal method to confirm the PK data generated by the ELISA format for the RG7652 clinical Phase I study.

Direct comparison of data generated using ELISA and Affinity Capture LC-MS/MS RG7652 clinical PK assays

RG7652 clinical Phase I study

The Phase I clinical study was a randomized, placebo-controlled, double-
blind, SAD and MAD study to evaluate the safety, tolerability, PK, and PD of subcutaneous administration of RG7652 in healthy subjects with elevated serum LDL-c (130–220 mg/dL). The study was designed to include 6 SAD cohorts and 4 MAD cohorts. The 6 SAD cohorts were dosed subcutaneously at 10, 40, 150, 300, 600 and 800 mg, respectively; the 4 MAD cohorts were dosed subcutaneously at 40 mg and 150 mg once weekly for 4 weeks with and without statin pre-treatment. Subjects within each cohort were to be randomized to receive RG7652 or placebo in an approximately 3:1 ratio.

Data comparison

The ELISA and LC-MS/MS assay (using mAb 1.2A4 for the enrichment step) demonstrated comparable assay performance by quantifying the total RG7652 concentrations, with similar assay sensitivity. These 2 assays were therefore chosen to analyze a subset of Phase I study samples (n = 123) for direct comparison. Samples utilized for direct comparison are from subjects in the 6 SAD cohorts. For each cohort, 50% of RG7652 treated subjects were randomly selected, which resulted in 3 subjects per cohort. The 2 data sets were comparable in all 6 cohorts (Fig. 7A). Overall, good correlation was observed between the 2 sets of data with a R² value of 0.983 (Fig. 7B).

Discussion

A target-binding ELISA clinical PK assay was developed to measure the RG7652 levels in human serum samples. Dose normalized PK profiles indicated a non-dose proportional increase in exposure. At the highest dose of 800 mg, the elimination half-life of RG7652 was calculated to be ~14 days vs. ~7 days at the lowest dose of 10 mg. Furthermore the half-life was similar at doses 300 mg and above indicating target-mediated clearance pathway is saturated. However, the half-life was shorter for a typical antibody therapeutic with linear pharmacokinetics (~21 days). Together these data represent an observation that is not consistent with a typical target mediated disposition characteristic of mAb therapeutics.10,11 Among the 60 subjects who received RG7652 treatments, 3 were tested positive for anti-RG7652 antibodies, including 1 subject from the SAD cohorts and 2 from the MAD cohorts. The signals of anti-RG7652 antibodies in the 3 positive subjects were low and did not have an effect on PK readout. This observation of non-dose proportional PK prompted a need to characterize the PK ELISA used to evaluate the samples.

Although target-binding assay formats are generally used to quantify free therapeutic, the actual therapeutic species being measured are affected by assay conditions such as sample dilution, incubation times and levels of soluble target in the samples. Therefore, in the presence of high concentrations of circulating target, the choice of reagents and assay conditions can have a significant effect on the observed PK profiles. In addition to technical considerations, the selection of the assay format also depends on factors such as reagent availability and project requirements. In this instance a target-binding ELISA was developed, due to presence of soluble PCSK9 and assay sensitivity requirement, the assay conditions were altered so that total RG7652 could be detected. This was done primarily by extending the sample incubation time of the assay from 2 hours to overnight, allowing PCSK9 bound RG7652 to exchange from binding with endogenous PCSK9 (in solution) to the coat rhuPCSK9 (on the plate) to be detected. In addition, samples were diluted at the minimum dilution of 1:100, which could further promote the dissociation of RG7652/PCSK9 complexes. With combined

![Figure 5. Characterization of affinity capture LC-MS/MS assays. The assay using mAb 1.2A4 for capture has better sensitivity (A) and accuracy (B) in the presence of rhuPCSK9.](image)

![Figure 6. RG7652 measurements of Phase I post dosing Day 29 samples from 150 mg (A) and 600 mg (B) cohorts using 2 affinity capture LC-MS/MS methods.](image)
During the characterization of ELISA format, 2 affinity capture LC-MS/MS RG7652 clinical PK assays were developed, using either Protein A or anti-CDR mAb 1.2A4 for capture, to verify the ELISA PK results. MAb 1.2A4 is specific to the CDR portion of RG7652 and was also utilized...
for detection in the ELISA format. The in vitro characterization data demonstrated that the mAb 1.2A4 recognized RG7652 in the presence of biologically relevant levels of PCSK9, therefore the LC-MS/MS assay using mAb 1.2A4 for analyte enrichment would be able to capture total RG7652 (free or PCSK9-bound). Protein A is known to have high binding affinity for the Fc portion of human IgG1, which should bind to all forms of RG7652 in the clinical serum samples. However, because of the non-specific nature of Protein A, using it as capture reagent resulted in less desirable sensitivity. As expected, the LC-MS/MS method using mAb 1.2A4 for capture achieved better assay sensitivity (100 ng/mL) due to its specificity. Representative Day 29 samples from one lower (150 mg) and one higher (600 mg) dosing cohorts were selected to compare the 2 LC-MS/MS methods and the data obtained was comparable. This comparison gave us the confidence to use mAb1.2A for the immunocapture step of the LC-MS/MS assay to quantify total RG7652 with the needed sensitivity.

A direct comparison of the ELISA and LC-MS/MS (using mAb 1.2A4 for immunocapture) assays was conducted by analyzing a subset of Phase I PK samples. The 2 sets of data were comparable and showed good correlation. The direct comparison data confirmed that the ELISA PK assay detected total RG7652 and the dose-normalized PK profile difference observed was not due to assay artifacts.

To properly interpret PK data and assess the relationship between therapeutic exposure and clinical outcomes (e.g., efficacy, safety), it is critical to understand what form of the therapeutic is being measured in the PK assay. Although assay reagents and formats can in theory be designed to measure the “total” or “free” forms of a therapeutic, factors such as reagent limitations, sample dilutions, assay conditions and biological factors (e.g., soluble target levels) can raise uncertainty regarding the actual form of the therapeutic being measured. When unexpected results are observed, the bioanalytical methods and reagents are often called into question. It is therefore important to well characterize the bioanalytical methods and reagents in order to understand the actual drug species being measured by the assay. In these situations it is also beneficial to have a second orthogonal method to verify the results and to exclude any bioanalytical artifacts.

In this case study, an unexpected nonlinear dose normalized exposure was observed in the RG7652 Phase I study. This observation prompted characterization of the ELISA PK assay and reagents to understand whether the bioanalytical method used had contributed to the PK observations. Our work confirmed that the ELISA used in this study detected total RG7652 as it was intended. In addition, we were able to confirm our ELISA results by directly comparing them with results generated using an orthogonal LC-MS/MS assay. This strategy provided the project team with the data needed regarding the reliability of the ELISA results. In this case study, it was concluded that the nonlinear PK observations were not caused by assay artifacts.

Materials and Methods

Materials
RG7652 is a human IgG1 mAb therapeutic generated at Genentech. Pooled NHS and individual serum samples from healthy volunteers were purchased from Bioreclamation and BioChemed. Recombinant human PCSK9 (rhuPCSK9) and 1.2A4, a mouse mAb raised specifically to the CDR of RG7652 were also produced at Genentech. The other materials are horseradish peroxidase (HRP) conjugated anti-mouse IgG2b (Jackson ImmunoResearch, 115-035-207); 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories, 50-76-03); illustra™ Nap-10 columns (GE Healthcare, 17-0854-01); bovine serum albumin (BSA) (Equitech-Bio Inc., BAH-1000) for the ELISA and (Sigma, 0547) for the LC-MS/MS assay; CHAPS (Research Organics, C3023–100G); ProClin 300 (Supelco, 48914-U); streptavidin (SA)-coated microplates (StreptaWell High bind 96 well) (Roche Diagnostics, 11989685001); stable isotope labeled peptide (Midwest Bio-Tech; customized order). Acetonitrile (EM-AX0145-1), methanol (BJ230–4), hydrochloric acid (EM-HX0608-7), isopropyl alcohol (BJ323–4), and HPLC grade water (JT9831–3) were obtained from VWR Scientific. Formic acid (94318) was purchased from Fluka; Trizma® hydrochloride (15506-017), iodoacetamide (D-6003), dithiothreitol-DL (D-1532), Tween 20 (00–3005), trifluoroethanol (AM9856), sodium chloride (AM9760G) and EDTA (AM9261) were obtained from Ambion. Other reagents used in the LC-MS/MS assays include ammonium bicarbonate (J.T. Baker, 3003-01); sodium hydroxide (Fisher Scientific, S719932); RapiGest SF surfactant (Waters, 186001861); sequencing grade modified trypsin (Promega, V5111); Pure Proteome Protein A magnetic beads (Millipore, LSKMAGA10) and SA coated paramagnetic beads (Invitrogen, 60210).

Preparation of conjugates of RG7652, rhuPCSK9 and 1.2A4
RG7652, rhuPCSK9 and mAb 1.2A4 were buffer exchanged into phosphate buffered saline (PBS) prior to conjugation using an illustra™ Nap-10 column. Experimental details were performed according to the manufacturers’ instructions (GE Healthcare). Buffer exchanged RG7652, rhuPCSK9 and mAb 1.2A4 were conjugated with biotin at a challenge ratio of 10:1 (biotin to RG7652, rhuPCSK9 or 1.2A4) using EZ-Link SulfoNHS-LC-Biotin (Pierce, 21338). All conjugations were prepared according to the manufacturer’s instructions followed by a buffer exchange step into storage buffer (20 mM sodium phosphate, 0.35 M sodium chloride, 6% sucrose, 0.25% Polysorbate 20, 0.25% CHAPS, 0.05% Proclin 300, 0.5% BSA, pH 5.2 ± 0.2).

ELISA clinical PK assay

Assay format
SA-coated microtiter plates were coated with 100 μL of biotin-rhuPCSK9 at 1 μg/mL and incubated at room temperature (RT) for 1 hour with agitation. The plates were washed 3 times with wash buffer (PBS, 0.05% Polysorbate 20, pH 7.2 ± 0.2).
blocked with 200 μL per well of blocking buffer (PBS, 0.5% BSA, 0.05% Polysorbate 20, 15 ppm Proclin 300, pH 7.2 ± 0.2), and incubated at RT for 1 to 2 hours with agitation. Human serum samples and controls were prepared at 1:100 dilutions in the assay buffer (PBS, 0.5% BSA, 0.05% Polysorbate 20, 0.05% Proclin 300, 0.25% CHAPS, 5 mM EDTA, 350 mM sodium chloride, pH 7.4). A RG7652 standard curve, ranging from 0.78 to 50 ng/mL (in-well concentrations) at 1:2 serial dilutions, was prepared in the standard diluent (assay buffer plus 1% pooled NHS). Diluted samples, standards, and controls were applied to the coated plate at 100 μL for incubation at RT overnight with agitation. After 4 washes, 100 μL of 100 ng/mL mAb 1.2A4 was added for detection and incubated at RT for 1 hour with agitation. An aliquot of 100 μL of HRP conjugated anti-mouse IgG2b of 25 ng/mL was applied subsequently and incubated at RT for 1 hour with agitation. A peroxidase substrate, TMB was added at 100 μL for color development and the reaction was stopped by adding 100 μL of 1 M phosphoric acid. The plates were read on a BioTek ELx800 plate reader using 450 nm for detection absorbance and 620 or 630 nm for reference absorbance.

Characterization of the detection reagent mAb 1.2A4

Specificity of mAb 1.2A4

A Nunc Maxisorp 96-well ELISA plate was coated with 1 μg/mL of human IgG, rhuPCSK9 or RG7652 at 2–8°C overnight. The next day, the plate was incubated with blocking buffer at RT for 1 hour. An aliquot of 125 ng/mL mAb 1.2A4 was added for detection and incubated at RT for 1 hour with agitation. An aliquot of 100 μL of HRP conjugated anti-mouse IgG2b of 25 ng/mL was applied subsequently and incubated at RT for 1 hour with agitation. A peroxidase substrate, TMB was added at 100 μL per well for color development and the reaction was stopped by adding 100 μL of 1M phosphoric acid to each well. Absorbance was measured at 450 nm using 650 nm as reference on a Molecular Devices SpectraMax M5 plate reader.

Effect of PCSK9 on mab1.2A4 binding to RG7652

RG7652 was labeled with biotin at a challenge ratio of 10:1 (biotin: RG7652). In vitro samples were prepared in PBS buffer by spiking 75 μL of 0.5 μg/mL biotin-labeled RG7652 plus 75 μL of rhuPCSK9 at 0, 0.5, 1, 2, 4 and 8 μg/mL, which resulted in 6 samples with molar ratios of RG7652 to biotin-labeled rhuPCSK9 at 1:0, 1:1, 1:2, 1:4, 1:8 and 1:16. Each sample was split into 2 aliquots and incubated in a polypropylene round-bottom plate for either 2 hours or overnight at RT with agitation. After incubation, samples were transferred to a SA-coated plate at 100 μL per well and incubated for 1 hour at RT with agitation. After 3 washes, 150 ng/mL of 1.2A4 and 25 ng/mL of HRP conjugated anti-mouse IgG2b were applied subsequently for detection with 1 hour incubation at each step. TMB was added at 100 μL per well for color development and the reaction was stopped by adding 100 μL of 1 M phosphoric acid. The plates were read on a Tecan reader, using 450 nm for detection absorbance and 620 or 630 nm for reference absorbance.

Affinity Capture LC-MS/MS clinical PK assay

Assay format

Affinity capture of RG7652 in human serum was done by incubating 25 μL sample diluted in 300 μL loading buffer (0.1% Tween 20, 5.0% Trizma® hydrochloride (1 M), 3.0% sodium chloride (5 M), 0.2% EDTA (0.5 M) and 91.7% water with 0.1% in weight of BSA) with 10 μg of biotin-labeled mAb1.2A4 in a 96-well plate at RT for 2 hours with agitation. Subsequently, 1 mg SA-coated paramagnetic beads (Invitrogen), re-suspended in 100 μL loading buffer, were added to the plate followed by an additional 2 hour incubation at RT. The beads were separated and washed by 400 μL washing buffer (1M Trizma® hydrochloride, 5 M sodium chloride, 0.5 M EDTA) for 3 times. The second immunocapture approach using 25 μL diluted serum samples (1:2 v/v dilution with loading buffer) incubated with Protein A magnetic beads (25 μL; Millipore) was also evaluated, which theoretically allowed the capture of all forms of RG7652 and compared with the mAb1.2A4 capture assay to confirm that it was a total antibody PK assay as intended. A KingFisher 96 magnetic particle processor (Thermo Scientific)
was used for the immunocapture steps. The enriched samples, with subsequent addition of 25 μL SIL IS (50 ng/mL in 20% acetonitrile), underwent typical steps of denaturation by 75 μL RapiGest (Waters), reduction by 10 μL of 0.1 M dithiothreitol-DL (Sigma), and alkylation by 25 μL of 0.1M iodoacetic acid (Sigma) before 10 μL trypsin (0.25 mg/mL, Promega) was added to perform proteolytic digestion at 37°C overnight. Digestion was then quenched by 15 μL of 2M HCl. After removing the magnetic beads by filtration, samples were ready for LC-MS/MS analysis.

An aliquot of 25 μL processed sample was injected onto a Shimadzu LC-20ADvp binary system coupled with a reversed phase Kinetex C18 column (2.1 × 50 mm, 2.6 μm; Phenomenex) maintained at 50°C with a flow rate of 300 μL/min. Mobile phase consisted of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in 75% acetonitrile/25% methanol). The gradient was operated between 5% and 100% buffer B with a total run time of 7.5 minutes. Detection was accomplished by a QTRAP 5500 mass spectrometer (AB Sciex) equipped with a turbo ionspray source operating in the positive ion mode with the source temperature set at 600°C and the ionspray voltage at 5500 V. Details of the MRM transitions for the signature peptide and the corresponding SIL IS are listed in Table 1. Data processing was performed using Intelliquan (AB Sciex).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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