Microdosing of a Carbon-14 Labeled Protein in Healthy Volunteers Accurately Predicts Its Pharmacokinetics at Therapeutic Dosages

MLH Vlaming[1], E van Duijn[1], MR Dillingh[2], R Brands[3], AD Windhorst[4], NH Hendrikse[5], S Bosgra[1], J Burggraaf[2], MC de Koning[1], A Fidder[1], JAJ Mocking[1], H Sandman[1], RAF de Ligt[1], BO Fabriek[1], WJ Pasman[1], W Seinen[3,6], T Alves[7], M Carrondo[7], C Peixoto[7], PAM Peeters[2] and WHJ Vaes[1]

Preclinical development of new biological entities (NBEs), such as human protein therapeutics, requires considerable expenditure of time and costs. Poor prediction of pharmacokinetics in humans further reduces net efficiency. In this study, we show for the first time that pharmacokinetic data of NBEs in humans can be successfully obtained early in the drug development process by the use of microdosing in a small group of healthy subjects combined with ultrasensitive accelerator mass spectrometry (AMS). After only minimal preclinical testing, we performed a first-in-human phase 0/phase 1 trial with a human recombinant therapeutic protein (RESCuing Alkaline Phosphatase, human recombinant placental alkaline phosphatase [hRESCAP]) to assess its safety and kinetics. Pharmacokinetic analysis showed dose linearity from microdose (53 µg) [^{14}C]-hRESCAP to therapeutic doses (up to 5.3 mg) of the protein in healthy volunteers. This study demonstrates the value of a microdosing approach in a very small cohort for accelerating the clinical development of NBEs.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC? ☑ New biological entities are an upcoming class of drug compounds. Microdosing is a technique that can reveal human PK data very early in the drug development process. WHAT QUESTION DID THIS STUDY ADDRESS? ☑ We examined the applicability of microdosing to determine the PK of carbon-14 labeled human recombinant placental alkaline phosphatase (hRESCAP), an endogenous anti-inflammatory protein. Dose linearity, safety, and tolerability of hRESCAP were determined in a phase 0/I single ascending dose study. • WHAT THIS STUDY ADDS TO OUR KNOWLEDGE ☑ The hRESCAP exhibits dose-proportional pharmacokinetics across the doses administered. The increased half-life of hRESCAP may trigger the treatment of an array of chronic inflammatory diseases. • HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS ☑ This study suggests that microdosing can be used to provide early human PK data, not only for small molecule compounds, but also for new biological entities. A microdosing approach may reduce drug development costs and shorten the timelines to introduce new drugs onto the market.

New biological entities (NBEs), such as recombinant proteins, monoclonal antibodies, and oligonucleotides, are increasingly being developed for therapeutic and diagnostic purposes. Currently, NBEs make up 42% of the research pipelines of pharmaceutical companies, and 8% of currently marketed drugs are biotech products.[1] Developing NBEs is particularly challenging as human biopharmaceuticals are often intrinsically immunologically incompatible with preclinical (animal) models.[2,3] Further, because of the lack of predictive preclinical models to study pharmacokinetic (PK) profiles, biologicals have a relatively high failure rate at late stages of drug development.[4] Microdosing, a relatively new approach, is proposed to accelerate the overall drug development process. Although it is otherwise difficult, or impossible, to predict the PK of new drugs in humans, microdosing offers the superior advantage to obtain data with high statistical power, prior to a phase 1 study in a small group of volunteers and after limited preclinical safety testing.[5–9] Thus, this approach is appealing to candidate drugs with a potentially undesirable PK profile, as these can be excluded before entering costly clinical trials.

In a phase 0 microdosing trial extremely low doses of carbon-14 (^{14}C) labelled compounds are administered to healthy

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[1]TNO, Zeist, The Netherlands, UK; [2]Centre for Human Drug Research, Leiden, The Netherlands, UK; [3]AMRIF BV, Wageningen, The Netherlands, UK; [4]Department of Radiology and Nuclear Medicine, VU University Medical Center, Amsterdam, The Netherlands, UK; [5]Department of Pharmacy and Clinical Pharmacology, VU University Medical Center, Amsterdam, The Netherlands, UK; [6]Utrecht University, Utrecht, The Netherlands, UK; [7]GenIBET/IBET, Oeiras, Portugal. Correspondence: E van Duijn (Esther.vanduijn@tno.nl)

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volunteers.[10,11] Typically, a microdose would be <1% of the therapeutically active dose, ≤100 μg, or ≤30 nmol for proteins.[10,12,13] The resulting minute drug concentrations in the body after microdoses are unlikely to elicit pharmacodynamic or toxicological effects and do not impose radiological risks. The detection of the extremely low amounts of carbon-14 labelled drugs in biological matrices is enabled by the highly sensitive analytical technique accelerator mass spectrometry (AMS).[14–16]

Until now, microdosing has been used only for small molecule drugs.[8] The opinion prevails that microdosing cannot be applied to research on biologicals. This may be correct for biopharmaceuticals that target a specific tissue, resulting in a non-linear PK profile.[9] However, for a (non)-endogenous NBE that resigns in the systemic compartment, the PK is expected to show dose linearity.

Another advantage of microdosing is that the very small starting dose allows safe generation of safety and PK data in humans. In this view, the clinical trial, including the monoclonal antibody TGN1412, could have ended less disastrous than it did in 2006. In this particular study, 0.1 mg/kg TGN1412 was administered,[17] which, for the average person, would result in a dose of ∼7 mg or 47 nmol. In the present study, we show a microdose application starting with a 100-fold lower dose of an NBE.

Alkaline phosphatase (ALP) is an endogenous anti-inflammatory protein that is suggested to neutralize potentially harmful substrates, such as damage-associated molecular pattern molecules, like extracellular nucleotides,[18] pathogen-associated molecular pattern molecules, and lipopolysaccharides.[19] A bovine homologue of ALP has previously been investigated for treatment of acute ischemia-mediated and hypoxia-mediated inflammatory responses during invasive surgery and sepsis, and was shown to be well-tolerated in humans.[20,21] Because of its short plasma residence time, bovine ALP is not compatible with chronic disease management. The recent development of human recombinant ALP, human recombinant placental alkaline phosphatase (hRESCAP), which is expected to have a similar plasma residence time as the endogenous sialylated placental protein *in vivo*, may boost the treatment of an array of chronic inflammatory diseases. As ALP is endogenously expressed in humans at relatively high levels, dose linearity is expected.

In this study, we report, to our knowledge for the first time, on a clinical microdosing study to determine the PKs of a carbon-14 labelled hRESCAP. In phase 0 the PKs of a single i.v. microdose of [^{14}C]-hRESCAP were determined, whereas phase 1 focused on the safety, tolerability, and PKs at increasing doses. The current work also summarizes the required conditions for generalized execution of microdosing studies with biopharmaceuticals. A microdosing study using an NBE was never performed in humans before and these results may revolutionize the drug development of biotherapeutics.

RESULTS

Production and analysis of hRESCAP and [^{14}C]-hRESCAP

A good manufacturing practice-compliant method to incorporate a [^{14}C] label into hRESCAP was developed and various batches of [^{14}C]-hRESCAP with 100% radiochemical purity were produced. On average, 0.4–0.6 [^{14}C] atoms were present per hRESCAP dimer of ∼110 kDa. [^{14}C]-hRESCAP and hRESCAP were extensively characterized *in vitro* for: appearance, pH, radiochemical purity, chemical purity, radiochemical identity, glycosylation, stability, aggregation, enzymatic activity, and protein concentration (see **Supplemental Table S1** online). Both products were similar, if not identical, and met the predefined specifications. In addition, a two-week repeated dose toxicity test in hRESCAP-tolerized mice was performed. Up to the highest dose tested (1 mg/kg or 750 U/mg daily for 14 days), there were no signs of toxicity observed. The collected data, combined with knowledge obtained from clinically tested bovine ALP[20,21] resulted in approval from the Medical Ethics Review Board of the Foundation "Evaluation of Ethics in Biomedical Science," Assen, The Netherlands. A clinical phase 0/phase 1 first-in-human study with [^{14}C]-hRESCAP and hRESCAP was performed in accordance with the Declaration of Helsinki and Guideline for Good Clinical Practice.

STUDY DESIGN AND SUBJECT CHARACTERISTICS

The clinical study with hRESCAP and [^{14}C]-hRESCAP was designed as a two-phase study, starting with an open label, single dose study to assess the PKs of a microdose of hRESCAP (phase 0), followed by a randomized, double-blind, placebo-controlled, parallel, single ascending dose first-in-human study to assess PKs, safety, and tolerability of hRESCAP at various therapeutically relevant doses (phase 1). A schematic overview of the study design is shown in **Figure 1**.

Fifteen (phase 0: n = 3; phase 1: n = 12) healthy male volunteers, aged 18–44 years with a body mass index of 18.1–28 kg/m^2 and a body weight of 64.2–91.5 kg, were included. After providing informed consent, subjects were medically screened within three weeks before participation. Among others, exclusion criteria included history of allergy or other inflammatory indications, ALP levels in plasma of <30 U/L or >115 U/L (range of normal physiological concentrations), and clinically relevant abnormal laboratory results, electrocardiogram findings, vital signs, or physical findings that would interfere with the study objectives or subject safety.

Phase 0 microdosing study with [^{14}C]-hRESCAP

After administration of a microdose of [^{14}C]-hRESCAP to three healthy volunteers, blood samples were collected at various timepoints up to 35 days after administration. A direct total ^{14}C-count analysis was performed on 1.5 μL plasma samples using AMS. In addition, the ALP enzymatic activity of all plasma samples was determined. Because of the endogenous ALP plasma levels of the subjects, administration of a microdose (∼44 U total) led to only slightly elevated ALP levels in plasma (**Figure 2b**, upper panels).

AMS analysis of the plasma samples showed a background radioactivity of 9.62–10.5 mBq/mL, originating from the natural presence of carbon-14. Upon administration of [^{14}C]-hRESCAP, the plasma concentration vs. time curve exhibited three distinct elimination phases (**Figure 2a, and Supplemental Figure S1** online), similar to the profile previously reported for bovine ALP in humans and animals.[21,22] The interindividual differences were negligible. A terminal half-life ($t_{1/2}$) of ∼116 hours

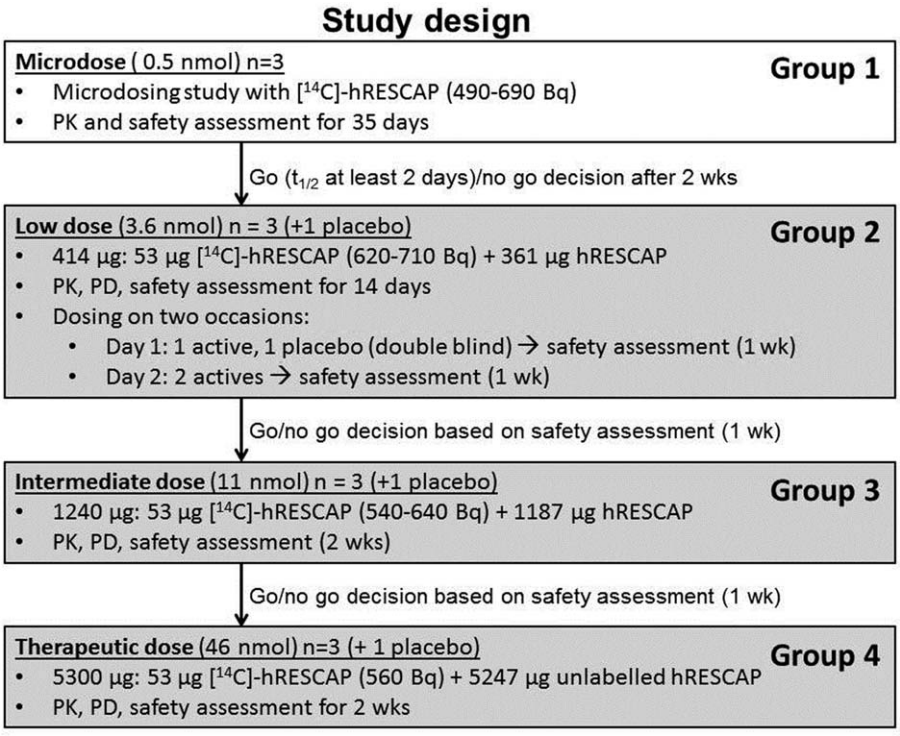

Figure 1 Study design of the phase 0/phase 1 first-in-human study with [^{14}C]-human recombinant placental alkaline phosphatase (hRESCAP). Phase 0 is indicated in white, phase 1 is indicated in grey.

(4.8 days) for hRESCAP was observed, which allowed continuation of the study to phase 1 (requirement to continue to phase 1: $t_{1/2} \geq 2$ days; **Figure 1**).

Phase 1 single ascending dose study of hRESCAP supplemented with microdose [^{14}C]-hRESCAP

The single i.v. microdose was well tolerated in all healthy male subjects and did not result in clinically significant changes from pre-dose values. In phase 1, increasing dosages of 361–5247 µg hRESCAP (~350–5300 enzymatic units, respectively), supplemented with 53 µg [^{14}C]-hRESCAP (540–710 Bq), were administered. After administration, increased ALP enzymatic activity levels in plasma were detected (**Figure 2b**), again displaying three-phase elimination kinetics. After registration of minor adverse effects (fatigue, headache) at the low dose, the medium dose was reduced by 50% to 1240 µg hRESCAP in total. Overall, the safety assessment showed that hRESCAP was well tolerated at all administered dosages. Administration of hRESCAP did not result in clinically significant changes in physiological parameters (data not shown).

Pharmacokinetic analysis

Radioactivity and enzyme activity vs. time data were first analyzed by noncompartmental methods. PK parameters were derived for each subject, and then averaged for each dose group. The results are shown in **Table 1**. The plasma enzyme activity vs. time showed an initial concentration (peak plasma concentration (C_{max})) above baseline of 1.5 U/mL and area under the curve $(AUC)_{0\text{-inf}}$ above baseline of 66 h*U/mL at the highest dose of 5300 µg hRESCAP (5260 U), an (initial) volume of distribution of 3.1 l, and a terminal half-life of ~110 hours. The dose-normalized parameters remain within a factor of 0.8–1.25, indicating dose-proportional kinetics. No striking differences between radioactivity and enzyme activity were observed. To further substantiate these findings and to obtain a more detailed interpretation of the PKs of hRESCAP, we analyzed the data by nonlinear mixed effects modeling. The three-phase elimination was described by a tri-exponential model. In its common interpretation, the compound is dosed in a central compartment (plasma) and equilibrates with two peripheral compartments (extravascular spaces) at different rates. However, for hRESCAP, a more plausible explanation relates to the distinct degrees and patterns of glycosylation displaying different elimination kinetics. Protein analysis of produced hRESCAP showed two prominent peaks, the mass difference of which could be attributed to a glycan. Of the glycosylated hRESCAP, ~20% has a sialylated protein core. Animal studies with nonsialylated ALP demonstrated rapid plasma clearance that could be prevented by asialo protein being supplemented as competitor for binding to asialo glycoprotein receptor (proprietary data). Human ALPs of different origin (liver, bone, and placenta) show different sialylation patterns associated with different plasma residence times. Based on this, we expected that hRESCAP, appearing to be heterogeneous in its degree and pattern of sialylation in the protein analysis, would display a variation in the elimination rates.

The estimated coefficients of the best fit model to the separate and simultaneous data sets are given in **Supplemental Tables S2 and S3** online. The individual and population predictions of

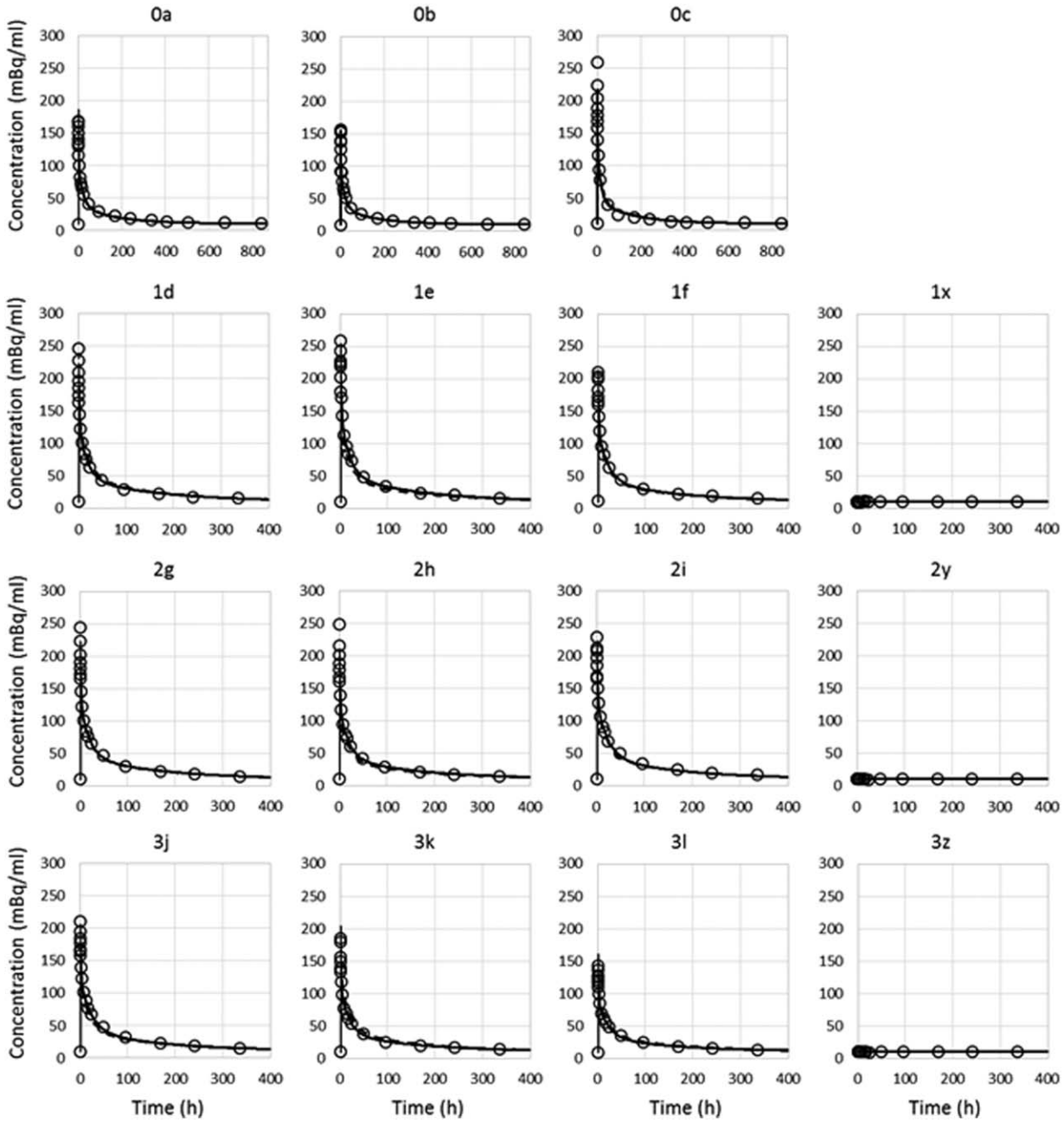

Figure 2 Plasma concentration-time in individual healthy volunteers after i.v. administration of a microdose of 53 μg [^{14}C]-human recombinant placental alkaline phosphatase (hRESCAP) (45 U, ~592 Bq) alone (first row of panels), or in combination with increasing doses of unlabeled hRESCAP (subsequent rows of panels, respectively 414, 1240, and 5300 μg or 391, 1224, and 5260 U, in total). The fourth column of panels shows the placebos. Symbols indicate the observed radioactivity in mBq/mL (**a**) or ALP activity in mU/mL (**b**) solid and dashed curves show the individual and population average simulations by a fitted nonlinear mixed effects model.

plasma radioactivity and enzyme activity (simultaneously) are plotted in **Figure 2**. The obtained population mean coefficients are consistent with the results of the noncompartmental analysis. The obtained terminal half-life was 116 hours. The population average volume of distribution was 3.2 L with some dependency on body weight, in line with human plasma volume. The within-subject coefficient of variation of background radioactivity and ALP activity in placebos were 1.7% in both cases, with no apparent trend in time (**Figure 2**). The respective protein fractions that showed different elimination kinetics made up ~40%, 40%, and 20% of the dose, and their contributions to the AUC were calculated to be 1.1%, 17%, and 82%, respectively (see **Supplemental Figure 1** online). The slowly eliminated fraction corresponds to ~20%, which is in line with the fraction of fully sialylated hRESCAP, supporting our interpretation of the observed kinetics.

Table 1 Summary of pharmacokinetics of hRESCAP, established by noncompartmental analysis unless reported otherwise

	Microdose	Low	Mid	High
Radioactivity	556 Bq	649 Bq	606 Bq	558 Bq
Background (mBq/mL)	10.0 (4.7)	11.1 (6.0)	11.0 (1.2)	10.3 (8.0)
C_{max} (mBq/mL)[a]	194 (9.6)	208 (12)	226 (13)	180 (20)
C_{max}/dose	0.35 (9.6)	0.32 (12)	0.37 (13)	0.32 (20)
V_d (L)	2.9 (9.6)	3.1 (12)	2.7 (13)	3.1 (20)
$AUC_{0\text{-}336}$ (h*mBq/mL)[a]	6,457 (22)	6,518 (18)	6,820 (5.1)	6,225 (21)
$AUC_{0\text{-}inf}$ (h*mBq/mL)[a]	7,834 (27)	7,401 (19)	7,630 (6.3)	6,984 (23)
$AUC_{0\text{-}336}$/dose	11 (22)	11 (18)	12 (5.1)	11 (21)
$AUC_{0\text{-}inf}$/dose	13 (27)	13 (19)	13 (6.3)	12 (23)
Terminal $t^1/_2$ (h)	136 (17)	118 (1.7)	117 (6.6)	119 (6.0)
Enzyme activity	45 U	391 U	1224 U	5260 U
Baseline (mU/mL)	72.7 (2.6)	90.2 (25)	65.2 (18)	77.2 (18)
C_{max} (mU/mL)[a]	15.3 (35)	127 (6.3)	407 (14.0)	1516 (23)
C_{max}/dose	0.35 (35)	0.33 (6.3)	0.33 (14)	0.29 (23)
V_d (L)	2.9 (35)	3.0 (6.3)	3.0 (14)	3.4 (23)
$AUC_{0\text{-}336}$ (h*mU/mL)[a]	475[b]	3,842 (29)	14,473 (1.1)	59,570 (21)
$AUC_{0\text{-}inf}$ (h*mU/mL)[a]	531[b]	4,536 (41)	15,737 (3.2)	65,913 (22)
$AUC_{0\text{-}336}$/dose	10.7[b]	10 (29)	12 (1.1)	11 (21)
$AUC_{0\text{-}inf}$/dose	11.9[b]	12 (41)	13 (3.2)	13 (22)
Terminal $t^1/_2$ (h)	108[b]	108[b]	104 (19)	121 (7.2)

[a]C_{max} and area under the curves (AUCs) reported are the areas above background radioactivity or baseline alkaline phosphatase (ALP). [b]Estimates based on the model fit are given, as the variation was too high to establish these values reliably by noncompartmental methods. Values reported represent means and coefficients of variation (between brackets) of three subjects in each dose group.

The model fitted to the radioactivity and enzyme activity vs. time data separately with and without dose as covariate showed a small but significant difference in the elimination rates λ_1 and λ_2 only for the enzyme activity data ($P = 0.0011$ by analysis of variance). The model fit to the datasets simultaneously with and without quantification method as covariate showed small but significant differences in the protein fraction α_1 and in λ_1 and λ_2 ($P < 0.0001$). A possible explanation for the differences between radioactivity and enzyme activity may be that the radiolabels may not have been evenly distributed among the different protein fractions. The impact of these differences on the dose-corrected AUCs is minor, because of the minimal contributions of these rates to the overall AUC (1.1% and 17%, respectively). This is illustrated in **Figure 3**, showing that predictions of the enzymatic activity in the phase 1 ascending dose study based on model fits of the microdose radioactivity data were accurate. Based on the fitted coefficients (see **Supplemental Table 2** online), a deviation between microdose and high dose AUC of 8% would be expected. Compared to prediction of human PKs from animal data based on allometric scaling, in which predictions within a factor of two are commonly considered acceptable,[24] this deviation can be considered very small.

Summarizing these results, the analysis shows that hRESCAP displays dose-proportional PKs over the dose range tested, and that the microdose radioactivity levels in healthy volunteers could be used to accurately predict the enzyme activity levels at the intended therapeutic doses.

DISCUSSION

Since the introduction of microdosing studies, there has been discussion on how predictive a microdose can be for the PK at therapeutic doses. Validation studies addressed this issue by selecting well-known small molecule drugs, as well as compounds that failed in traditional phase 1 clinical trials.[7,24] About 80% of these drugs showed dose-linear PKs.[7,24] Consequently, microdosing proved to offer great potential to select the appropriate drug candidates. Although, currently, biologics make up an important part of the research pipelines in the pharmaceutical industry, here the application of microdosing considerably lags behind. Typically for proteins, concern exists about the predictive value of a microdose for the PKs at therapeutic doses. Only a very limited number of protein microdosing studies have been performed.[25,26] These showed promising results, but none of these studies were performed in humans. As one of the major advantages of

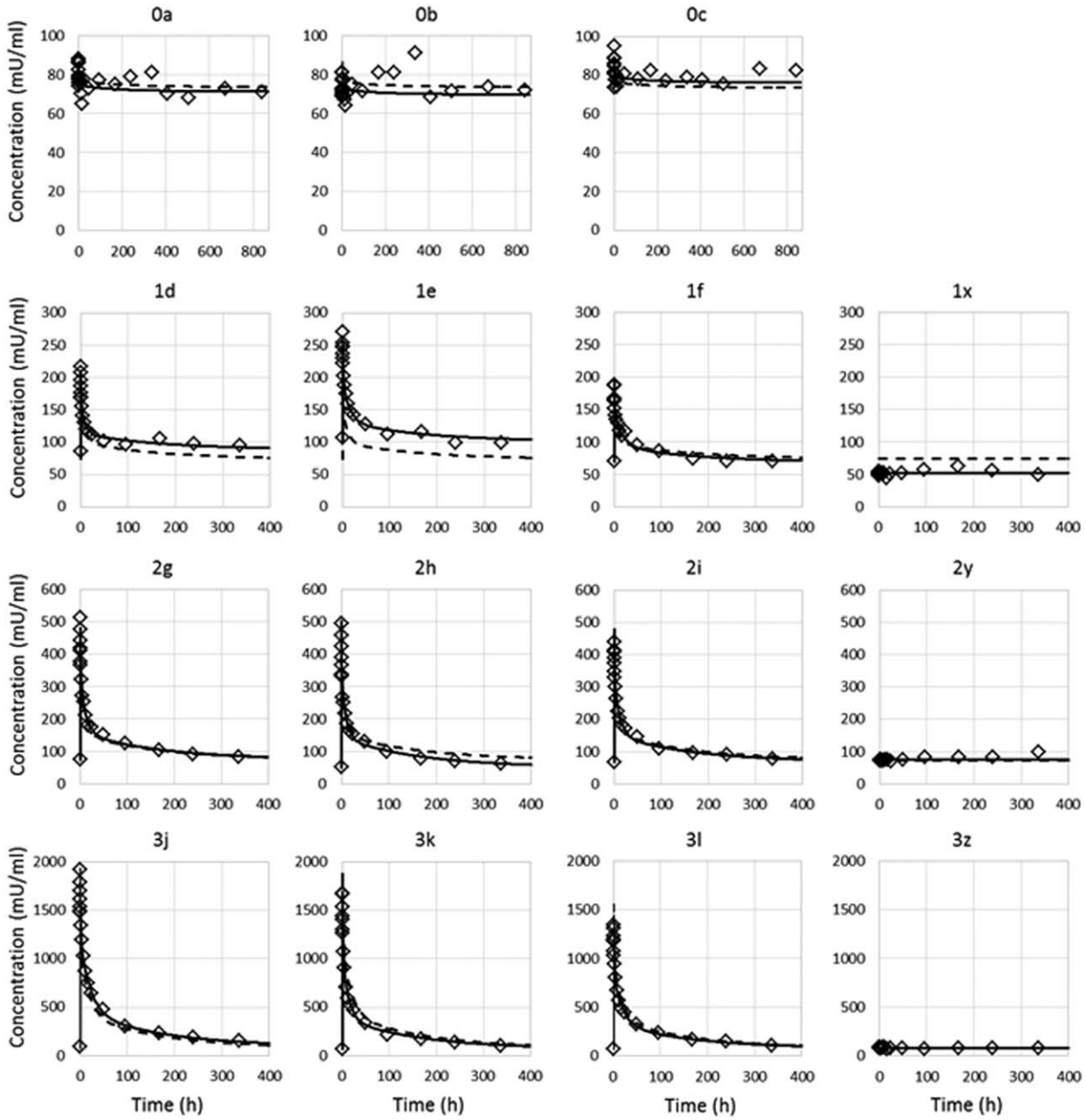

Figure 3 (**a**) Observed plasma radioactivity (mean and SD, in mBq/mL) after a microdose of 557.9 Bq [14C]-human recombinant placental alkaline phosphatase (hRESCAP) (symbols) and fitted population average (curve). (**b**) Observed plasma alkaline phosphatase (ALP) activity (mean and SD, in mU/mL) after a dose of 391 U (left: 414 μg), 1,224 U (middle: 1,240 μg), and 5,260 U (right: 5,300 μg). The dotted curve represents model predictions based on the model fit of the microdose radioactivity.

microdosing is the potential to directly obtain human data, an important application area of microdosing had until now been left unaddressed. However, it is important to realize that microdosing cannot automatically be applied to any biological directly. For proteins that show target-mediated disposition, microdosing data in combination with binding affinities determined *in vitro* and physiologically based pharmacokinetic modeling may allow the prediction of the PKs at therapeutic levels from a microdose.[27]

This first microdosing study with hRESCAP showed dose proportionality, in line with our expectations for this particular NBE, as the protein is endogenously present in the systemic circulation. In this study, microdosing immediately provides an added-value, and these results may encourage the exploration of a wider range of applications of microdosing with AMS to further assess clinical PK parameters at earlier stages of biopharmaceutical development. Additionally, this study demonstrates that a microdose can be used as a safe starting dose of biotherapeutics for first-in-human studies. This particular approach could have been helpful for the study, including the compound TGN1412. The microdose would have been about 100 times lower than was given in the study.[17] After this conservative dosing regimen,

consequences are minimized as these low drug concentrations will likely not induce toxicological effects nor impose any radiological risks. Still, the pivotal information will become available relatively early, speeding up overall timelines in drug development.

Another advantage is that microdosing studies can be performed with a relatively low number of subjects (cost reduction of clinical studies). Our data showed almost negligible interindividual differences in the PKs (**Figure 2a**). In addition, the sensitive detection by AMS allows individuals to be their own controls (e.g., for baseline measurements before hRESCAP dosage), which improves statistical power of the data. The between-subject variation in ALP activity vs. time was larger than in radioactivity (**Figure 2b**), but also limited in this study. This may be due to ALP being an endogenous protein and inclusion of subjects with baseline ALP within normal range.

To obtain approval from the regulatory authorities for a microdosing study with a biotherapeutic, limited preclinical studies are required (14-day single dose study one species, rodent, and SAR assessment).[10] A clear difference with traditional small molecule microdosing studies lies in the incorporation of the carbon-14 label into the molecule of interest, reductive amination vs. chemical derivatization, respectively. Although the labeling approach is generally applicable for proteins, the product characterization strategy after labeling will be protein-specific. Comprehensive analysis of hRESCAP before and after labeling showed no differences illustrating the feasibility of this approach.

In summary, microdosing holds the promise that selected compounds may be developed more rationally and faster, by the early availability of human PK data. Important concomitant advantages will be significant reductions in costs of drug development and the use of laboratory animals.

METHODS

Animal studies

Mice animal studies were performed to determine the toxicity and investigate possible abnormalities in body or organ weight. No toxicity or abnormalities were observed (details: see the **Supplemental Material** online).

hRESCAP production

hRESCAP is expressed in the human amnion-derived production CAP9 cell line (CEVEC Pharma, Köln, Germany). Good manufacturing practice production of hRESCAP was performed at the GenIbet facilities (Oeiras, Portugal). Specific chemical and physical viral clearance studies, such as formaldehyde, diafiltration, and nanofiltration, have been performed on the process that was used to inactivate putative extraneous viruses.

hRESCAP characterization

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration chromatography were performed to determine the molecular weight and purity for hRESCAP. The enzymatic activity of hRESCAP was tested *in vitro* (see below). Mass spectrometric analysis of the glycosylated protein and deglycosylated product indicated a G0-GlcNAc as the most prominent glycan structure, located at N271 (data not shown, Abundnz B.V., Woerden, The Netherlands). No protein aggregation was determined for the bulk product and [^{14}C]-hRESCAP by SEC-UV (Abundnz B.V.). The hRESCAP was supplied as a clear to slightly opalescent, colorless, sterile, essentially pyrogen-free solution in a Hyclone bag. The protein concentration was determined using a Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The mean protein concentration was 3.3 mg/mL. Stability studies on one batch of hRESCAP showed an initial loss of enzymatic activity from 1446 U/mg after production → 1064 U/mg after two months. The activity remained stable during the following months at 992 U/mg. The hRESCAP was stored in an aqueous buffer containing 10 m*M* Tris-HCl, 5 m*M* magnesium chloride, 0.1 m*M* zinc chloride, and pH 8.3, at 2–8°C.

[^{14}C]-hRESCAP synthesis and quality control

A good manufacturing practice-produced and certified hRESCAP batch was radiolabeled with carbon-14 by reductive amination with [^{14}C]formaldehyde of lysine residues (see **Supplemental Material** online). The product was characterized by radio-high performance liquid chromatography with a beta-flow-through detector, via comparison of retention times for the radiolabeled product and nonradioactive hRESCAP. The requirement for radiochemical purity of the batch was set at ≥95%. Furthermore, bacterial endotoxin content, filter integrity, and sterility were tested for each produced batch. Requirements for the enzymatic activity of the produced [^{14}C]-hRESCAP batches were specified to be ≥70% of the original enzymatic activity. Protein concentrations of the batches were 8.8–11.4 μg/mL and the specific activities varied between 86 and 120 Bq/mL.

Clinical study and data analysis

The clinical study with hRESCAP and [^{14}C]-hRESCAP was designed as a two-phase study in healthy male volunteers (**Figure 1**). Blood samples were taken at regular time intervals after administration, and one predose sample was obtained for each volunteer.

Determination of enzymatic activity

The enzymatic activity of the plasma samples was determined using an Olympus AU 400 chemical analyzer (Goffin Meyvis) at 410/480 nm. The instrument was calibrated using the system calibrator kit (66300 Goffin Meyvis) and deionized water as a blank. The method is based on the recommendations of the "International Federation for Clinical Chemistry." As a quality control, the starting material hRESCAP was included.

AMS total count analysis

A novel AMS sample introduction method was used.[16] Briefly, 1.5 μL plasma samples were transferred to tin foil cups, evaporated to dryness, and combusted using an elemental analyzer (Vario Micro, Elementar, Germany). The resulting CO_2 was captured on a zeolyte trap. CO_2 was released by heating of the trap and transferred to a vacuum syringe using helium. The resulting 6% v/v gas mixture of CO_2 with helium was infused at a pressure of 1 bar at 60 μL/min into the titanium target in the SO110 ion source of a 1 MV Tandetron AMS (High Voltage Engineering Europe B.V., The Netherlands).[28,29] As the AMS solely counts [^{14}C] atoms, the analysis is universal. No study, matrix, or compound method development is required.

LC+AMS

Enzymatically active hRESCAP was purified from plasma using a 4 mL high performance liquid chromatography column (prepared in-house) containing mimetic ligand adsorbent for ALP (Prometic Life Sciences, Québec, Canada). The affinity chromatography specifically isolated enzymatically active ALP from plasma samples, including [^{14}C]-hRESCAP (see **Supplemental Material** online). Method validation was based on the European Bioanalysis Forum recommendation for AMS analysis.[30] Calibration standards and quality controls were prepared in blank human pooled heparin plasma (Bioreclammation IVT, New York, NY). Activity levels ranged from 10–250 mBq/mL, and from 30–200 mBq/mL for the calibration standards and quality controls, respectively. All samples of two subjects, one receiving a microdose and one high-dose,

were analyzed by liquid chromatography accelerator mass spectrometry (LC+AMS). The LC+AMS data are in line with the total count and enzymatic activity analysis (>90% agreement between AUCs). As no additional advantage is provided by the LC+AMS data, the remaining subject samples were only analyzed for total count and enzymatic activity.

AMS data processing

For total control analysis, ratios were calibrated using the standard reference material oxalic acid II (OXII, SRM 4990c; National Institute of Standards and Technology, USA).[31] Anthracite was used as a zero-reference sample (Certified Reference Material, BCR-460 Coal). Australian National University sucrose (ANU sucrose, IAEA-CH-6, Reference Material 8542) was used for quality control.[32] For LC+AMS data, linear regression on calibration standards with weighting factor 1/× was applied.

PK modeling and statistics

Subjects showed a nonzero background radioactivity and a baseline endogenous enzyme activity. Upon administration at time 0.5 hours, the concentration-time curve exhibited three distinct elimination phases. This profile was described by a linear combination of three exponential terms (Eqs. 1–4):

$$\tau = t - T_D \tag{1}$$

$$\varphi = \ln(\lambda) \tag{2}$$

$$c = C_{0\ \tau<0} \tag{3}$$

$$c = C_0 + D/V \times \left\{ \begin{array}{l} \alpha_1 \times \exp[-\exp(\varphi_1) \times \tau] + \\ \alpha_2 \times \exp[-\exp(\varphi_2) \times \tau] + \\ \alpha_3 \times \exp[-\exp(\varphi_3) \times \tau] \end{array} \right\}_{\tau>=0} \tag{4}$$

where τ is the time after dosing T_D (0.5 h), ϕ are defined as the natural log of the elimination rate constants λ (h^{-1}), D is the administered dose (Bq or U), C_0 is the background radioactivity (mBq/mL) or baseline ALP activity (mU/mL), V is the apparent volume of distribution (L), and the multipliers α represent fixed but unknown proportions of the dose belonging to the different protein fractions.

The natural logs ϕ of the elimination rate constants λ (Eq. 2) were estimated to enforce positive values without constraining the optimization problem. The exponential terms were ordered by their rate constants: $\phi_1 > \phi_2 > \phi_3$. The background was included in the model to account for between-subject and within-subject variation. For this purpose, placebo-treated subjects were also included in the dataset.

An equal volume of distribution V was assumed for all protein fractions. The multipliers α_1, α_2, and α_3 represent the fixed but unknown proportions of dose D corresponding to the different protein fractions, of which the first two were estimated and the last is set at $\alpha_3 = 1 - \alpha_1 - \alpha_2$.

Log-normal distributions about the means were considered appropriate. The log of the right hand side of Eq. 4 was fit to the log-transformed plasma concentration time data with the nonlinear mixed effects library *nlme*[33] in R (version 3.0.2.1), by the first order conditional estimation algorithm. Random effects were considered at the subject level in all coefficients, except for the background radioactivity.

To explicitly study dose-proportionality in PK across the doses administered, the model was fit separately to the radioactivity vs. time data and enzyme activity vs. time data with and without dose as a covariate for all coefficients. To exclude a significant effect of the radiolabel on the PK of the protein, the model was fit to all data with and without method (enzyme activity or radioactivity) as a covariate for all parameters. The significance of each random effect term and covariate was evaluated by analysis of variance.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

E.V.D., M.L.H.V., A.D.W., S.B., W.S. and W.H.J.V. wrote manuscript; E.V.D., M.L.H.V., R.B., A.D.W., N.H.H., R.A.F.d.L., B.O.F., W.J.P., W.S., P.A.M.P. and W.H.J.V. designed research; E.V.D., M.L.H.V., M.R.D., J.B., M.C.d.K., A.F., J.A. J.M., H.S., W.J.P. and W.S. performed research; E.V.D. and M.L.H.V. analyzed data; E.V.D., M.L.H.V., R.B., A.D.W., J.A.J.M.T.A., M.C. and C.P. contributed new reagents/analytical tools.

Additional Supporting Information may be found in the online version of this article.

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