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Pharmacokinetics and pharmacodynamics of PEGylated truncated human cystathionine beta-synthase for treatment of homocystinuria

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ABSTRACT

Aims: PEGylated human truncated cystathionine beta-synthase, lacking the C-terminal regulatory domain (PEG-CBS), is a promising preclinical candidate for enzyme replacement therapy in homocystinuria (HCU). It was designed to function as a metabolic sink to decrease the severely elevated plasma and tissue homocysteine concentrations. In this communication, we evaluated pharmacokinetics (PK), pharmacodynamics (PD) and sub-chronic toxicity of PEG-CBS in homocystinuric mice, wild type rats and monkeys to estimate the minimum human efficacious dose for clinical trials.

Main methods: Animal models received single or multiple doses of PEG-CBS. Activity of PEG-CBS and sulfur amino acid metabolites were determined in plasma and used to determine PK and PD.

Key findings: The plasma half-lives of PEG-CBS after a single subcutaneous (SC) injection were approximately 20, 44 and 73 h in mouse, rat and monkey, respectively. The SC administration of PEG-CBS resulted in a significant improvement or full correction of metabolic imbalance in both blood and tissues of homocystinuric mice. The PD of PEG-CBS in mouse was dose-dependent, but less than dose-proportional, with the maximal efficacy achieved at 8 mg/kg. PEG-CBS was well-tolerated in mice and monkeys, but resulted in dose-dependent minimal-to-moderate inflammation at the injection sites and vacuolated macrophages in rats. Allometric scaling of animal data was linear and the estimated human efficacious dose was determined as 0.66 mg/kg administered once a week.

Significance: These results provide critical preclinical data for the design of first-in-human PEG-CBS clinical trial.

1. Introduction

Classical homocystinuria (HCU; OMIM# 236200) is a metabolic disorder caused by an inherited deficiency of cystathionine beta-synthase (CBS; EC# 4.2.1.22). CBS condenses homocysteine with serine to cystathionine and its deficiency leads to accumulation of homocysteine and methionine and subsequent clinical symptoms [25]. The disease manifests in four major organ systems: the eye (severe myopia, dislocated lens), the skeletal and connective tissues (osteoporosis), the vasculature (thromboembolism, stroke) and the central nervous system (mental retardation, seizures) [25]. Symptoms vary in both severity and onset time, from severe multisystemic childhood onset to a nearly asymptomatic presentation in adulthood. The severity of HCU mostly depends on the amount of residual enzymatic activity driven by the type of mutation in the *cbs* gene. Severely affected patients require treatment with a methionine-restricted diet supplemented with cysteine and/or betaine to limit production of excess homocysteine. In some patients, very high doses of pyridoxine, a precursor of CBS cofactor, may increase the residual activity of some mutant CBS enzymes. Poor compliance with both the methionine-restricted diet and betaine is common when patients reach adolescence [24,33]. Although the methionine-restricted diet may avoid elevated homocysteine levels and therefore prevent negative clinical outcomes, substantial unmet need for alternative treatment remains for the majority of HCU patients.

Enzyme replacement therapy (ERT) represents an alternative treatment option to address the underlying enzymatic deficiency. Human CBS is a homotetrameric enzyme with a complex architecture and regulation involving three cofactors: heme of enigmatic function located at the N-terminus, catalytically active pyridoxal-5′-phosphate in the central core and allosteric activator S-adenosylmethionine (SAM) binding to the C-terminal regulatory domain of the CBS subunit [21]. In its native form, CBS is not suitable for ERT development due to its

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requirement for activation by SAM and its tendency to aggregate. Removal of the C-terminal domain yields a SAM-independent, highly active dimeric human truncated enzyme with high-yield recombinant expression and no aggregation, making it an excellent candidate for development as an ERT for HCU [4]. However, the plasma half-life of the injected truncated enzyme in a mouse model of HCU was 2.7 h and thus the truncated enzyme was conjugated with polyethylene glycol (i.e. PEGylated) to increase its half-life and enable long-term treatment [4]. PEGylation is a well-recognized technique to improve chemical/physical properties of therapeutic peptides and proteins and to enhance the bioactivity of the conjugates, namely to prolong half-life and to decrease immunogenicity of the biologically active component [26,31]. Indeed, PEGylation of the truncated human CBS with 2 kDa linear and 40 kDa 4-branch maleimide PEG targeting accessible sulfhydryl groups resulted in 6.2- and 11.3-times longer half-life of the conjugates compared to the unmodified enzyme after intravenous administration to HCU mouse [4]. In order to identify the most promising PEG-CBS as a lead candidate for clinical development, we rank-ordered several conjugates with sulfhydryl-targeting maleimide PEGs or amino group-targeting N-hydroxysuccinamide (NHS) ester-activated PEG in vivo, characterized the PEGylation sites and compared reproducibility of manufacturing [20]. The enzyme modified with 20 kDa linear NHS ester PEG (further referred to as PEG-CBS) showed excellent potency in vivo after repeated administration. This PEG-CBS conjugate was observed to have an average five PEG chains attached to each protein subunit with no reduction in enzyme's catalytic activity compared with the unmodified protein [20].

In this study, we determined pharmacokinetics (PK) of PEG-CBS in three animal models: homocystinuric mice, wild type (WT) Sprague-Dawley rats and Cynomolgus monkeys. The use of an HCU mouse model instead of WT mice permitted the assessment of pharmacodynamics (PD) effects and the PK-PD relationship for PEG-CBS. Allometric scaling of the PK parameters was conducted across species to estimate the human efficacious dose (HED) and the PK parameters in human clinical trials. The sub-chronic toxicity (clinical signs, gross macroscopic pathology, organ histology and hematology) was evaluated in rats and monkeys during and after 2 weeks of repeated dosing to estimate the human safety margin relative to the HED.

2. Materials and methods

2.1. Chemicals

Unless stated otherwise, all materials were purchased from Sigma or Fisher Scientific. L-[U-^{14}C]-serine was obtained from Perkin Elmer Life Sciences.

2.2. Test compounds

Human truncated cystathionine beta-synthase carrying the C15S mutation and PEGylated with linear 20 kDa NHS ester PEG ME-200GS (NOF Corp.) was prepared as described elsewhere [4,20]. The average CBS specific activity of individual PEG-CBS batches was 1239 ± 121 U/mg of protein. The average specific activity of the unmodified htCBS C15S used for preparation of PEG-CBS was 1233 ± 201 U/mg of protein.

2.3. Animals and study design

Procedures involving mice were performed at the University of Colorado Denver under IACUC-approved protocol# B-49414(03)1E. The University is an AAALAC-accredited (#00235), Public Health Service-assured (#A 3269-01) and USDA-licensed (#84-R-0059) institution. Human Only (HO) mice were generated in our lab [15], the CBS knockout (KO) mice [35] were purchased from the Jackson Laboratory and both strains were propagated and genotyped as described previously [4,16]. Animals were maintained on extruded standard diet 2920X (Envigo). A single-use lancet for submandibular bleeding was used for blood collection into Capiject T-MLHG lithium heparin (12.5 IU) tubes with gel (Terumo Medical Corporation). The tubes were then centrifuged at 1,200 × *g* for 10 min, followed by transfer of plasma to 1.5 ml tubes and storage at −80 °C. The design studies using HO mice is summarized in Supplementary Table S1. For a multi dose study, PEG-CBS was administered once every 24 h via SC injection on study days 1–10 (total of 10 injections). Since the untreated KO mice do not readily survive into adolescence, metabolites in liver, kidney and brain tissues were determined in untreated 18 days old KO mice (n = 3) and compared their levels from tissues of age-matched untreated WT mice (n = 3) and PEG-CBS-injected KO mice (n = 3). The treated KO mice received 7.5 mg/kg PEG-CBS since birth via SC injection thrice a week.

Three separate studies using wild-type Sprague Dawley rats were executed (Supplementary Table S2). Two single dose studies were performed at Charles River Laboratories (Edinburgh, UK). In the original study (study #1), technical difficulties occurred during sample collection and high inter-subject variability was observed as well. For these reasons, a supplemental study was conducted (study #2) using only male rats; however, bioavailability was inexplicably lower compared to study #1. In both studies, the IV data were superimposable suggesting no difference in test item activity and thus sex-related differences in adsorption were the most likely explanation. As the comparison between males and females could not be made because of technical difficulties in study #1, an additional study #3 was designed to verify the apparent differences in absorption in male versus female rats. Although the multi dose study #3 executed at MPI Research (Mattawan, MI, USA) was designed to gain insight into the safety and tolerability of PEG-CBS, the study design was amended to allow extraction of relevant data after the first injection to complement incomplete data sets from studies performed at Charles River Laboratories. The vehicle (PBS) or PEG-CBS were administered in a multi dose study #3 once every 48 h on study days 1, 3, 5, 7, 9, 11, 13, 15 and 17 (total of 9 injections) via bolus SC injection administered between the skin and underlying layers of tissue in the scapular region on the back of each animal. Both vendors were AAALAC-accredited and the protocols were approved by the local IACUCs. Rats were housed 2–3 per cage in a controlled environment (19–23 °C, humidity 40–70%, 12 h of light and dark). Certified Rodent Diet #5002 (PMI Nutrition International, Shoreview, MN, USA) and tap water from public supply were available ad libitum.

Single and repeated dose studies on Cynomolgus monkeys (*Macaca fascicularis*) were performed at Charles River Laboratories (Edinburgh, UK) (Supplementary Table S3), which is an AAALAC-accredited, GLP-compliant facility certified for work with non-human primates. The protocols were reviewed and approved by the local IACUCs. Monkeys were socially housed in groups of up to 6 by sex in two-story gang pens (18–24 °C, humidity 40–70%, 12 h of light and dark). A 200 g ration of Certified Primate Diet #5S48 (PMI Nutrition International, Shoreview, MN, USA) was provided one daily to each monkey, while tap water from public supply was available ad libitum. PEG-CBS was administered in a single dose study #1 at time 0 via bolus IV or SC injection, while in a multi dose study #2 once every 72 h on study days 1, 4, 7, 10, 13 and 16 (total of 6 injections) via bolus SC injection into a pre-shaved site on the thoracic region of the back of the animal (two injection sites – right and left thoracic – were used alternatively in a multi dose study #2). Blood was collected at designated times from the femoral vein into lithium heparin tubes.

2.4. CBS activity assays

The CBS activity was determined by a previously described radioisotope assay using L-[^{14}C]-serine as the labeled substrate [4,11].

2.5. Determination of metabolite concentrations

Plasma metabolites were determined by stable-isotope-dilution gas chromatography mass spectrometry as previously described [2]. Metabolites in tissues, which homogenized in the presence of 5 mM DTT and deproteinized with 4 volumes of 0.1 M perchloric acid, were determined using stable-isotope-dilution liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) ([3,14].

2.6. PK calculations

The PK parameters were calculated using a non-compartmental curve stripping model, which resolved the curve into a series of exponential terms corresponding to the absorption, distribution and elimination phases in blood. The curve stripping approach assumes that the disposition phases of the drug follow apparent first-order kinetics, which is evidenced by the linearity in the terminal portion of a semi-log plot. The PK calculations were conducted using PK Solutions 2.0 (Summit Solutions, Montrose, CO, USA). The bioavailability was calculated by dividing the area under the curve per dose (AUC_{0-t}/dose) for the SC route by the AUC_{0-t}/dose for the IV route.

2.7. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical comparisons of 2 groups were conducted using an unpaired, two tailed Students *t*-test. Statistical analysis of 3 or more factor levels was conducted by ANOVA followed by Tukey's multiple comparison test to determine significance. For all the tests, a value of $p < 0.05$ was considered significant. Significance in figures is shown by letters at the top of the error bars with no letter indicating no significance.

3. Results

3.1. PK of PEG-CBS in HO mice

We showed in our previous work that the unmodified htCBS was rapidly cleared from plasma after IV injection to C57BL/6 mice with an elimination half-life of 2.7 h, while its PEGylation resulted in 6–11-fold increased residency of the CBS activity in plasma [4]. Here we determined PK of our clinical candidate PEG-CBS in the HO mouse model of HCU (Fig. 1, Table 1). Fig. 1A shows the levels of CBS activity in plasma over time following a bolus IV or SC injection of 10 mg/kg PEG-CBS. The calculated PK parameters are summarized in Table 1. Elimination half-life of PEG-CBS after IV dosing was about 2 days, which decreased to 17.5 h after SC dosing with absorption half-life of 12.7 h and bioavailability of 64.6%. The elimination phase of the semi-log plots was linear for either route of administration demonstrating that the clearance of PEG-CBS from plasma conformed to first order kinetics (Supplementary Fig. S1). In addition, we injected HO mice subcutaneously with 0.5× and 1.5× of 10 mg/kg PEG-CBS to determine the dose response (Fig. 1B, Table 1). Although the peak plasma levels of CBS activity increased with an increasing dose, the differences were not dose proportional. This could likely be explained by a decreased absorption of PEG-CBS from SC compartment with an increasing dose as suggested by decreased bioavailability, thus leading to a lower exposure of PEG-CBS assessed by AUC_{0-t}/dose.

3.2. PK/PD relationship of PEG-CBS in HO mice

The use of homocystinuric HO mice for PK studies allowed us to determine the PK/PD relationship and correlate plasma CBS activity with the levels of sulfur amino acids (i.e. PD biomarkers), primarily total homocysteine (Hcy), cystathionine (Cth) and total cysteine (Cys).

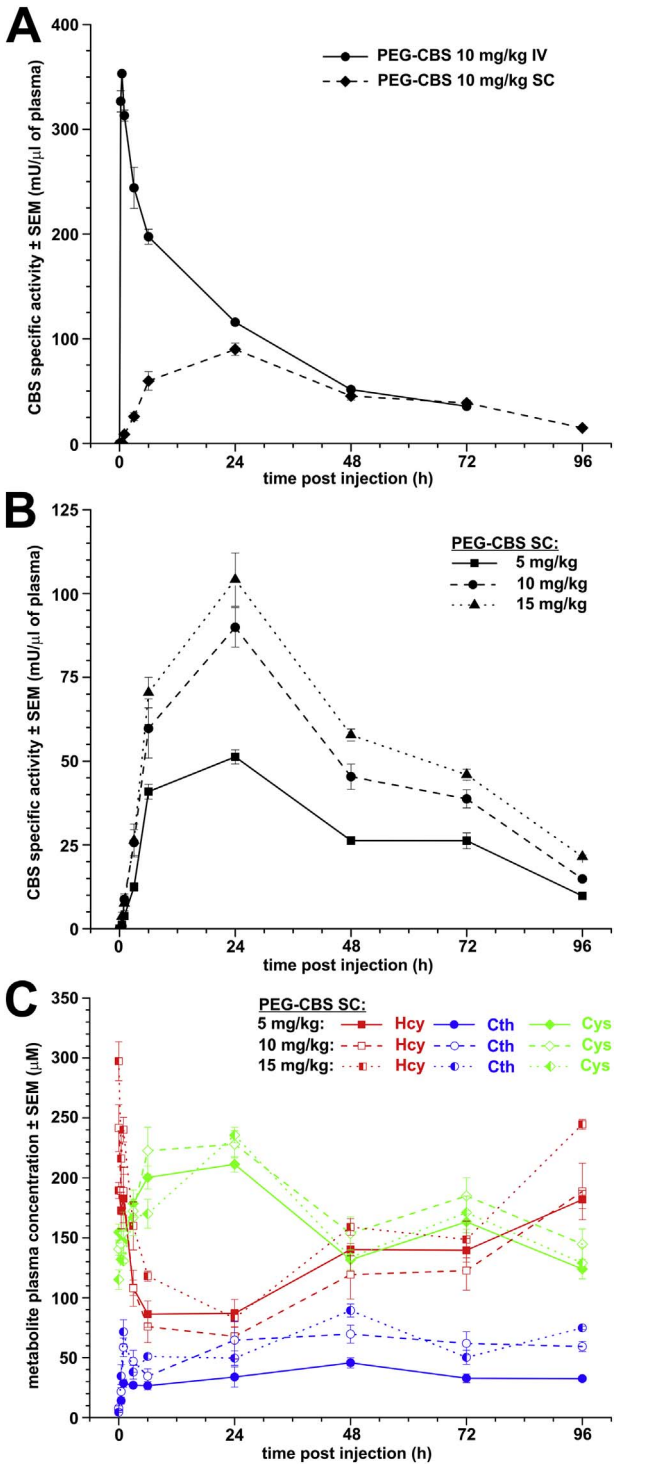

Fig. 1. Pharmacokinetics and PK/PD relationship after a single dose administration of PEG-CBS in HO mice.
A – CBS activity in plasma of HO mice (n = 6 each group) dosed with 10 mg/kg PEG-CBS via IV (solid line with circles) or SC administration (dashed line with diamonds). **B** – CBS activity in plasma of HO mice (n = 6 each group) dosed with 5 (solid line with squares), 10 (dashed line with circles) or 15 mg/kg (dotted line with triangles) of PEG-CBS via SC route. **C** – plasma levels of homocysteine (red), cystathionine (blue) and cysteine (green) following a single SC administration of 5 (solid line with closed symbols), 10 (dashed line with open symbols) and 15 mg/kg PEG-CBS (dotted line with half-filled symbols). Metabolites in panel C were determined in the same plasma samples used for CBS activities shown in panel B. The data points are average values and the error bars represent SEMs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Pharmacokinetic parameters of PEG-CBS in HO mice.

PK parameter	Unit	SC	SC	SC	IV
Dose	mg/kg	5	10	15	10
AUC_{0-t} (obs area)	mU-h/μl	3062 ± 158	4788 ± 626	5754 ± 160	7431 ± 154
AUC_{0-t}/dose	mU-h/μl/(mg/kg)	612	479	384	743.1
Bioavailability	%	82.6	64.6	51.6	100
$t_{1/2-A}$	h	5.3 ± 1.1	12.7 ± 0.6	11.9 ± 1.2	N/A
$t_{1/2-E}$	h	137 ± 95[a]	17.5 ± 1.27	22.0 ± 1.7	47.6 ± 14.6
t_{max} (obs)	h	24	24	24	N/A
c_{max}	mU/μl	51.3 ± 3.7	89.9 ± 10.2	104.2 ± 11.3	N/A
c_{p0}	mU/μl	N/A	N/A	N/A	371.6 ± 11.2
c_{max}/dose	mU/μl/(mg/kg)	10.3	9.0	6.9	N/A
MRT (area)	h	194 ± 128	44.8 ± 1.7	49.0 ± 1.4	52 ± 13
CL (obs area)	μl/h/kg	N/A	N/A	N/A	1346 ± 27.3

[a] Poor estimate of half-life (too few points in elimination phase).

The PD response to PEG-CBS administration was determined for 3 different SC doses. Fig. 1C shows that the changes in plasma levels of Hcy, Cth and Cys over time were close to maximal at 5 mg/kg of PEG-CBS such that higher doses of 10 or 15 mg/kg resulted in only incremental changes in peak efficacy. Thus, a single SC dose between 5 and 10 mg/kg produced the maximal efficacy in HO mice.

On the other hand, no significant differences were observed for different doses on plasma levels of Cth and Cys, which were in all cases markedly elevated and normalized, respectively (Supplementary Fig. S2). This result suggests that no further benefit on plasma metabolite balance can be achieved with a dose over 8 mg/kg in HO mice fed with a standard rodent chow.

3.3. Repeated administration of PEG-CBS to HO mice

Repeated SC administration of PEG-CBS to HO mice resulted in a dose-proportional and predictable PK up to a dose of 24 mg/kg. Fig. 2A shows plasma CBS activities over time in HO mice receiving ten SC injections every 24 h of 4, 8, 12 and 24 mg/kg PEG-CBS. Steady state was reached after 5–7 doses in all dose groups. The minimal and maximal plasma concentrations of PEG-CBS at steady state (i.e. prior to the 8th injection and 12 h after the 9th injection), corrected for the dose, ranged between 27–31 and 29–36 mU/μl, respectively, for all dose groups. We also determined plasma levels of PD biomarkers to assess PD response to a different repeatedly administered dose of PEG-CBS. Fig. 2B shows significant decrease in Hcy levels 24 h after the 1st injection for all doses ($p < 0.01$). The efficacy of PEG-CBS to decrease Hcy levels at the lowest dose (4 mg/kg) was significantly lower compared to 8 ($p = 0.019$), 12 ($p = 0.003$) and 24 mg/kg dose ($p < 0.001$), while the three higher doses achieved similar efficacy on Hcy. Interestingly, plasma Hcy levels were not entirely normalized even after ten injections of 24 mg/kg PEG-CBS with the lowest achieved value of 25 μM Hcy (compared to 2–7 μM in WT mice; data not shown).

3.4. PK of PEG-CBS in rats

PK of PEG-CBS was also determined in wild-type Sprague Dawley rats (Fig. 3, Table 2). As shown in Fig. 3A, the levels of plasma CBS activity over time after IV administration of 4 mg/kg PEG-CBS to male and female rats are nearly superimposable. Elimination half-life of PEG-CBS after IV dosing was between 35 and 40 h and the plasma exposure as estimated by area under the curve observed from 0 to 120 h (AUC_{0-t}) was not substantially different between sexes. On the other hand, Fig. 3B shows significant differences between males and females after SC injection. The fraction of PEG-CBS absorbed after SC dosing was nearly twice as much in female compared with male rats ($p < 0.05$). Specifically, the bioavailability in male rats after doses of 8 mg/kg and 24 mg/kg was 18.7% and 21.3%, respectively. In contrast, the bioavailability in female rats after doses of 8 and 24 mg/kg was 36.5% and 35.5%, respectively. As bioavailability of PEG-CBS was nearly identical at the different doses (8 and 24 mg/kg) for each gender, it suggests that the observed differences in absorption in rats were likely due to sexual dimorphism. The elimination phase of PEG-CBS was log-linear in all cohorts (Supplementary Fig. S3) and thus conformed to first

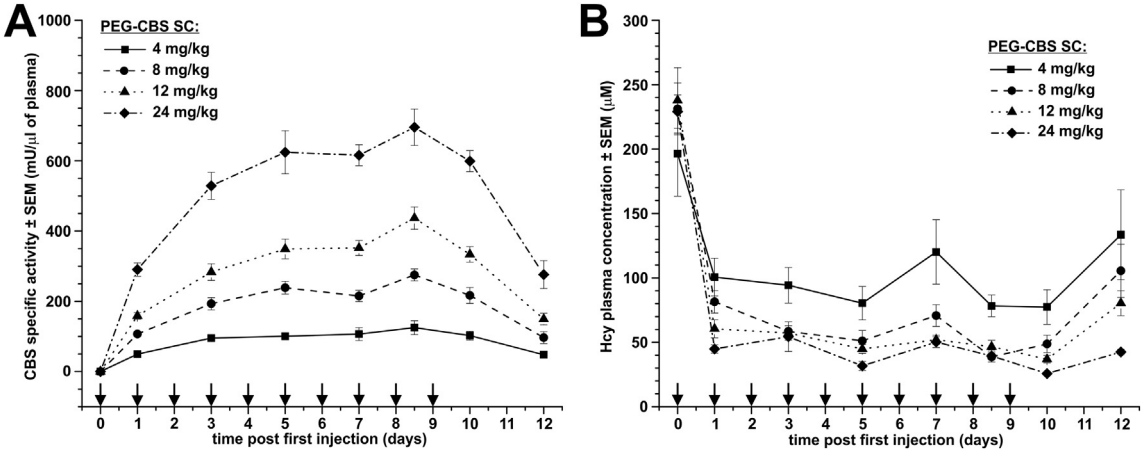

Fig. 2. PK and PD of a repeated administration of PEG-CBS in HO mice.
Plasma CBS activities (**A**) and total homocysteine levels (**B**) in HO mice (n = 3 M + 3F in each group) after repeated SC injections of 4 (solid line with squares), 8 (dashed line with circles), 12 (dotted line with triangles) and 24 mg/kg PEG-CBS (dash dotted line with diamonds). Arrows designate the dosing time points for a total of 10 injections administered once a day. The data points in all plots represent average values and the error bars show SEMs.

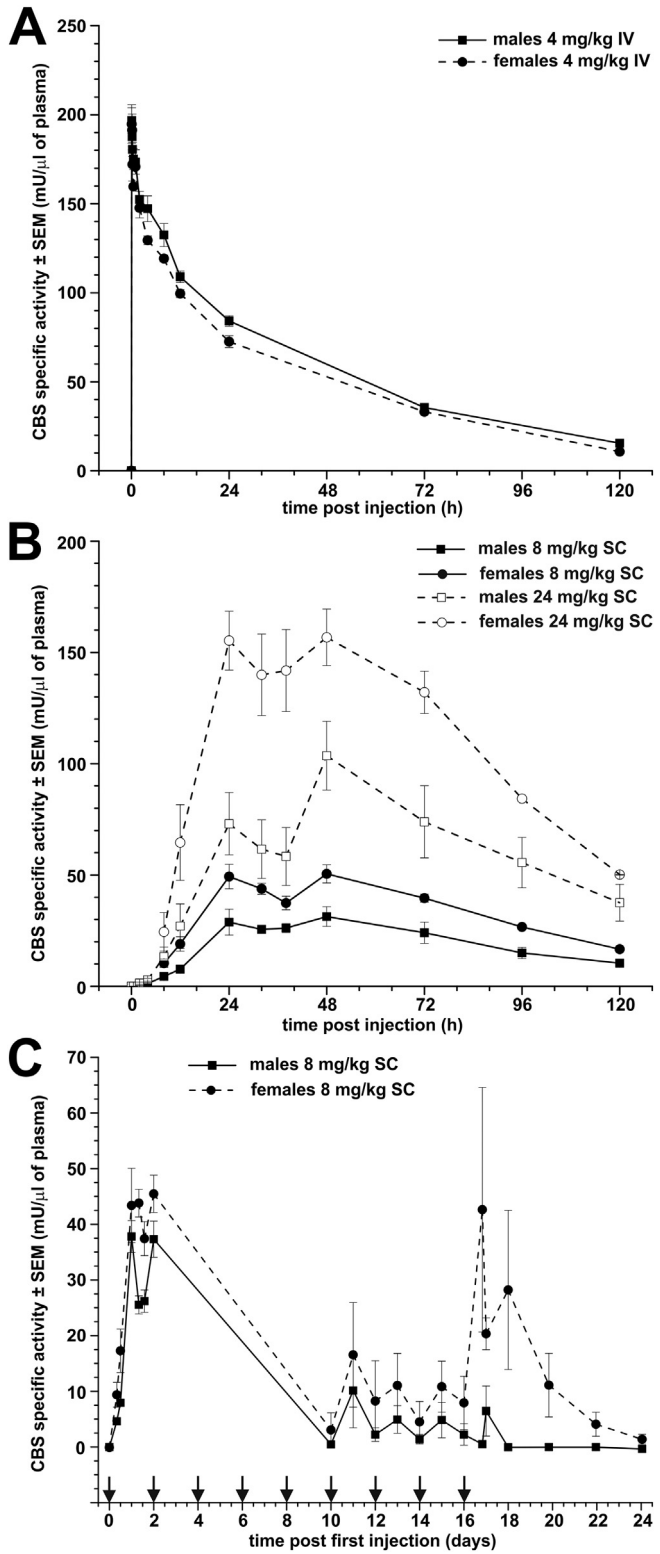

Fig. 3. Pharmacokinetics of PEG-CBS in Sprague Dawley rats.
A – Plasma CBS activities in male (n = 5; solid line with squares) and female rats (n = 2; dashed line with circles) after a single IV injection of 4 mg/kg PEG-CBS. **B** – Plasma CBS activities in male (n = 11 each group; squares) and female rats (n = 8 each group, circles) dosed with 8 (solid lines with closed symbols) or 24 mg/kg PEG-CBS (dashed lines with open symbols) via SC route. **C** – Plasma CBS activities in male (n = 8; solid line with squares) and female rats (n = 8; dashed line with circles) after repeated SC injections of 8 mg/kg PEG-CBS. Arrows designate the dosing time points for a total of 9 doses administered every 2 days. The data points in all plots represent average values and the error bars show SEMs.

order kinetics.

Differences due to sexual dimorphism in rats were also apparent in a repeated dosing study, where the rats received a total of 9 injections 48 h apart of 4, 8 and 24 mg/kg PEG-CBS (Fig. 3C, Supplementary Fig. S4). In general, initial as well as steady state levels of PEG-CBS activity in plasma were higher in female than male rats at the same dose level correlating well with a similar observation in a single dose study. More importantly, plasma levels of PEG-CBS after repeated dosing demonstrated little or no accumulation at any dose level, even though the doses were administered when slightly more than a half of the PEG-CBS has cleared from the plasma (48 h dosing interval versus average elimination half-life after SC dosing of 44 h). To aid in the understanding of these unexpected observations, the predicted steady state CBS plasma levels were calculated after hypothetical repeated dosing using the simulation function in the PK software. After multiple doses, the observed plasma PEG-CBS activity was 15–52% and 0–35% of the predicted values for the peaks and troughs at steady state, respectively (Supplementary Table S4).

3.5. PK and PK/PD relationship of PEG-CBS in monkeys

Lastly, PK of PEG-CBS was determined in wild-type Cynomolgus macaques. Fig. 4A shows plasma CBS activities over time in monkeys injected with a single dose of 2 or 6 mg/kg PEG-CBS via IV or SC, while Table 3 summarizes the calculated PK parameters. Elimination phase of the semi-log plots was linear for both the IV and the SC dose-groups demonstrating that the clearance of enzyme activity from plasma conformed to first order kinetics (Supplementary Fig. S5). No significant differences between males and females were observed. The elimination half-life of PEG-CBS after the IV dose was 66.7 h, while it increased slightly to ~73 h after the SC dose. The bioavailability of PEG-CBS after 2 and 6 mg/kg SC dose was essentially identical (80.8% versus 79.9%).

We hypothesized that the PD response after a single dose to wild type monkeys might not be observed due to very low baseline plasma Hcy levels compared to e.g. HO mice (4.1 ± 0.2 versus 217 ± 9 μM). Indeed, no significant differences were observed for Hcy and Cys after a single injection of PEG-CBS (data not shown). However, significant increases in plasma levels of Cth 8 h after the 2 mg/kg IV ($p = 0.019$) and 8 and 48 h after the 2 mg/kg SC dosing ($p = 0.010$ and 0.002, respectively) compared to pre-dose levels, but not for the 6 mg/kg SC dose group, were clearly observed (Fig. 4B). However, the lack of a vehicle-treated control group in combination with diurnal variation made further interpretation of this result difficult.

In stark contrast to rats, repeated SC administration of PEG-CBS to monkeys resulted in a dose-proportional and predictable PK up to a dose of 10 mg/kg. Fig. 4C shows plasma CBS activities over time in monkeys receiving six consecutive SC injections every 3 days of 1, 3 and 10 mg/kg PEG-CBS. Steady state was reached after 3–4 doses in all dose groups. The minimal and maximal plasma concentration of PEG-CBS at steady state corrected for dose ranged between 23–28 and 36–39 mU/μl, respectively, for all dose groups. Interestingly, the peak and trough plasma levels of PEG-CBS were accurately predicted from the single dose PK study using modeling to range between 30 and 40 mU/μl at steady state.

After observing significant spikes in Cth plasma levels after a single dose, we determined plasma levels of selected sulfur amino acid metabolites in the same plasma samples from the multiple injection study used for assessment of CBS activity. Similar to a single dose PK/PD relationship, no clear differences between dose groups were found for plasma Hcy and Cys after repeated administration (data not shown). However, a clear dose-proportional response was observed for plasma Cth levels (Fig. 4D). The plasma levels of PEG-CBS activity fluctuated and correlated with peaks and troughs of Cth levels (Fig. 4C).

Table 2
Pharmacokinetic parameters of PEG-CBS in Sprague Dawley rats.

PK Parameter	Unit	SC males	SC females	SC males	SC females	IV males	IV females
Dose	mg/kg	8	8	24	24	4	4
AUC_{0-t} (obs area)	mU-h/μl	2703	4505	9269	13,135	7231	6165
AUC_{0-t}/dose	mU-h/μl/(mg/kg)	338	563	386	547	1808	1541
Bioavailability	%	18.7	36.5	21.3	35.5	100	100
$t_{1/2-A}$	h	14.6	8.9	13.6	13.0	N/A	N/A
$t_{1/2-E}$	h	44.2	40.1	51.2	41.1	40.0	34.8
t_{max} (obs)	h	48	24	38	48	N/A	N/A
c_{max}	mU/μl	32.7	58.2	110.0	166.3	N/A	N/A
c_{p0}	mU/μl	N/A	N/A	N/A	N/A	182.1	186.2
c_{max}/dose	mU/μl/(mg/kg)	4.1	7.3	4.6	6.9	N/A	N/A
MRT (area)	h	65.6	76.8	66.1	79.8	52.9	45.5
CL (obs area)	μl/h/kg	N/A	N/A	N/A	N/A	553	649

3.6. PEG-CBS toxicity

Repeated administration of PEG-CBS allowed us to ascertain the potential sub-chronic toxicity in wild-type rats and monkeys. Assessment of toxicity was based on mortality, clinical observations, body weight, food consumption and clinical and anatomic pathology. There were no PEG-CBS-related effects noted for mortality, clinical observations, body weight, organ weight or macroscopic evaluations in either male or female rats at any dose level. Female rats injected with PEG-CBS decreased their food consumption 9–13% at termination compared to PBS-injected controls. However, changes lacked dose-dependency and did not result in changes in body weights, thus were not considered as adverse effects. Microscopic examination of injection sites showed dose-dependent minimal to moderate accumulation of vacuolated macrophages accompanied by minimal to moderate subacute/chronic inflammation in both rat sexes. These changes persisted during the two-week recovery period. More importantly, there were no treatment-related clinical observations noted during treatment or the recovery period in monkeys. Based on these findings, the no-observed-adverse-effect-level (NOAEL) established in rats and monkeys for PEG-

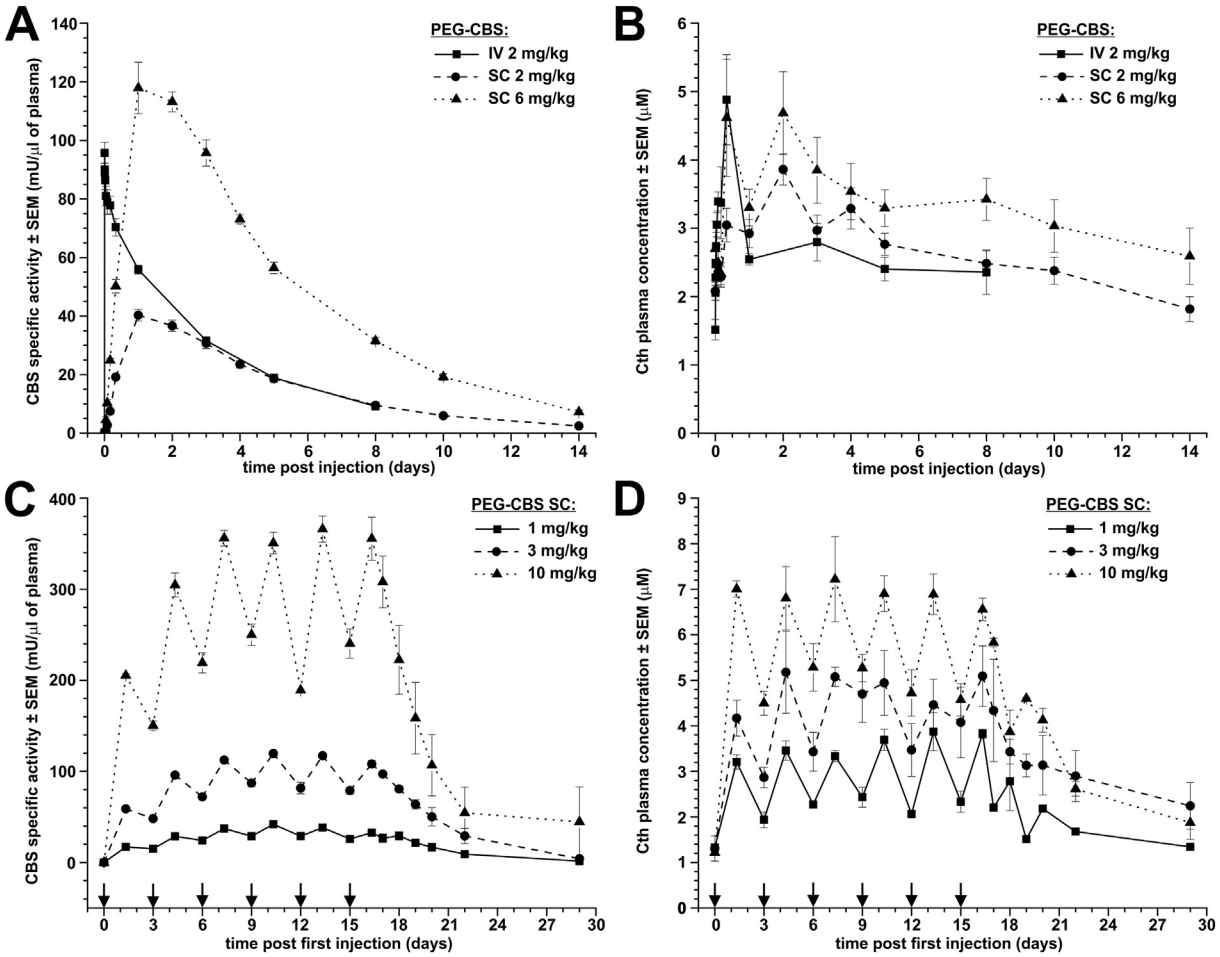

Fig. 4. Pharmacokinetics and PK/PD relationship of PEG-CBS after administration to Cynomolgus monkeys.
A, B – Plasma CBS activities (**A**) and Cth levels (**B**) in Cynomolgus monkeys (n = 2 M + 2F in each group) after a single IV injection of 2 mg/kg PEG-CBS (solid line with squares) or a single SC administration of 2 and 6 mg/kg PEG-CBS (dashed line with circles and dotted line with triangles, respectively). **C, D** – Plasma CBS activities (**C**) and Cth levels (**D**) in Cynomolgus monkeys (n = 2 M + 2F in each group) after repeated SC injections of 1 (solid line with squares), 3 (dashed line with circles) and 10 mg/kg PEG-CBS (dotted line with triangles). Arrows designate dosing time points for a total of 6 doses administered every 3 days. Data points in all plots represent average values and error bars show SEMs.

Table 3
Pharmacokinetic parameters of PEG-CBS in Cynomolgus monkeys.

PK parameter	Unit	SC	SC	IV
Dose	mg/kg	2	6	2
AUC_{0-t} (obs area)	mU-h/μl	5263 ± 299	15,743 ± 629	5942 ± 156
AUC_{0-t}/dose	mU-h/μl/(mg/kg)	2761 ± 160	2727 ± 119	2971 ± 78
Bioavailability	%	80.8 ± 4.7	79.9 ± 3.5	100
$t_{1/2-A}$	h	7.9 ± 1.0	9.7 ± 1.0	N/A
$t_{1/2-E}$	h	72.9 ± 1.1	72.8 ± 1.7	66.7 ± 1.2
t_{max} (obs)	h	26.2 ± 2.6	32.4 ± 2.4	N/A
c_{max}	mU/μl	40.8 ± 2.0	114.9 ± 7.5	N/A
c_{p0}	mU/μl	N/A	N/A	96.5 ± 2.8
c_{max}/dose	mU/μl/(mg/kg)	20.4 ± 1.0	19.2 ± 1.3	N/A
MRT (area)	h	116.3 ± 2.4	116.2 ± 5.0	89.9 ± 2.4
CL (obs area)	μl/h/kg	N/A	N/A	933 ± 53

The data represents mean ± SEM.

CBS was 24 and 10 mg/kg, respectively.

3.7. Allometric scaling and estimation of HED

Interspecies comparison was conducted based on body weight scaling in order to estimate PK in humans and HED. Bioavailability of PEG-CBS after the SC administration was ~80% in mice and monkeys, but only 19–37% in rats. The allometric scaling assumed the corrected for an 80% bioavailability in rats for the estimates of c_{max} and AUC in humans. When the selected PK parameters, such as $t_{1/2-E}$, c_{max}/dose and AUC_{0-t}/dose, were plotted against the body weight on a log-log scale, a linear correlation was observed suggesting that these parameters are likely predictive of human values (Fig. 5, Supplementary Table S5). Thus, in a human patient weighing 70 kg, the PEG-CBS elimination half-life $t_{1/2-E}$, c_{max}/dose and AUC_{0-t}/dose were predicted to be 176.4 h, 33.2 mU/μl/(mg/kg) and 7236 mU-h/μl/(mg/kg), respectively. PEG-CBS was absorbed slowly into the bloodstream after a single SC administration in all 3 species with adsorption half-lives similar across species and ranging between 5.3 and 12.7 h in HO mice, 8.9–14.6 h in rats and 7.9–9.7 h in monkeys (Tables 1–3). These values lead to t_{max} values that ranged from 24 to 48 h across 3 species allowing to reasonably assume a range between 48 and 72 h in humans. These predicted PK parameters in human allowed for construction of a hypothetical PK curve after a single SC administration in humans, which subsequently enabled modeling of a multiple dose PK curves assuming first order elimination kinetics in humans. Table 4 summarizes predicted peak and trough plasma PEG-CBS activities in humans after receiving SC doses of 0.33, 0.66 and 1 mg/kg administered once or twice a week assuming 80% bioavailability. From these calculations, an SC dose of 0.66 mg/kg PEG-CBS administered to a human patient once a week is predicted to give plasma levels that range between 30.8 and 45.2 mU/μl at steady state. Similarly, an SC dose of 0.33 mg/kg PEG-CBS administered twice a week is predicted to give plasma levels that range between 34.5 and 41 mU/μl at steady state. These doses and regimens are thus expected to produce peak and trough plasma levels at steady state that are in the efficacious range for PEG-CBS as determined in HO mice (peak level of 51.3 mU/μl after a single 5 mg/kg SC dose, Table 2). Taken together, a calculated PEG-CBS dose of 0.33 mg/kg administered 2× weekly or 0.66 mg/kg administered 1× weekly will likely be effective doses in human clinical trials.

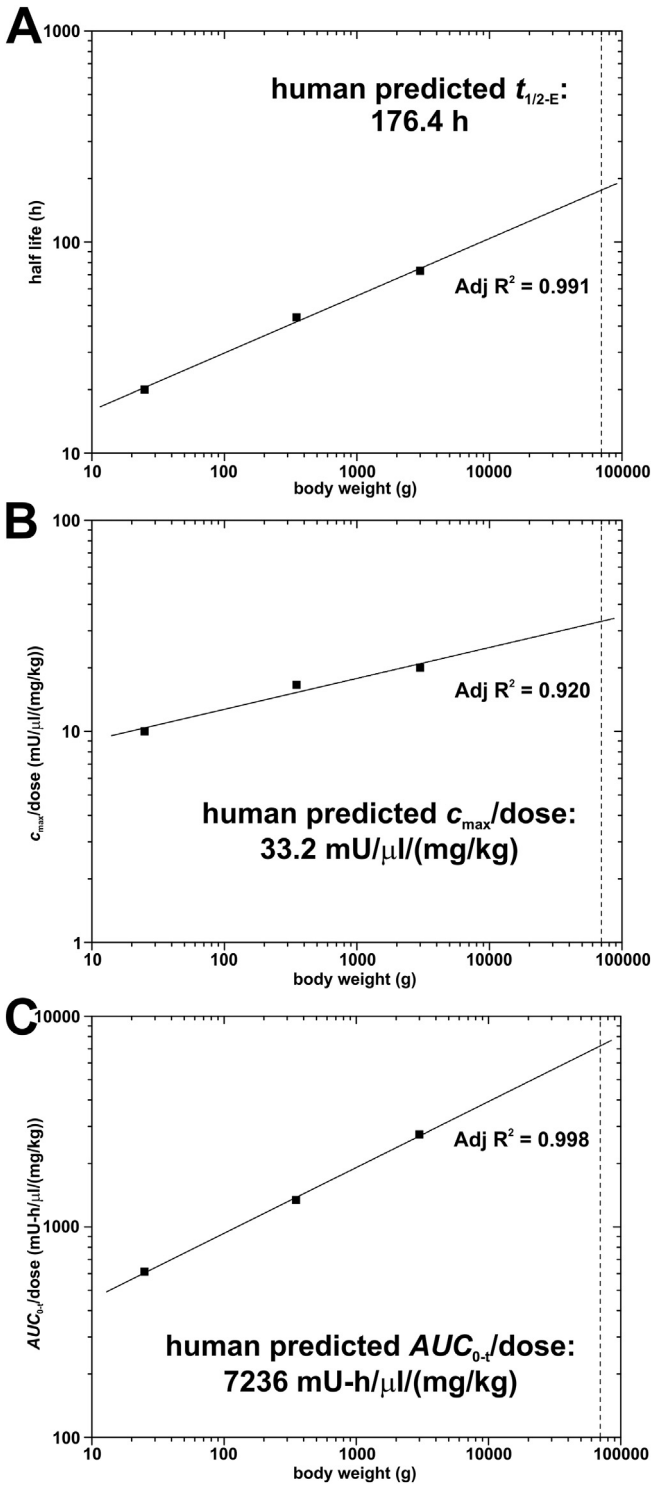

Fig. 5. Allometric scaling of PK parameters determined in animal models to humans. The extrapolated elimination half-life (**A**), c_{max}/dose (**B**) and AUC_{0-t}/dose (**C**) of PEG-CBS predicted in humans after a single SC injection. Plot axes are in logarithmic scale. Dotted line in all panels represents an average 70 kg human patient. Solid line in each of the panel designate a linear fit used for extrapolation of PK parameters in humans with noted adjusted R^2 values to illustrate the quality of the fitting. The 80% bioavailability of PEG-CBS after a single SC dose was assumed in all the species.

3.8. Effect of PEG-CBS on tissue metabolite levels

The calculated volume of distribution at steady state in monkey after a single IV dose of 2 mg/kg PEG-CBS was 73 ± 4 ml, which is roughly equivalent to plasma volume in a 3 kg Cynomolgus monkey (109 ml) [1] suggesting that PEG-CBS resides in circulation with low tissue penetration. However, the repeated administration of PEG-CBS improved or entirely normalized metabolic balance in tissues of the most severely affected HCU mouse model, the KO mice (Fig. 6, Supplementary Table S6). Hcy was greatly accumulated in tissues of the KO mice compared to the WT controls (75/4/23-fold in liver/kidney/

Table 4
Predicted initial, maximal and minimal PEG-CBS plasma levels at steady state in humans after SC administration.

Huma dose	1 mg/kg		0.66 mg/kg		0.33 mg/kg	
Dosing interval	1×/ week (168 h)	2×/ week (84 h)	1×/ week (168 h)	2×/ week (84 h)	1×/ week (168 h)	2×/ week (84 h)
Initial c_{max} (mU/μl)	34	34	22.6	22.6	11.3	11.3
$c_{max\text{-}SS}$ (mU/μl)	67.9	123	45.2	82	22.6	41
$c_{min\text{-}SS}$ (mU/μl)	46.2	109	30.8	69	15.4	34.5

brain), but the treatment resulted in its significant reduction in liver ($p < 0.01$) and normalization in kidney and brain (Fig. 6A). Interestingly, Cys levels were not markedly different among WT controls, untreated KO mice and PEG-CBS-injected KO mice (Fig. 6B). Tissue Cth was 8–18-fold diminished in the KO mice compared to the WT controls (Fig. 6C). The administration of PEG-CBS resulted in its normalization in liver, a 60-fold accumulation in kidney compared to WT levels ($p < 0.01$) and a 4-fold increase in brain compared to the untreated KO levels ($p < 0.01$). Similarly to Hcy, Met was markedly elevated in KO mice tissues compared to the WT mice, but the ERT essentially normalized its levels in all tissues (Fig. 6D). Interestingly, highly elevated Met levels in tissues of KO mice resulted in a 13-fold accumulation of

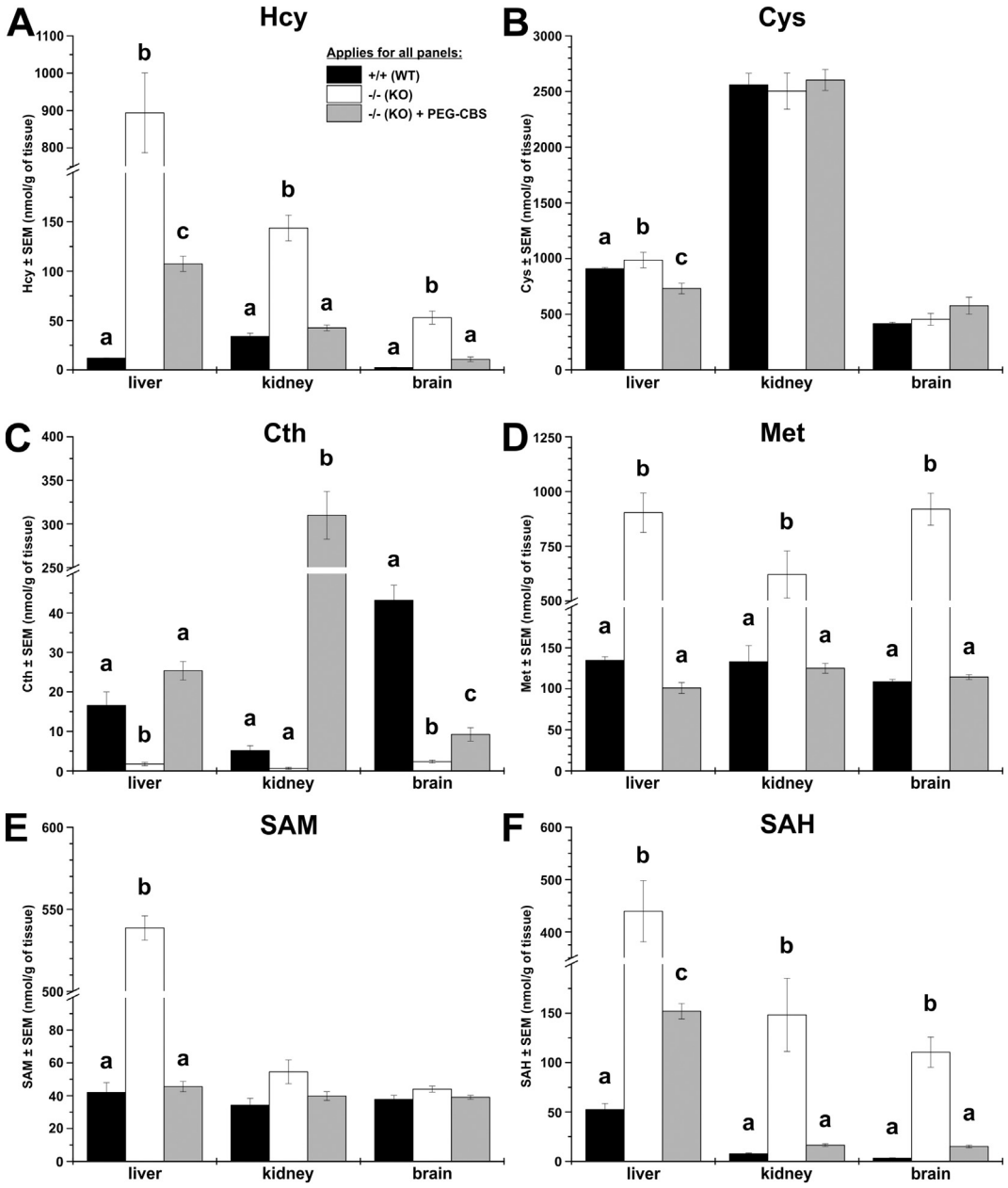

Fig. 6. Administration of PEG-CBS to KO mice since birth improves or normalizes metabolic balance in tissues.
Panels **A-F** show tissue levels of total homocysteine (Hcy), total cysteine (Cys), cystathionine (Cth), methionine (Met), S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). Newborn KO mice (n = 3, grey bars) were treated since birth to 18 days of age with PEG-CBS (3× a week, SC, 7.5 mg/kg) and compared to age-matched untreated positive (n = 3, white bars) and negative controls (n = 3, black bars). Data represent average values and error bars show SEMs. Statistical significance is indicated by the letter above respective bars with no letter indicating non-significance.

SAM only in their livers compared to WT mice, while SAM levels in kidney and brain remained unchanged (Fig. 6E). Treatment normalized SAM levels in livers of KO mice. On the other hand, S-adenosylhomocysteine (SAH) was elevated in all studied tissues of the KO mice compared to the WT controls (Fig. 6F). Treatment of KO mice with PEG-CBS resulted in a substantial reduction of SAH ($p < 0.05$), but not a normalization compared to WT ($p < 0.05$). Taken together, PEG-CBS, which predominantly distributes in blood circulation, formed a catabolic sink for plasma Hcy leading to a restoration of the metabolic balance in the examined tissues.

4. Discussion

The translation of pharmacokinetic, pharmacodynamic and toxicological findings from animal studies to human estimates serves as a guide to design optimal Investigational New Drug (IND) program enabling long term GLP toxicology studies and subsequent phase I/II clinical trials ([29,36]. We evaluated PK of PEG-CBS after single and repeated dosing at 3 different dosing levels in HCU mice, WT rats and Cynomolgus monkeys in non-GLP preliminary studies. Executed multiple dose studies were designed to monitor sub-chronic toxicity in parallel with repeated dose PK, to estimate the NOAEL in non-GLP rat and monkey studies and to evaluate the suitability of available animal models.

PEG-CBS is a clinical candidate for ERT against HCU. PEGylation of CBS increased the residence time of the enzyme in circulation by 11-fold without decreasing its catalytic activity [4]. The target destination for PEG-CBS is the blood and therefore the plasma PK studies directly represent the levels at the site of action allowing for uniquely relevant PK/PD studies. This approach represents an advantage over intracellularly targeted ERTs, such as those approved for lysosomal storage disorders, which depend on their glycosylation pattern enabling the ERT to be internalized by the cells and directed to the lysosomes (reviewed in [32]).

PEG-CBS has been identified as the most promising conjugate with a great efficacy profile in treating murine HCU and its production is scalable for commercial manufacturing [16,20]. PEG-CBS was found to rapidly and effectively decrease the toxic load of Hcy in blood, liver, kidney and brain tissues of HCU mice and was well tolerated over many months of dosing from early days after birth. We showed here (Fig. 6) and in our previous work [4,16] that the circulating PEG-CBS significantly improves or normalizes the underlying metabolic imbalance associated with HCU in target tissues, such as liver, kidney and brain. The correction of the tissue metabolites is made possible by lowering Hcy in plasma by PEG-CBS with a subsequent outflow of Hcy out of the tissues. Condensation of Hcy with serine by PEG-CBS leads to an elevation of Cth in plasma and kidney (most likely due to its urinary excretion), similar to the levels seen in hereditary cystathioninuria, which may be a benign biochemical finding with no adverse clinical effects [6,12,34]. In addition to Cth, PEG-CBS was found to affect plasma and tissue levels of related thioethers lanthionine (Lth) and homolanthionine (Hlth) in healthy Cynomolgus monkeys and HCU mice [18]. The biological functions of Lth and Hlth are unknown; however, they represent relevant surrogate markers of hydrogen sulfide (H_2S) biosynthesis by the enzymes of transsulfuration pathway [10,18]. In particular, Hlth and simultaneous H_2S production from Hcy by cystathionine gamma-lyase was 32-times higher in HCU patients compared to healthy population controls [10]. When HCU mice were treated with PEG-CBS, levels of Hlth were fully normalized in liver, where the majority of transsulfuration pathway activity occurs, and significantly decreased in kidney and brain [18]. H_2S was shown to exert a multitude of physiological effects including those on vasculature [9] and hepatic functions [22]. In addition, PEG-CBS treatment normalized plasma concentration of Cys (Supplementary Fig. S2B) [16,17,20], which is substantially decreased in HCU [25]. This effect is most likely indirect as similar finding was observed in HCU mice fed with Met-restricted diet [7]. It has been hypothesized that any treatment (e.g. dietary or PEG-CBS), which results in the decrease of plasma Hcy levels, normalizes plasma Cys, which is otherwise lost due to an increased urinary excretion of Hcy-Cys disulfides [13]. In stark contrast to HCU patients, plasma Met levels in mouse models of HCU are normal or only slightly elevated compared to controls [19]. However, severely elevated Met concentrations in plasma and tissues were observed in ~3-weeks old KO mice (Fig. 6D) [16] and they were totally normalized with the treatment. Taken together, by acting on plasma Hcy, PEG-CBS in circulation partially or fully normalizes sulfur amino acid metabolites above as well as below the block in plasma and tissues of several mouse models of HCU.

In addition to the ability of PEG-CBS to correct the clinical manifestation of HCU in model mice, we showed here that (i) the PK profile of PEG-CBS activity was predictable and dose-proportional in mice and monkeys, (ii) the PD response was rapid and coincided with plasma levels of the enzyme and (iii) the PEG-CBS was well tolerated with no adverse effects following a repeated dosing in monkeys. The PK properties of PEG-CBS in rats were different than that observed in mice and monkeys. The absorption of PEG-CBS from the SC compartment was lower in rat (< 30%) compared to mouse and monkey (> 80%), and sexual dimorphism was observed with nearly 50% lower absorption of PEG-CBS in male compared to female rats. Furthermore, lack of PEG-CBS accumulation in plasma after repeated dosing at all dose levels was surprising and unexpected, especially compared to the results in the mice and primates. In addition, minimal to moderate accumulation of vacuolated macrophages and inflammation of the injection site was associated with repeated administration of PEG-CBS in rats. It is possible to conclude, however, that rat is not a suitable species to conduct long-term GLP toxicology studies with PEG-CBS. All these observations can be most likely explained by an immune response to PEG-CBS, its neutralization and increased clearance from the circulation of rats. Similar to our observation, preclinical testing of PEGylated human interferon (IFN)-beta-1a also showed substantially lower bioavailability after SC administration in rats compared to monkeys (28 versus 100%) [27]. Cellular vacuolation, particularly of skin macrophages at the SC injection site, has been observed in approximately half of the non-clinical toxicology studies of the approved PEGylated drugs [8]. Since removal of foreign material from circulation is a normal function of phagocytic cells, the resulting vacuolation is typically considered a normal physiological response, which was found to be PEG size-, dose- and time-dependent [30]. More importantly, no functional impact of vacuolation was reported in toxicology studies and no indication of PEG-related adverse effects has been reported from clinical trials or post-marketing surveillance with any of the approved PEGylated drugs [8].

Here we used allometry and PK- and PK/PD-guided modeling approach to estimate HED for FIH clinical trial. Subcutaneous administration of PEG-CBS is intended to occur roughly every half-life, which has been estimated to be approximately 7 in humans. Compared to ERTs for lysosomal disorders, which are typically clinically administered IV infusions weekly or every two weeks [32], the proposed SC administration of PEG-CBS would allow self-administration at home with no need to travel to an outpatient clinic. To lessen the burden on patients and their families, some patients with lysosomal disorder receiving ERT may be transitioned from well-controlled settings of outpatient infusion clinics to home therapy, where the ERT administration is conducted under the care of trained infusion personnel [23]. In addition, compliance with treatment regimen was improved in patients on home therapy [5]. The HED estimated here based on PK/PD relationship studies in mice and monkeys is approximately 0.66 mg/kg PEG-CBS with weekly SC administration. Pending the NOAEL of long-term toxicity studies in monkeys, the predictable and dose-dependent PK profiles and PK/PD relationships of PEG-CBS in mouse and monkey provides enough confidence to use herein described HED forward into phase I clinical trial.

Our data suggest that HO mice on a standard lab chow can to achieve significant reductions in Hcy concentrations, but not a complete normalization even with a substantially increased dose of PEG-CBS (Fig. 2B). Reactive nature of Hcy leads to its strong binding to free sulfhydryls forming (mixed) disulfides and often displacing Cys from its binding to proteins. PEG-CBS has the capability to alter the balance between protein-bound and free forms of Hcy (either as free reduced or in a form of disulfides) [28], thus leading to a substantial decrease of total Hcy in plasma of treated HO mice. Thus, the lack of complete normalization of plasma Hcy levels by PEG-CBS in HO mice most likely stems from other factors, such as slow protein turnover, balance between vascular and extravascular pools of Hcy or constant flux of Hcy from intracellular compartment. It is noteworthy, that the HO mice used here were maintained on a standard diet (19% protein), i.e. with no restriction of dietary Met intake. It is plausible that partial Met restriction may result in a complete normalization of Hcy levels combined with PEG-CBS treatment. Interestingly, even severe Met restriction (i.e. 0.5 g/kg Met compared to a regular diet containing 4–6 g/kg Met) did not normalize plasma Hcy levels in HCU mice yielding ~80 μM Hcy [7]. The opportunity to fully normalize Hcy levels or dramatically reduce diet restrictions with PEG-CBS would be tremendously beneficial to patients and caregivers.

5. Conclusion

The animal studies provide the first systematic in vivo evaluation of PEG-CBS with respect of PK, PK/PD relationship and sub-chronic toxicity after repeated administration. The findings demonstrate that PEG-CBS is well tolerated and readily adsorbed from the SC compartment to bloodstream in mice and monkeys, where it serves as a metabolic sink to positively impact the levels of sulfur amino acid biomarkers in tissues as well. These results provide critical preclinical data for the design of FIH PEG-CBS trial in patients with HCU.

Abbreviations

AUC	area under the curve
$t_{1/2\text{-A}}$	adsorption half-life
$t_{1/2\text{-E}}$	elimination half-life
c_{max}	maximal plasma concentration
t_{max}	the time of c_{max}
c_{p0}	theoretical plasma concentration extrapolated to time 0 after IV administration
MRT	mean residency time (average time a drug unit circulates in plasma)
CL	clearance (volume of plasma cleared of drug per unit of time)
FIH	first-in-human
NOAEL	no-observed-adverse-effect-level

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Conflict of interest

The research was funded by Orphan Technologies Ltd., a private pharmaceutical company developing an enzyme replacement therapy for CBS-deficient homocystinuria. TM, EMB and JPK are inventors on patents related to the processes and products referred here (US patents 9,034,318 and 9,243,239).

Authorship contribution

TM designed and performed mouse studies, prepared and analyzed PEG-CBS conjugates, analyzed all the data, coordinated the project, prepared figures and wrote the manuscript. EMB and FG designed and coordinated studies on rats and monkeys. IP took care of mouse colony and executed studies on mice. AE and TB performed metabolite analyses on tissues. JPK conceived the overarching idea and coordinated the project. All authors reviewed the manuscript, contributed to its revisions and approved its final form.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.03.018.

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