Received: 21 June 2017 | Revised: 28 February 2019 | Accepted: 8 March 2019

DOI: 10.1111/bcp.13942

ORIGINAL ARTICLE

Pharmacokinetics, safety and tolerability of the novel β-hCG derived immunomodulatory compound, EA-230

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Funding information
Exponential Biotherapy Incorporation

Aims: EA-230 is a newly developed synthetic linear tetrapeptide (AQGV) derived from the chorionic gonadotropin hormone (β-hCG). We investigated the pharmacokinetics, safety and tolerability of EA-230 in healthy subjects using different administration strategies.

Methods: Double-blind, randomized, placebo-controlled, dose-escalating phase I studies in healthy subjects using intravenous administration were conducted. In the single dosage study, 32 subjects were assigned to four single dosage groups (1, 3, 10 or 30 mg/kg). In the multiple dosage study, 24 subjects were assigned to three dosage groups (10, 20 or 30 mg/kg, thrice daily for 3 days). In the continuous dosage study, 24 subjects were assigned to three dosage groups (15, 30, or 90 mg/kg/hour for 2 hours). Pharmacokinetics, safety and tolerability assessments were performed up to 14 days.

Results: The highest dosage of EA-230 (continuous infusion of 90 mg/kg/hour for 2 hours) showed more than proportional increases in exposure (C_{max}136%; $AUC_{0\text{-}last}$137%), a large volume of distribution (geometric mean and 95% CI: 13 [3–58] L/kg), a high clearance rate (26 [15–43] L/h/kg), and a short half-life (0.35 [0.13–1.0] minutes). EA-230 was well tolerated and no safety concerns were observed.

Conclusion: These dose-escalating phase I studies with different administration strategies reveal a pharmacokinetic profile of EA-230 with a large volume of distribution and a short half-life. Furthermore, EA-230 was well tolerated and no safety issues emerged. These results have enabled further clinical development in a phase IIa trial assessing the pharmacodynamics of this compound during systemic inflammation described elsewhere in this issue.

KEYWORDS

EA-230, immunomodulation, pharmacokinetics, phase I, randomized clinical trial, tolerability

1 | INTRODUCTION

Systemic inflammation plays a detrimental role in various autoimmune diseases, but also during critical illness, such as sepsis, trauma and major surgery. In the latter group, an injurious systemic inflammatory response to a variety of inflammatory stimuli may occur, often resulting in pronounced tissue damage with associated organ failure and mortality rates up to 30%.[1-3] Despite its tremendous impact, current intensive care consists of supportive treatment, as no pharmaceutical interventions have proven effective in regulating the systemic inflammatory response to prevent organ injury.[4-6] Therefore, new therapeutic strategies are warranted.

The adaptation of the maternal immune system during pregnancy has provided a basis for research into new immunomodulatory strategies. Pregnancy represents a unique immunologic situation in which the maternal immune system tolerates the semi-allogeneic fetus, while maintaining pathogen clearing capacity.[7-9] This immune-tolerant anti-inflammatory phenotype is also exemplified by the fact that various autoimmune diseases like rheumatoid arthritis (RA), multiple sclerosis (MS) and psoriasis show attenuated disease activity during pregnancy and often relapse following delivery.[10-14]

The hormonal milieu, in particular the release of human chorionic gonadotropin (hCG), is thought to play a pivotal role.[15] Produced throughout pregnancy, hCG is already present at a very early stage and has been shown to exert immunomodulatory effects.[16-19] In addition to the integral hCG molecule, nicked fragments originating from the β-loop of hCG, which are abundantly present in the circulation during pregnancy, exert immunological effects.[20] Recent studies in animal models of systemic inflammation have shown that these oligopeptides exert immunomodulatory effects and limit organ failure and mortality.[21-29] Of particular interest is the linear tetrapeptide alanine-glutamine-glycine-valine (AQGV), which has shown the most promising effects up till now. This peptide preserved kidney function and substantially reduced mortality in murine models of renal ischaemia and reperfusion.[22] Furthermore, it attenuated the release of inflammatory mediators during haemorrhagic and endotoxemia-induced shock in mice and monkeys.[23,24,28]

AQGV is currently developed under the product name EA-230 as a potential novel immune modulatory compound. The present work describes three phase I studies in which the first-in-human pharmacokinetics, safety and tolerability of EA-230 are investigated, using escalating single and multiple dosages as well as escalating continuous dosages.

What is already known about this subject

- Systemic inflammation can result in pronounced tissue damage and is associated with organ failure and high mortality rates.
- EA-230 is a new, β-hCG-derived immunomodulatory compound developed to modulate systemic inflammation and to protect organs.

What this study adds

- These first phase I studies using single, multiple and continuous dosage administration demonstrate that EA-230 has a non-proportional dose-exposure relationship, a large volume of distribution and a very high clearance rate.
- EA-230 is well tolerated by healthy volunteers without any safety concerns.

2 | METHODS

2.1 | General

Double-blind, randomized, placebo-controlled, phase I studies in healthy subjects were conducted to evaluate pharmacokinetics, safety and tolerability of EA-230 in escalating single dosages, multiple dosages and single continuous dosages. Dosages were selected based on the effective immunomodulatory dose in pre-clinical studies, ranging from 5–50 mg/kg, without exceeding the established maximum tolerated dose of 200 mg/kg/day.[22-25,28] Animal pharmacokinetic (PK) data are summarized in the Supplemental data file. The studies were approved by the Ethics Committee ZNA/OCMW in Antwerp and of the Radboud University Medical Center, Nijmegen, and registered at clinicaltrial.gov (NCT02629874). All studies complied with the Declaration of Helsinki and in compliance with the International Conference on Harmonisation E6 Guideline for Good Clinical Practice (CPMP/ICH/135/95). All healthy volunteers who participated in the study provided written informed consent before the start of any study-related procedures. Quality assurance, data management with full data validation, and monitoring of all source documents and study procedures were performed by contract research organizations (SGS Life Sciences Clinical Research Services [Antwerp, Belgium] and QPS [Groningen, The Netherlands]).

2.2 | Study medication

EA-230 and placebo were supplied as solution for injection in identical sterile single-use vials. EA-230 vials contained 11 ml of 40 mg/ml (single and multiple dosage studies) or 5 ml of 300 mg/ml (continuous dosage study) active substrate, and placebo vials contained an equivalent osmolar dose of sodium chloride solution. Vials were manufactured by Octoplus and HAL allergy BV, and quality controlled by PROXY Laboratories BV (both based in Leiden, The Netherlands). Manufacturing, packaging, quality control and preparation were described in an Investigational Medicinal Product Dossier (IMPD) and complied with Good Manufacturing Practice (GMP) requirements.

Randomization, using a pre-determined randomization list, and preparation of study medication were performed by independent

research personnel, who were not involved in data sampling, analysis or any other study-related activity.

2.3 | Subjects

Following written informed consent, healthy Caucasian adult males and females with a body mass index between 18 and 30 kg/m^3 were included. Before participation, health status was determined by medical history, physical examination, electrocardiogram (ECG) and routine laboratory blood tests. Female subjects were also required to have a negative pregnancy test result (urine hCG). Exclusion criteria included atopic constitution, presence and/or history of clinically significant allergies, use of any medication, significant blood loss and participation in any other clinical trial within 90 days prior to the study. The use of tobacco, recreational drugs, alcohol and/or caffeine 7 days prior to or during the study was not allowed. All subjects were fasted from the evening before (midnight) until 4 hours after the start of study drug administration, to exclude any dietary effects on the PK of the compound and for safety reasons.

2.4 | Study procedures

A schematic overview of the study procedures is presented in Figure 1.

2.4.1 | Single dosage study

Thirty-two male subjects were assigned to one of four dosage groups: 1, 3, 10 and 30 mg/kg body weight. Each dosage group consisted of eight subjects, randomly assigned to receive either EA-230 or placebo (n = 6 active study drug, n = 2 placebo).

The study drug was administered as an i.v. bolus injection in 2 minutes for dosage groups 1 and 2 (1 and 3 mg/kg), and in 15 minutes for dosage groups 3 and 4 (15 and 30 mg/kg). Frequent blood samples for PK analyses were collected and subjects were monitored for 24 hours.

2.4.2 | Multiple dosage study

In this subsequent 3-day multiple dosage study, 24 healthy male volunteers were assigned to three escalating dosage groups: 10, 20 and 30 mg/kg body weight. Each dosage group consisted of eight subjects who were randomly assigned to receive either EA-230 (n = 6) or placebo (n = 2). The study drug was administered as an i.v. bolus infusion in 10 minutes thrice daily for three subsequent days at intervals of 8 hours (nine dosages per subject in total). Serial blood samples for PK analyses were collected and subjects were monitored until discharge 12 hours after the last dose administration. Subjects returned at Day 14 for follow-up.

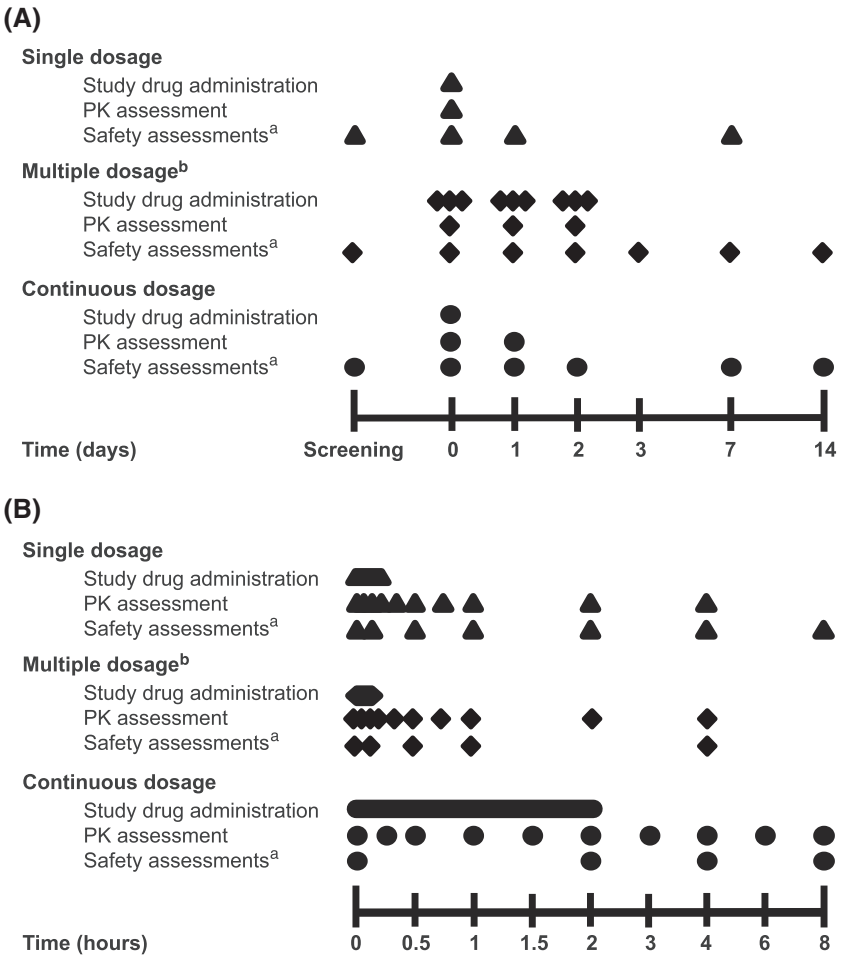

FIGURE 1 Schematic overview of study procedures. A, Total study period. B, Day of study drug administration. [a]Safety assessments including vital parameters, ECG, routine haematology and biochemistry and injection site inspection. [b]For the multiple dosage study, study drug administration and safety assessments were performed thrice daily for 3 days, whereas PK assessments were performed following every first study drug administration of the day

2.4.3 | Continuous dosage study

Twenty-four subjects were assigned to three dosage groups: 15, 45 or 90 mg/kg/hour EA-230, for the duration of 2 hours. Each dosage group consisted of eight subjects (four males and four females) and within each dosage group subjects were randomly assigned to receive either EA-230 (n = 6) or placebo (n = 2), with equal numbers of males and females within active and placebo groups. The study drug was administered by 2-hour continuous i.v. infusion. Serial blood samples for PK analyses were collected and subjects were monitored until release from the research unit 8 hours after start of study drug administration. Subjects returned at Days 1, 2, 7 and 14 after study drug infusion for follow-up.

2.5 | Pharmacokinetic analyses

Blood samples for measurement of EA-230 concentrations were collected from the arm opposite to the one where EA-230 was administered. A schematic overview of the sampling time-points is provided in Figure 1. For the single dosage study, samples were collected at the following time points: prior to and at 5, 10, 15, 20, 30, 45, 60, 120 and 240 minutes after the start of study drug administration. For the multiple dosage study, PK samples were only collected after the first study drug administration on each day, at the same time points as in the single dosage study. For the continuous dosage study, PK samples were collected prior to and at 15, 30, 60, 90, 120, 180, 240, 360, 480 and 1440 minutes after start of study drug administration.

Immediately following withdrawal of 3 mL ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood, Protease Inhibitor Cocktail (P8340, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) was added to stabilize EA-230 by preventing proteolysis. Blood samples were centrifuged at 2000–2700g for 5–15 minutes at 4°C and plasma samples were stored at −20–80°C until analysis.

EA-230 concentrations were determined by a validated liquid chromatography–tandem mass spectrometry (LS-MS/MS) assay. Briefly, a stable isotope-labelled internal standard of EA-230 (A*QGV; Caslo, Lyngby, Denmark) was added to 100 µL plasma sample, followed by the addition of 300 µL of acetonitrile. Five µL supernatant, obtained by passing the mixture through an OstroTM 96-well plate, was injected for chromatographic separation using a high performance liquid chromatography (HPLC) system. The retention time of EA-230 and its stable isotope-labelled internal standard was 2.2 min. A tandem mass spectrometer was used for the detection of the compounds, and quantification was based on the peak area ratios of EA-230 and its stable isotope labelled internal standard. The detection range of the method was 0.5–100 ng/mL with low, medium and high quality control (QC) concentrations of 1.5, 10 and 75 ng/mL. Concentrations below the limit of quantification were not included in the PK analyses. Inter-run and intra-run precision coefficients of variation (CV) and accuracy relative error (RE) were determined for the low, intermediate and high concentration standards. Inter-run and intra-run CV were between 4.8–8.6% and 2.1–11.4%, respectively. RE were between −2.5–8.0% and −10.6–11.2%, respectively. EA-230 concentrations were shown to be stable at room temperature for 17 hours and at −80°C for up to 183 days.

The highest observed plasma concentration was defined as C_{max}. The area under the plasma concentration *vs*. time curve from t = 0 to the time of the last measured concentration ($AUC_{0\text{-last}}$) was calculated using the linear-log trapezoidal rule, with extrapolation to infinity (using C_{last}/β) to obtain the AUC from t = 0 to infinity ($AUC_{0\text{-inf}}$). The log-linear period (log concentration *vs*. time) was defined by visual inspection of data points. The absolute value of the slope (β/2.303) was calculated by least squares linear regression analysis, where β is the first-order elimination rate constant. Elimination half-life ($t_{1/2}$) was calculated by the equation 0.693/β. Clearance (Cl) was calculated by dividing dose by $AUC_{0\text{-inf}}$ and volume of distribution (Vd) by dividing Cl by β.

2.6 | Safety and tolerability assessments

On the study drug administration days of each study, frequent safety and tolerability assessments were performed until discharge and rechecked during the follow-up visits (Figure 1). Safety parameters included vital signs (blood pressure and heart rate), 12-lead ECG and routine haematology and biochemistry laboratory tests. Adverse events (AEs) were recorded throughout the study, until the final study visit. All AEs were judged by the investigator with regard to severity (mild, moderate or severe) according to Common Terminology Criteria for Adverse Events (CTCAE) guidelines 4.0,[30] and their relation to the study drug (definitely, probably, possibly or unrelated/unlikely to be related). Serious adverse events (SAEs) included death, life-threatening disease, persistent and/or significant disability and/or incapacity and hospitalization and/or prolongation of inpatient hospitalization. In order to minimize risks in these studies, dosage groups were tested sequentially if the previous dosage was well tolerated without relevant adverse effects. Safety parameters were reported to an independent Data Safety Monitoring Board (DSMB) after completion of each dosage group.

2.7 | Statistical analysis

Demographic data of each study are expressed as mean ± SD and compared using one-way analysis of variance (ANOVA). Adverse events are summarized by treatment group, preferred term, severity and relation to the study drug. PK parameters are presented according to treatment group using geometric mean and 95% confidence intervals (CI). Dose proportionality was assessed using unpaired Student's t-tests or one-way ANOVA followed by a Bonferroni post-hoc test on dose-normalized, log-transformed data. Dose accumulation in the multiple dosage study was assessed by repeated measures one-way ANOVA on log-transformed data. A p-value of <0.05 was considered statistically significant. Statistical calculations were performed using GraphPad Prism version 5.03 (GraphPad Software), The PK analysis was performed with non-compartmental methods using WinNonLin/Phoenix version 6.3 (Pharsight Corporation, St. Louis, MO, USA).

3 | RESULTS

3.1 | Subject disposition

All subjects were Caucasian and there were no differences in baseline characteristics between groups (Table 1). All subjects received study medication as intended and were deemed compliant to the study protocol. In the multiple dosage study, one participant was replaced after 1 day (three dosings) for personal reasons and the replacing participant withdrew after 2 days (six dosings) for work-related reasons. The two subjects that did not complete the study were included in the safety analysis as they received study medication. However, because of incomplete PK data, these subjects could not be used for the PK analysis.

3.2 | Single dosage study

3.2.1 | Pharmacokinetics

Plasma EA-230 concentration–time profiles are presented in Figure 2 A and PK parameters are summarized in Table 2a. In the two lowest dosage groups (1 and 3 mg/kg), the maximum concentration observed was at the first time point (at t = 5 minutes, 3 minutes after administration had stopped). In the highest dosage groups (10 and 30 mg/kg), C_{max} was reached before the end of infusion (at t = 10 minutes, 5 minutes before administration had stopped). A rapid decline in plasma concentration was observed; concentrations decreased below the limit of quantification for the four dosage groups at 15, 20, 45 and 120 minutes, respectively. With regard to this rapid decline in plasma concentrations, the elimination rate constant β and other β-dependent PK parameters ($AUC_{0\text{-inf}}$, $t_{1/2}$, Vd, Cl) could not be determined for the lowest dosage groups. In the two highest dosage groups, a high volume of distribution (geometric means of 3 and 33 L/kg) and fast clearance rate (geometric means of 57 and 61 L/h/kg) were observed. Dose proportionality could not be assessed across all dosages as the duration of administration differed between the 1 and 3 mg/kg groups (2 minutes) and the 15 and 30 mg/kg groups (15 minutes). Nevertheless, the dosage increase from 1 to 3 mg/kg as well as that from 10 to 30 mg/kg resulted in a proportional increase in exposure parameters C_{max} and $AUC_{0\text{-last}}$ (Figure 4A).

3.2.2 | Safety and tolerability

Administration of a single dose of EA-230 was well tolerated by all subjects in every dosage group, and did not result in any safety concerns. No SAEs were reported. Six subjects (25%) treated with EA-230 and two placebo-treated subjects (25%) reported one or more AEs (Table 3a). All AEs were mild and transient, and no dose-dependent increase in number or intensity of AEs was observed. Most of the AEs were deemed unrelated to the study drug. Two subjects reported AEs which were considered possibly related to the study

TABLE 1 Demographic characteristics

	Placebo	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg	
a. Single dosage	***n* = 8**	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 6**	***P*-value**
Gender (m), *n*	8	6	6	6	6	
Age, years	32 ± 6	34 ± 7	40 ± 4	38 ± 9	37 ± 7	0.22
BMI, kg/m^2	23.6 ± 1.7	24.5 ± 2.1	23.1 ± 2.5	22.7 ± 1.4	25.4 ± 1.3	0.11
Weight, kg	80 ± 6	79 ± 10	76 ± 10	79 ± 5	82 ± 6	0.74
Height, cm	184 ± 6	180 ± 5	181 ± 4	175 ± 6	179 ± 6	0.08
	Placebo	**10 mg/kg**	**20 mg/kg**	**30 mg/kg**		
b. Multiple dosage	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 7**		***P*-value**
Gender (m), *n*	6	6	6	7		1
Age, years	29 ± 11	36 ± 11	27 ± 7	34 ± 8		0.34
BMI, kg/m^2	22.3 ± 2.0	23.9 ± 3.0	23.7 ± 1.9	22.7 ± 3.7		0.70
Weight, kg	70 ± 9	79 ± 10	77 ± 10	72 ± 7		0.31
Height, cm	177 ± 8	182 ± 6	180 ± 6	179 ± 6		0.62
	Placebo	**15 mg/kg/h**	**45 mg/kg/h**	**90 mg/kg/h**		
c. Continuous dosage	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 6**		***P*-value**
Gender (m), *n*	3	3	3	3		1
Age, years	22 ± 3	22 ± 2	21 ± 4	21 ± 2		0.91
BMI, kg/m^2	23.1 ± 1.5	21.4 ± 1.8	21.9 ± 4.1	22.4 ± 1.4		0.67
Weight, kg	71 ± 11	66 ± 6	67 ± 16	70 ± 7		0.78
Height, cm	176 ± 8	175 ± 5	174 ± 5	178 ± 12		0.90

Parameters were determined during screening visit. BMI, body mass index. (a) Single dosage study. (b) Multiple dosage study. (c) Continuous dosage study. Data are presented as mean ± SD.

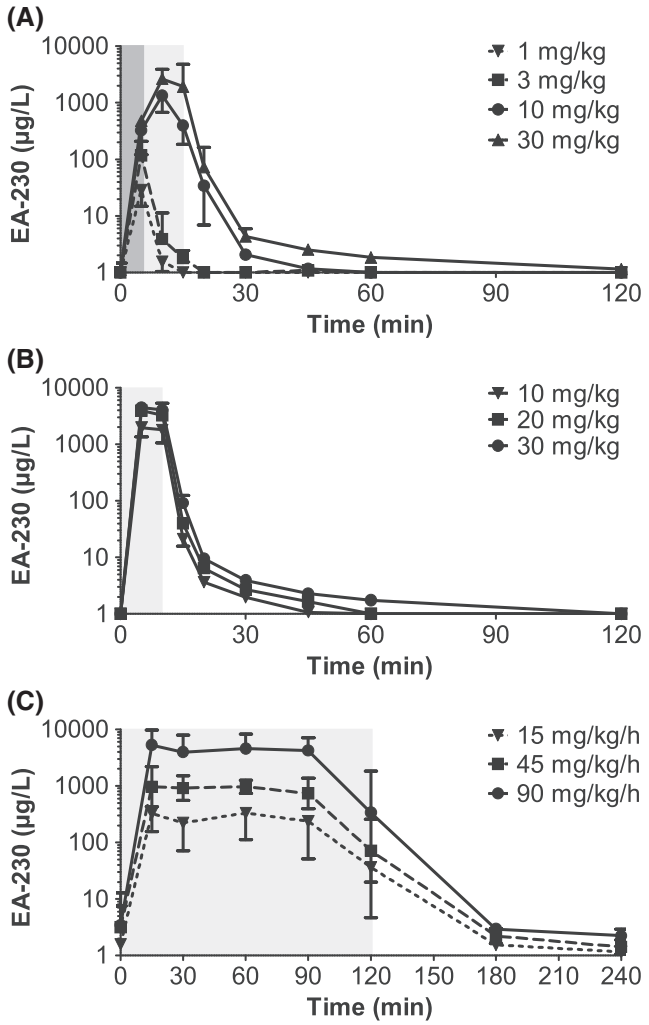

FIGURE 2 Plasma concentration–time profiles of EA-230. A, Single dosage study. B, Multiple dosage study. C, Continuous dosage study. Data are expressed as geometric means and 95% CI. The grey areas indicate the study drug administration periods. (For panel A: Dark grey indicates administration period for dosage groups 1 mg/kg and 3 mg/kg, light grey for dosage groups 10 mg/kg and 30 mg/kg)

drug; one subject in the placebo group with mild headache and one subject in the lowest dosage group with mild headache and dizziness for <1 hour. Other non-related AEs are shown in Table 4a. All variations in laboratory parameters, vital signs and 12-lead ECG were considered not clinically significant.

3.3 | Multiple dosage study

3.3.1 | Pharmacokinetics

Plasma EA-230 concentration–time profiles are presented in Figure 2 B and PK parameters are summarized in Table 2b. All subjects that were administered EA-230 had quantifiable values of EA-230 5 minutes after the start of every study drug administration. C_{max} was reached both during (t = 5 min) and at the end of infusion (t = 10 min), independent of dosage group, subject and/or day of administration. In line with the single dosage study, a high volume of distribution (geometric means across the three dosage groups ranging from 4 to 21 L/kg) and high clearance rate (geometric means across the three dosage groups ranging from 35 to 54 L/h/kg) was observed, resulting in a rapid decline in plasma concentration with quantifiable concentrations up to 30 minutes in the 10 mg/kg group, up to 45 minutes in the 20 mg/kg group, and up to 1 h in the 30 mg/kg group. No accumulation in this multiple dosage study was observed as C_{max} and $AUC_{0\text{-last}}$ were similar on all three days for all three dosage groups (Figure 3). A proportional increase in exposure parameters $AUC_{0\text{-last}}$ and C_{max} was observed with the dose increase from 10 mg/kg to 20 mg/kg, whereas the dose increase to 30 mg/kg resulted in a more than proportional increase (C_{max} 126%; $AUC_{0\text{-last}}$ 123%; Figure 4B).

3.3.2 | Safety and tolerability

All dosages of EA-230, up to 90 mg/kg daily for 3 days, were well tolerated by all subjects and did not result in any safety concerns. No SAEs were reported, and no subjects discontinued the study for safety reasons. Twelve subjects (61%) treated with EA-230 and three placebo-treated subjects (50%) reported one or more AEs, and no relevant dose-dependent increase in number or intensity of AEs was observed (Table 3b). All AEs were mild and transient (with the exception of one moderately severe AE based on a venous injection site haemorrhage which was deemed unrelated to the study drug). Four AEs (17%) were considered possibly related to study drug treatment; two subjects (33%) in the lowest treatment group reported short-lasting postural dizziness and two subjects (33%) in the placebo group reported headaches.

Other non-related AEs are shown in Table 4b. All variations in laboratory parameters, vital signs and 12-lead ECG were considered not clinically significant.

3.4 | Continuous dosage study

3.4.1 | Pharmacokinetics

In the lowest dosage group (15 mg/kg/h), one subject was excluded from all PK analyses because of an abnormal pattern of plasma concentrations of EA-230, with high concentrations at baseline, probably due to an interchange of tubes.

Plasma EA-230 concentration–time profiles are presented in Figure 2C and PK parameters are summarized in Table 2c. In all three dosage groups, stable plasma concentrations were attained 15 minutes after start of EA-230 administration. A very rapid decline in plasma concentrations was observed after cessation of study drug administration in all groups, resulting in few measurable EA-230 concentrations beyond the 2-hour time point. As a result, the elimination rate constant β (and $t_{1/2}$, Cl and Vd) could only be estimated in a limited number of subjects, revealing a large volume of distribution (geometric means across the three dosage groups ranging from 13 to 21 L/kg)

TABLE 2 Pharmacokinetic parameters of EA-230

a. Single dosage	*n*	1 mg/kg	*n*	3 mg/kg	*n*	10 mg/kg	*n*	30 mg/kg
$AUC_{-0\text{-last}}$ (h*µg/L)	6	2 (1–4)	6	8 (5–13)	6	175 (98–312)	6	490 (363–660)
$AUC_{0\text{-inf}}$ (h*µg/L)		–		–	6	175 (98–312)	6	490 (364–660)
C_{max} (µg/L)	6	27 (14–55)	6	115 (64–208)	6	1336 (672–2656)	6	3071 (2133–4421)
$t_{1/2}$ (h)		–		–	6	0.04 (0.02–0.07)	6	0.37 (0.16–0.85)
CL (L/h/kg)		–		–	6	57 (32–102)	6	61 (45–82)
Vd (L/kg)		–		–	6	3 (2–5)	6	33 (12–87)
b. Multiple dosage	***n***	**10 mg/kg**	***n***	**20 mg/kg**	***n***	**30 mg/kg**		
$AUC_{0\text{-last}}$ (h*µg/L)	6		6		5			
Day 1		270 (161–454)		506 (387–662)		645 (357–1167)		
Day 2		241 (98–592)		393 (227–682)		560 (364–861)		
Day 3		273 (100–752)		565 (421–760)		572 (436–751)		
$AUC_{0\text{-inf}}$ (h*µg/L)	6		6		5			
Day 1		271 (162–453)		506 (387–662)		646 (357–1167)		
Day 2		242 (98–592)		393 (227–682)		560 (365–862)		
Day 3		273 (100–752)		566 (421–760)		572 (437–751)		
C_{max} (µg/L)	6		6		5			
Day 1		2295 (1279–4118)		4390 (3207–6008)		5816 (3618–9349)		
Day 2		2242 (945–5323)		3504 (2243–5474)		4807 (3080–7501)		
Day 3		2416 (855–6826)		4540 (3382–6093)		5096 (4050–6412)		
$t_{1/2}$ (h)	6		6		5			
Day 1		0.09 (0.04–0.20)		0.14 (0.11–0.17)		0.24 (0.22–0.26)		
Day 2		0.06 (0.04–0.11)		0.14 (0.08–0.25)		0.25 (0.21–0.31)		
Day3		0.07 (0.05–0.11)		0.13(0.11–0.16)		0.27 (0.22–0.36)		
CL (L/h/kg)	6		6		5			
Day 1		36 (22–62)		40 (30–52)		46 (26–84)		
Day 2		41 (17–102)		51 (29–88)		54 (35–82)		
Day3		37 (13–100)		35 (26–48)		52 (40–69)		
Vd (L/kg)	6		6		5			
Day 1		5 (2–14)		8 (5–12)		16 (9–28)		
Day 2		4 (1–11)		10 (6–18)		19 (12–30)		
Day3		4 (1–11)		7 (5–10)		21 (13–35)		
c. Continuous dosage	***n***	**15 mg/kg/h**	***n***	**45 mg/kg/h**	***n***	**90 mg/kg/h**		
$AUC_{0\text{-last}}$ (h*µg/L)	5	502 (207–1217)	6	1511 (1094–2088)	6	7344 (4458–12098)		
$AUC_{0\text{-inf}}$ (h*µg/L)	2	553 (–)	2	1534 (–)	2	7050 (–)		
C_{max} (µg/L)	5	441 (193–1011)	6	1385 (849–2261)	6	6785 (4409–10441)		
$t_{1/2}$ (h)	2	0.24 (–)	5	0.25 (0.14–0.45)	4	0.35 (0.13–1.0)		
CL (L/h/kg)	2	54 (–)	5	59 (38–90)	4	26 (15–43)		
Vd (L/kg)	2	19 (–)	5	21 (12–37)	4	13 (3–58)		

Data expressed as geometric means and 95% CI (No 95% CI for data with $n = 2$). $t_{1/2}$, elimination half-life; C_{max}, highest observed plasma concentration; $AUC_{0\text{-last}}$, the area under the plasma versus concentration time curve from $t = 0$ to the time of the last measured concentration; $AUC_{0\text{-inf}}$, the area under the plasma versus concentration time curve from $t = 0$ to infinity extrapolated; Cl, plasma clearance; Vd, volume of distribution.

with a rapid clearance rate (geometric means across the three dosage groups ranging from 26 to 54 L/h/kg). The dosage increase from 15 mg/kg/h to 45 mg/kg/h resulted in proportional increase in C_{max} and $AUC_{0\text{-last}}$, but a more than proportional increase in these exposure parameters occurred upon a dosage increase to 90 mg/kg/h (C_{max} 136%; $AUC_{0\text{-last}}$ 137%; Figure 4C).

TABLE 3 Summary of adverse events

	Placebo (*n* = 8)		1 mg/kg (*n* = 6)		3 mg/kg (*n* = 6)		10 mg/kg (*n* = 6)		30 mg/kg (*n* = 6)		Overall (*n* = 32)	
a. Single dosage	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**
Any AE	2	(25)	1	(16.7)	2	(33.3)	2	(33.3)	1	(16.7)	8	(25)
Any SAE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Discontinued due to (S)AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Concomitant medication given	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
AE of mild intensity	2	(25)	1	(16.7)	2	(33.3)	2	(33.3)	1	(16.7)	8	(25)
AE of moderate intensity	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
AE of severe intensity	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Definitely related AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Probably related AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Possibly related AE	1	(12.5)	1	(16.7)	0	(0.0)	0	(0.0)	0	(0.0)	2	(6.3)
Unlikely related/unrelated AE	1	(12.5)	0	(0.0)	2	(33.3)	2	(33.3)	1	(16.7)	6	(18.7)
	Placebo (*n* = 6)		**10 mg/kg (*n* = 6)**		**20 mg/kg (*n* = 6)**		**30 mg/kg (*n* = 7)**		**Overall (*n* = 25)**	
b. Multiple dosage	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**
Any AE	3	(50.0)	3	(50.0)	4	(76.7)	5	(71.4)	15	(60.0)
Any SAE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Discontinued due to AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Concomitant medication given	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
AE of mild intensity	3	(50.0)	3	(50.0)	3	(50.0)	5	(71.4)	15	(60.0)
AE of moderate intensity	0	(0.0)	0	(0.0)	1	(16.7)	0	(0.0)	0	(0.0)
AE of severe intensity	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Definitely related AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Probably related AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Possibly related AE	2	(33.3)	2	(33.3)	0	(0.0)	0	(0.0)	4	(16.0)
Unlikely related/unrelated AE	1	(16.7)	1	(16.7)	4	(76.7)	5	(71.4)	11	(44.0)
	Placebo (*n* = 6)		**15 mg/kg/h (*n* = 6)**		**45 mg/kg/h (*n* = 6)**		**90 mg/kg/h (*n* = 6)**		**Overall (*n* = 24)**	
c. Continuous dosage	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**	***n***	**(%)**
Any AE	1	(16.7)	1	(16.7)	3	(50.0)	4	(66.7)	9	(37.5)
Any SAE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Discontinued due to AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Concomitant medication given	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
AE of mild intensity	1	(16.7)	1	(16.7)	3	(50.0)	4	(66.7)	9	(37.5)
AE of moderate intensity	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
AE of severe intensity	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Definitely related AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Probably related AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Possibly related AE	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Unlikely related/unrelated AE	1	(16.7)	1	(16.7)	3	(50.0)	5	(83.3)	10	(41.7)

3.4.2 | Safety and tolerability

Continuous infusion of EA-230 was well tolerated by all subjects in every dosage group, and did not result in any safety concerns and/or discontinuation of study drug administration. No SAEs were reported. Eight subjects (44%) treated with EA-230 and one placebo-treated subject (17%) reported one or more AEs (Table 3c). All AEs were mild and transient, and considered unlikely to be or not related to the study drug. Short-lasting dizziness was observed in two subjects in the highest dosage group (33%), and ceased after eating in one subject. Flu-like symptoms were reported by two subjects in the intermediate (17%) and highest dosage group (17%), both starting at least 24 hours

TABLE 4 Summary of adverse events by system organ class and preferred term

a. Single dosage	Placebo (n = 8)			1 mg/kg (n = 6)			3 mg/kg (n = 6)			10 mg/kg (n = 6)			30 mg/kg (n = 6)			Overall (n = 32)		
System organ class and preferred term	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***
Number of subjects with at least one AE	2	(25.0)	4	1	(16.7)	2	2	(33.3)	4	2	(33.3)	2	1	(16.7)	1	8	(25.0)	13
General disorders	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	1	(16.7)	1	1	(16.7)	1	3	(9.4)	3
Catheter site related reaction	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	1	(16.7)	1	2	(6.3)	2
Nasopharingitis	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	1	(3.1)	1
Nervous system disorders	1	(12.5)	1	1	(16.7)	2	2	(33.3)	3	0	(0.0)	0	0	(0.0)	0	4	(12.5)	6
Dizziness postural	0	(0.0)	0	1	(16.7)	1	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	2	(6.3)	2
Headache	1	(12.5)	1	1	(16.7)	1	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	3	(9.4)	3
Paresthesia	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	1	(3.1)	1
Gastrointestinal disorders	1	(12.5)	2	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(3.1)	2
Abdominal pain	1	(12.5)	1	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(3.1)	1
Flatulence	1	(12.5)	1	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(3.1)	1
Musculoskeletal disorders	1	(12.5)	1	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	2	(6.3)	2
Back pain	1	(33.3)	1	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	1	(3.1)	1
Chest pain	1	(12.5)	1	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(3.1)	1

b. Multiple dosage	Placebo (n = 6)			10 mg/kg (n = 6)			20 mg/kg (n = 6)			30 mg/kg (n = 7)			Overall (n = 25)		
System organ class and preferred term	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***
Number of subjects with at least one AE	3	(50.0)	5	3	(50.0)	6	4	(76.7)	6	5	(71.4)	6	15	(60.0)	23
General disorders	1	(16.7)	1	1	(16.7)	1	4	(76.7)	4	3	(42.9)	3	8	(32.0)	8
Catheter site related reaction	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	1	(14.3)	1	2	(8.0)	2
Injection site haemorrhage	1	(16.7)	1	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	2	(8.0)	2
Fatigue	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	1	(14.3)	1	2	(8.0)	2
Nasopharingitis	0	(0.0)	0	0	(0.0)	0	2	(33.3)	2	1	(14.3)	1	3	(12.0)	3
Nervous system disorders	2	(33.3)	2	2	(33.3)	2	1	(16.7)	1	0	(0.0)	0	5	(20.0)	5
Dizziness postural	0	(0.0)	0	2	(33.3)	2	0	(0.0)	0	0	(0.0)	0	2	(8.0)	2
Headache	2	(33.3)	2	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	3	(12.0)	3
Musculoskeletal disorders	2	(33.3)	2	0	(0.0)	0	0	(0.0)	0	2	(28.6)	2	4	(16.0)	4
Back pain	2	(33.3)	2	0	(0.0)	0	0	(0.0)	0	2	(28.6)	2	4	(16.0)	4
Gastrointestinal disorders	0	(0.0)	0	1	(16.7)	1	1	(16.7)	1	1	(14.3)	1	3	(12)	3
Abdominal pain	0	(0.0)	0	1	(16.7)	1	1	(16.7)	1	0	(0.0)	0	2	(8.0)	2
Constipation	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(14.3)	1	1	(4.0)	1
(Sub)cutaneous disorders	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	1	(4.0)	1
Eczema	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	1	(4.0)	1
Vascular disorders	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	1	(4.0)	1
Peripheral coldness	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	1	(4.0)	1

c. Continuous dosage	Placebo (n = 6)			15 mg/kg/h (n = 6)			45 mg/kg/h (n = 6)			90 mg/kg/h (n = 6)			Overall (n = 24)		
System organ class and preferred term	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***	***n***	**(%)**	***e***
Number of subjects with at least one AE	1	(16.7)	1	1	(16.7)	1	3	(33.3)	4	4	(66.7)	8	8	(33.3)	14
General disorders	0	(0.0)	0	0	(0.0)	0	1	(16.7)	2	3	(50.0)	3	4	(16.7)	5
Catheter site related reaction	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	2	(33.3)	2	2	(8.3)	2
Influenza like illness	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	1	(16.7)	1	2	(8.3)	2
Infusion site reaction	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	1	(4.2)	1

(Continues)

TABLE 4 (Continued)

c. Continuous dosage	Placebo (*n* = 6)			15 mg/kg/h (*n* = 6)			45 mg/kg/h (*n* = 6)			90 mg/kg/h (*n* = 6)			Overall (*n* = 24)		
System organ class and preferred term	*n*	(%)	*e*	*n*	(%)	*e*	*n*	(%)	*e*	*n*	(%)	*e*	*n*	(%)	*e*
Nervous system disorders	1	(16.7)	1	1	(16.7)	1	0	(0.0)	0	2	(33.3)	2	4	(16.7)	4
Dizziness	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	2	(33.3)	2	2	(8.3)	2
Head discomfort	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(4.2)	1
Somnolence	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	0	(0.0)	0	1	(4.2)	1
Gastrointestinal disorders	0	(0.0)	0	0	(0.0)	0	1	(16.7)	2	1	(16.7)	2	2	(8.3)	4
Nausea	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	1	(16.7)	1	2	(8.3)	2
Upper abdominal pain	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	1	(4.2)	1
Soft faeces	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	0	(0.0)	0	1	(4.2)	1
Eye disorders	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	1	(4.2)	1
Ocular discomfort	0	(0.0)	0	0	(0.0)	0	0	(0.0)	0	1	(16.7)	1	1	(4.2)	1

AE, adverse events; *e*, number of events; *n*, number of subjects.

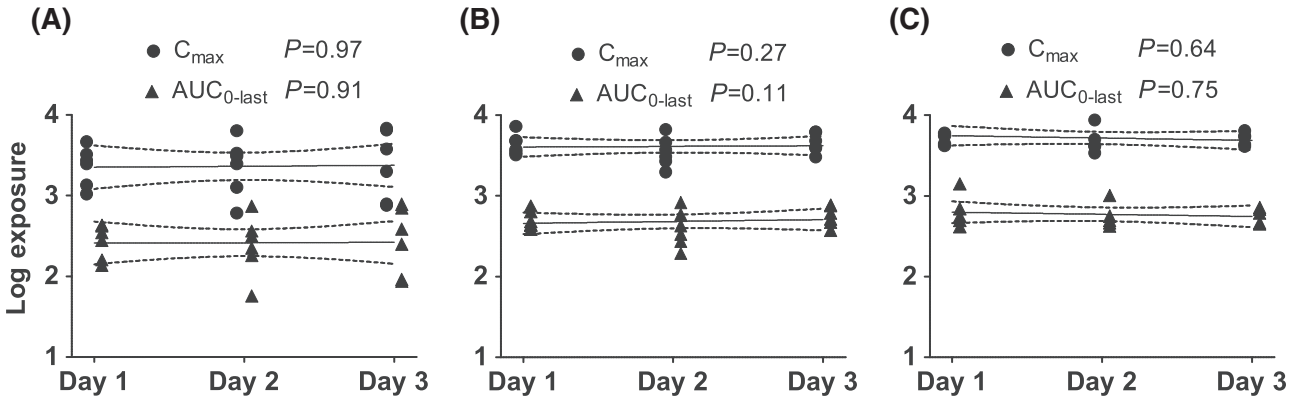

FIGURE 3 Dose accumulation of C_{max} and $AUC_{0\text{-last}}$ during the 3-day multiple dosage study. A, Dosage group 10 mg/kg (*n* = 6). B, Dosage group 20 mg/kg (*n* = 6). C, Dosage group 30 mg/kg (*n* = 5). Linear regression lines are shown, dotted lines indicate the 95% confidence interval. No accumulation was observed

after cessation of study drug administration and lasting for a maximum of 1 day. Gastrointestinal complaints (nausea and soft stool) were reported by two subjects in the intermediate (17%) and highest dosage group (17%), starting shortly after dinner >8 hours after cessation of study drug administration and disappeared the same evening. Other non-related AEs are summarized in Table 4c. All variations in laboratory parameters, vital signs and 12-lead ECG were considered not clinically significant.

4 | DISCUSSION

In this study, we describe the pharmacokinetics, safety and tolerability of EA-230 in healthy volunteers using different administration strategies and increasing dosages in three double-blind, randomized, placebo-controlled, phase I studies. Our data reveal that EA-230 has a large volume of distribution and a very rapid plasma clearance. A more than proportional increase in exposure with the highest dosages was observed and no accumulation occurred during the multiple dosage study. The drug was well tolerated and showed an excellent safety profile throughout the investigated dose range.

For the majority of subjects receiving dosages of 10 mg/kg or more in both the first and the second study, C_{max} was reached before the end of infusion, while in theory C_{max} is expected to be reached at the end of infusion. This observation implies a very rapid distribution of EA-230 and/or clearance rates that may have exceeded the rate of infusion. This assumption is further supported by the PK profile observed during continuous infusion, where EA-230 reached steady state concentrations already at the first sampling point 15 minutes after the start of administration, indicating rapid distribution and a rapid systemic clearance rate.

All three studies showed a large volume of distribution and a very rapid clearance, resulting in a short half-life, confirming previous animal data (see Supplemental data file) . However, with regard to the short half-life, we were only able to provide an estimate of these variables, representing a limitation of this study. In the bolus infusion studies, low concentrations approaching the limit of detection were observed already early after administration, therefore only limited time points were available and concentrations close to the limit of

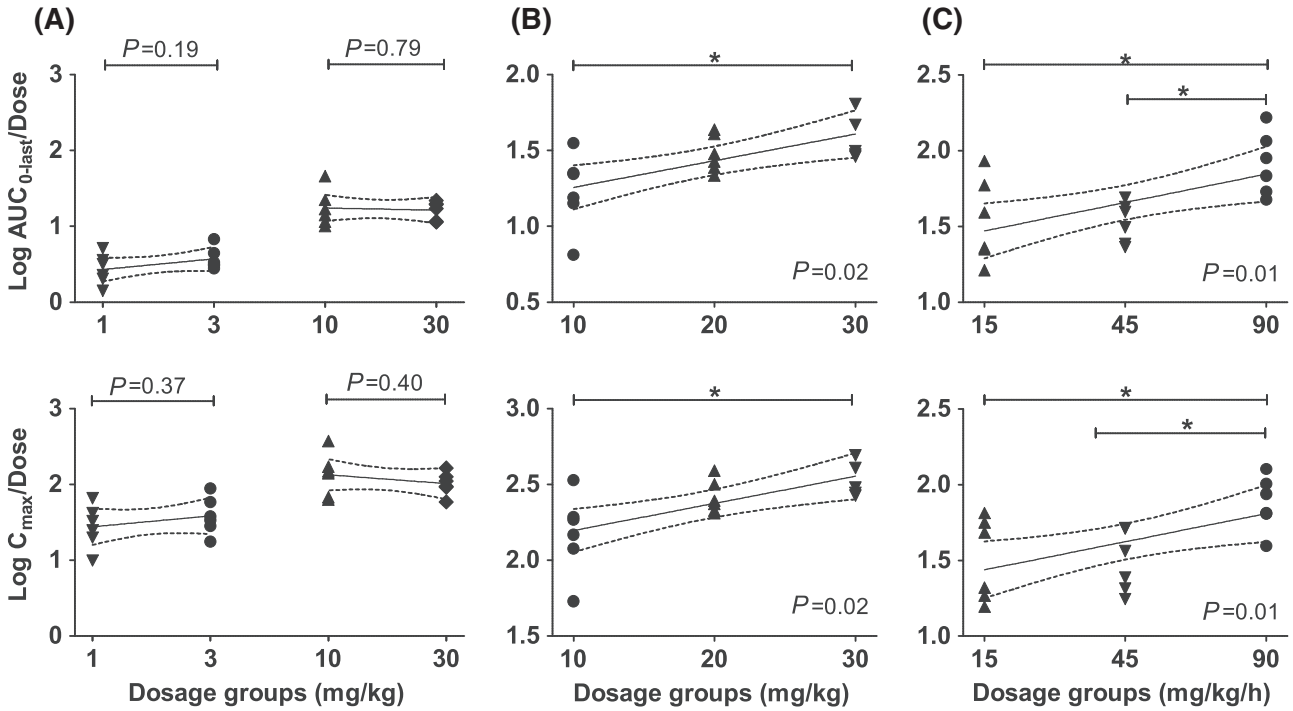

FIGURE 4 Dose proportionality of dose-normalized, log-transformed exposure parameters C_{max} and $AUC_{0\text{-last}}$. A, Single dosage study, dose proportionality could not be assessed across all dosages as the administration duration differed between the 1 and 3 mg/kg groups (2 minutes) and the 15 and 30 mg/kg groups (15 minutes). B, Multiple dosage study, as no dose accumulation was observed in this study (see Figure 3), the average values over the 3 days for each subject were used. C, Continuous dosage study. A *p*-value of <0.05 indicates non-proportionality. Linear regression lines are shown, dotted lines indicate the 95% confidence interval

detection may have been inaccurate. In the continuous infusion study, EA-230 concentrations were only measurable in a limited number of subjects and samples, also illustrative of the short half-life of the compound. As a result, we report the range related to the determinations of the elimination constant dependent PK parameters ($AUC_{0\text{-inf}}$, $t_{1/2}$, Vd, Cl). Nevertheless, consistent findings on $t_{1/2}$, Cl and Vd among all conducted studies with different administration strategies demonstrate a similar PK profile of EA-230 with a large distribution volume of at least 1 L/kg, a high clearance rate of at least 13 L/kg/h and a short half-life of less than 60 minutes. This PK profile indicates EA-230 to be very rapidly metabolized with plasma clearance exceeding both renal and portal flow, suggesting clearance of EA-230 through hydrolysis, proteolysis by systemic proteases and/or cellular uptake or tissue/protein binding. Of interest, Teftsin, a similar linear tetrapeptide with immunomodulatory properties that has been extensively investigated, is also characterized by fast degradation due to proteolysis in vivo with a half-life of 16 minutes.[31,32] Similar mechanisms of degradation might play a role in the fast elimination of EA-230; however, exact mechanisms of metabolism and drug clearance need to be further elucidated.

In the multiple and continuous dosage studies, a nonlinear dose–exposure relationship was observed with the highest dosage only, both for total exposure ($AUC_{0\text{-last}}$) and maximal exposure (C_{max}). This non-linear PK behaviour of EA-230 implies a saturation effect at a certain dosage. This could be caused by a dose-dependent shift in distribution, metabolism and/or elimination.

An excellent safety and tolerability profile were observed for EA-230 as no SAEs were reported, AEs were mild and transient, did not result in discontinuation of study drug administration and the vast majority of the AEs observed were deemed unlikely to be or not related to the study drug. Six AEs were considered possibly related to the study drug, but the evidence for the relationship between these AEs and study drug administration is unconvincing for several reasons. First, 50% of these AEs were observed in subjects treated with placebo. Second, the other AEs were all observed in the lowest dosage groups of each particular study. Noteworthy, there were no possible related AEs reported in the study where continuous infusion of EA-230 was employed, which resulted in the highest plasma concentrations and the longest exposure to the drug. Nevertheless, in this continuous infusion study, only one AE occurred in the placebo group compared with eight AEs in the treatment groups, with more AEs occurring in the higher dosage groups, indicating that a possible relation to the study drug cannot be entirely excluded. Upon review of the specific AEs, complaints of dizziness were reported in all studies. In the bolus studies, all possible related AEs in subjects with active treatment were complaints of (postural) dizziness. Also, in the continuous infusion study, although considered unlikely or not related, dizziness was observed in two subjects in the highest dosage groups. Although this pattern of complaints of dizziness might indicate a relation with administration of EA-230, similar complaints could well be explained by the long period of fasting by these subjects in all three studies. Furthermore, no changes in vital parameters between groups

were present in all studies (data not shown), nor did preclinical data indicate any effect of EA-230 on blood pressure or heart rate (unpublished data). Therefore, it appears unlikely that the reported dizziness would be related to haemodynamic effects. Taking into account all available data on safety and tolerability, no relevant safety issues for the i.v. administration of EA-230 to humans within the dosage range tested were observed. Although sample sizes were relatively small, and therefore side-effects that are rare might still go unnoticed, the proposed sample sizes were selected in line with generally used sample sizes for first-in-human phase I studies. The total of 80 volunteers in the three phase I studies with EA-230 would generally be regarded as sufficient. Based on these results, a phase IIa study in volunteers exposed to endotoxin has been conducted to investigate the immunomodulating properties of EA-230 (described elsewhere in this issue[33]) and a clinical trial in cardiac surgery patients has been initiated.[34]

In conclusion, these dose-escalating phase I studies with different administration strategies, describe a PK profile of EA-230 with a large volume of distribution and a short half-life, and demonstrate that i.v. administration is well tolerated without any safety issues emerging. These results enable further clinical development to assess safety, tolerability and immune modulating efficacy in humans during systemic inflammation.

ACKNOWLEDGEMENTS

The authors would like to thank G.P. Leijte, B. Koeneman and the research nurses for their assistance in the conduct of this study.

COMPETING INTERESTS

P.P. received consulting fees and travel reimbursements from EBI. All other authors have no competing interests to declare. This study was funded by Exponential Biotherapies Incorporation (EBI). The sponsor had no role in the study design, data collection, analysis or reporting.

CONTRIBUTORS

All authors participated in the conception, design, and/or coordination of study components. All authors have critically reviewed and approved the final manuscript for publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: van Groenendael R, Aarnoutse R, Kox M, van Eijk LT, Pickkers P. Pharmacokinetics, safety and tolerability of the novel β-hCG derived immunomodulatory compound, EA-230. *Br J Clin Pharmacol*. 2019;85:1572–1584. https://doi.org/10.1111/bcp.13942