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Research Paper

Toxicokinetic Study of Recombinant Human Heparin-Binding Epidermal Growth Factor-Like Growth Factor (rhHB-EGF) in Female Sprague Dawley Rats

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Purpose. To determine the toxicity and pharmacokinetics of recombinant heparin-binding epidermal growth factor-like growth factor in female Sprague Dawley rats following intra-bladder and intravenous administration.

Materials and Methods. rhHB-EGF was administered once daily for 6 or 27 days at doses of 3, 10, or 30 μg/kg. ^{125}I-rhHB-EGF was administered on day 7 or 28 for pharmacokinetic analysis. Toxicity was assessed by general appearance and behavior, gross necropsy, blood chemistry and microscopic evaluation.

Results. Plasma AUCss of [^{125}I] rhHB-EGF equivalents following IB administration for 7 days were 4.28±2.29, 7.75±2.70, and 7.11±1.42 ng ml^{-1} h^{-1} at doses of 3, 10, and 30 μg/kg, respectively. Following IV administration, the AUCss on day 7 increased from 27.0±2.66 to 124±5.09 and 385.11±7.57 ng ml^{-1} h^{-1} with increasing the dose from 3 to 10 and 30 μg/kg. Similar AUCss data was obtained after 28 day administration. No toxicity was evident upon gross examination. Histologic examination revealed subacute inflammation and lymphocytic infiltration of the urinary bladder in animals from all groups dosed by the IB route.

Conclusions. Plasma and bladder concentrations of recombinant human [^{125}I] rhHB-EGF equivalents were significantly lower following the IB route than following IV administration. Histologic tissue examination indicated no toxicity attributable to rhHB-EGF.

KEY WORDS: HB-EGF; interstitial cystitis; intrabladder instillation; intravenous administration pharmacokinetics; NONMEM; toxicity; toxicokinetics.

INTRODUCTION

Interstitial cystitis (IC) is a chronic bladder disorder. General symptoms of this disorder typically include increased daytime and nighttime urinary frequency, increased urinary urgency, and suprapubic pressure and/or pain with bladder filling. Cystoscopic abnormalities seen in the bladder of patients with this disorder include petechial hemorrhages called "glomerulations" and ulcers that extend into the lamina propria (Hunner's ulcers; 1,2). The most consistent histologic abnormalities include denudation or thinning of the bladder epithelium to one to two cell layers (3–5). These findings suggest that IC may be caused by an inhibition of normal bladder epithelial cell proliferation, resulting in a loss of epithelial barrier integrity. However, the precise cause of IC is still unknown, and there is no reliably effective treatment for this disorder.

Due to the limitation of available drugs for IC treatment, it is important to understand the pathology of the disease and search for new types of medicines that may treat the underlying bladder defects. Keay *et al.* (6) previously reported the discovery of a novel antiproliferative factor ("APF") peptide that is made uniquely by bladder epithelial cells from IC patients and profoundly inhibits normal bladder epithelial cell growth. Purified native and synthetic APF have both been shown to downregulate HB-EGF production while upregulating EGF production by primary bladder epithelial cells *in vitro*, and recombinant human HB-EGF can overcome the inhibitory effects of purified native APF on bladder cell proliferation *in vitro* at normal physiologic concentrations (6,7). These findings coupled with thinning of the bladder epithelium commonly found in biopsy specimens from patients in the IC data base study suggest that APF and

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its regulation of HB-EGF production may be involved in the pathogenesis of IC, and that rhHB-EGF is a potential therapy for this disorder (8). In addition, because HB-EGF is an endogenous substance that is produced in many tissues (including the reproductive tract, gastrointestinal tract, vasculature, urinary tract, lung, skeletal muscle, brain, and heart; 9–17), it would be expected to have little or no toxicity at normal physiologic levels.

Given the previously described *in vitro* activity of rhHB-EGF and its putative site of action in the bladder epithelium, this study was designed to determine the toxicity and pharmacokinetics of rhHB-EGF following intravenous (IV) and intrabladder (IB) administration to female Sprague Dawley rats. Three dose levels of recombinant human HB-EGF were given over a 28 day period by either IV or IB administration. The current pharmacokinetic study was carried out to support the subchronic toxicology assessments of rhHB-EGF following the two modes of administration. Intrabladder (IB) administration was chosen because this route of administration was thought to be a potential route for therapeutic intervention in the clinical setting based on the disease and the nature of the protein being administered. rhHB-EGF was also administered via the IV route to appropriately define the toxicity of this agent at escalating doses, and to compare plasma and tissue levels of rhHB-EGF given by different routes.

MATERIALS AND METHODS

Chemicals

Recombinant human HB-EGF was purchased from R&D Systems (Minneapolis, MN). [^{125}I] rhHB-EGF was prepared by Amersham Biosciences (Woburn, MA). NaCl injection of 0.9%, USP was purchased from Abbott Laboratories (North Chicago, IL). Xylazine was obtained from Sigma-Aldrich Corp. (St. Louis, MO). Ketamine was from Phoenix Pharmaceutical Inc. (St. Joseph, MO). Phosphate buffer saline (PBS) pH 7.4 was purchased from Invitrogen Corporation (Carlsbad, CA).

Animal Study

Female Harlan Sprague Dawley rats were purchased from Harlan Industries, Inc. (Indianapolis, ID). This protocol was reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC). Animals were housed, cared for, and used strictly in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All animals were kept on a 12 h light-dark cycle and had access to food and water *ad libitum*.

Animals for toxicology assessment were dosed with 3, 10, or 30 μg/kg of rhHB-EGF once daily for 7 or 28 days by either the IV or IB route. Six animals for each dose and each route of administration were treated. All animals were anesthetized with ketamine and xylazine (35 and 3.5 mg/kg) 15 min prior to all drug administration. For IV dosing, rhHB-EGF was administered by lateral tail vein injection at the appropriate concentration and 200 μl of rhHB-EGF in phosphate buffered saline was administered. Animals receiving IB dosing were likewise administered 200 μl rhHB-EGF diluted to the appropriate concentration with phosphate buffered saline. The drug solution was administered directly into the bladder through an Intramedic™ polyethylene tube (Becton Dickenson, Sparks, MD) and the urethral orifice was occluded using water-insoluble material (Mehron Inc, Chestnut Ridge, NY) for 1 h to prevent urination. On day 7 or 28 the animals were euthanized using carbon dioxide asphyxiation, blood was collected via cardiac puncture, and organs (brain, heart, liver, lung, spleen, kidneys, and bladders) were removed. All organs were weighed and examined and gross appearance noted. Standard hematology parameters including complete blood count, hematocrit, hemoglobin,

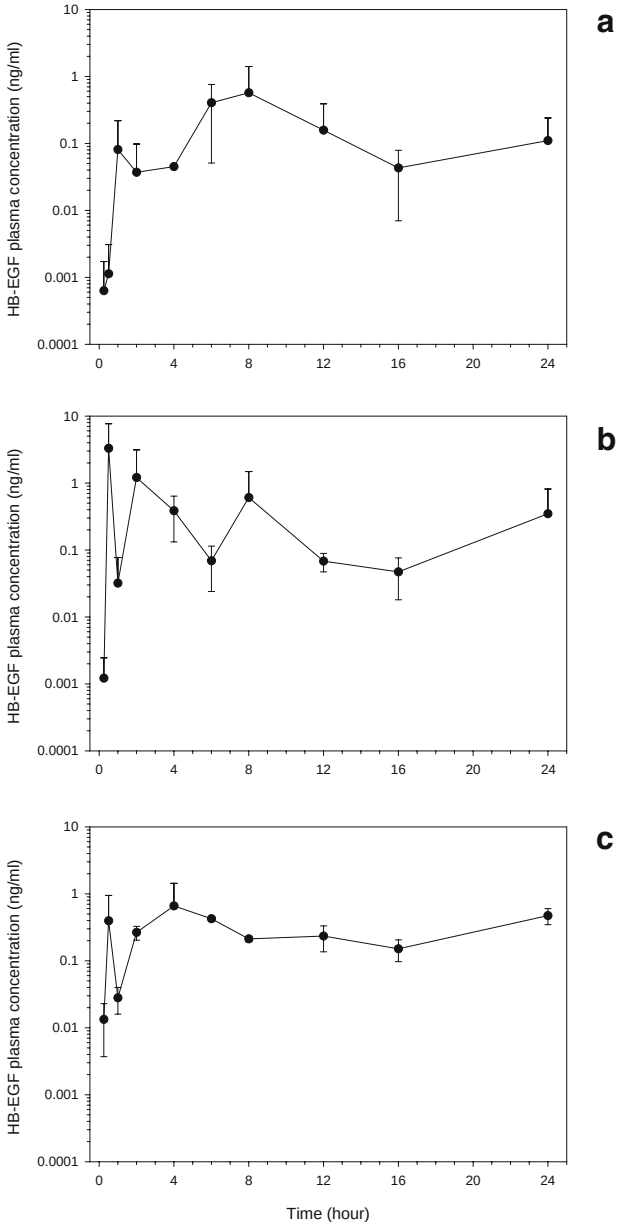

Fig. 1. Mean plasma concentration *versus* time plot for rhHB-EGF obtained following intrabladder (IB) administration of 3 (**a**), 10 (**b**), and 30 (**c**) μg/kg. *Solid circles* represent the mean concentration at each time and the *error bars* represent the standard deviation (s.d.) of the mean.

Table I. Area Under the Curve (AUCss; ng h^{-1} ml^{-1}) for Plasma and Tissue Levels of [^{125}I] rhHB-EGF Equivalents After IB Administration at 3, 10, and 30 μg/kg Once Daily for 7 Days

Plasma/Tissue	3 μg/kg		10 μg/kg		30 μg/kg	
	AUCss±SD	Tissue/Plasma Ratio	AUCss±SD	Tissue/Plasma Ratio	AUCss±SD	Tissue/Plasma Ratio
Plasma	4.28±2.29		7.75±2.70		7.11±1.42	
Brain	1.22±0.99	0.29	1.80±1.05	0.23	2.08±0.74	0.29
Heart	2.49±1.80	0.58	3.73±1.64	0.48	2.74±0.84	0.39
Lung	4.57±2.39	1.07	9.56±2.65	1.23	6.24±1.41	0.89
Liver	7.21±2.84	1.69	14.7±2.87	1.90	7.85±2.63	1.10
Spleen	4.40±2.04	1.03	14.9±2.74	1.92	8.06±1.42	1.13
Kidney	13.3±3.95	3.10	25.5±3.76	3.29	18.6±2.65	2.61
Bladder	62.7±6.36	14.7	79.6±3.78	10.3	707±16.51	99.4

Data based on Bailer estimate

and serum chemistry determinations were performed by Antech Diagnostics (Lake Success, NY). The organs were preserved in 10% formalin in phosphate buffer. Sections of each organ tissue (~5 μm) were embedded in paraffin, sectioned and stained with hemotoxylin and eosin for microscopic evaluation by a veterinary pathologist at Charles River Laboratories (Frederick, MD) under GLP conditions.

rhHB-EGF Sampling and Analysis

Animals for pharmacokinetic assessment were dosed as previously described once daily for 6 or 27 days with rhHB-EGF at doses of 3, 10, or 30 μg/kg. On day 7 or 28, [^{125}I] rhHB-EGF 3, 10, or 30 μg/kg (corresponding to 3, 10, or 30 μCi/kg) was administered and the rats (three rats per each time point) were sacrificed at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, and 24 h post administration. From each animal approximately 7 ml of blood was collected into Vacutainer® (Becton Dickenson, Franklin Lakes, NJ) heparinized tubes. Plasma was obtained by centrifugation at 2,500×g at 4°C for 10 min. Plasma and organs (brain, heart, liver, lung, spleen, kidney, and bladder) were then transferred into 3.5 ml-cryogenic vials (Evergreen Scientific, Los Angeles, CA) and stored at −80°C until analysis. The intensity of [^{125}I] rhHB-EGF in each sample was measured using a Beckman 5500 gamma counter within 24 h following the collection of the last sample.

Pharmacokinetic Analysis

[^{125}I] rhHB-EGF equivalent concentrations were determined for the pharmacokinetic study following IV and IB (intrabladder, or intravesical, instillation). A population approach was used to analyze the data due to the destructive sampling scheme. The plasma concentrations obtained after IV or IB administration of HB-EGF were analyzed using a two compartment model with the Nonlinear Mixed Effects Modeling Program (NONMEM) version V (Globomax Service Group, Hanover, MD, USA). Two and three compartment models were fit to the data using ADVAN3 TRANS3 and ADVAN4 TRANS4 for the IV and IB administration, respectively. The pharmacokinetic parameters estimated for IV and IB administration were clearance (CL or C/F, for IV and IB, respectively), volume of distribution of the central compartment (Vc or Vc/F for IV and IB, respectively), intercompartmental clearance (Q), peripheral volume after IV administration (V2), peripheral volume after IB administration (V3), and absorption rate constant (KA) after IB administration. Inter-animal variability was estimated by an exponential error model:

$$P_i = \theta * \exp(\eta_i) \quad (1)$$

where θ is the population mean value for parameter P, P_i is the individual parameter estimate, and η_i is a random variable

Table II. Area Under the Curve (AUCss; ng h^{-1} ml^{-1}) for Plasma and Tissue Levels of [^{125}I] rhHB-EGF Equivalents After IB Administration at 3, 10, and 30 μg/kg Once Daily for 28 Days

Plasma/Tissue	3 μg/kg		10 μg/kg		30 μg/kg	
	AUCss±SD	Tissue/Plasma Ratio	AUCss±SD	Tissue/Plasma Ratio	AUCss±SD	Tissue/Plasma Ratio
Plasma	1.21±1.55		7.88±3.73		23.0±5.88	
Brain	2.51±2.25	2.07	1.43±0.848	0.18	4.03±1.82	0.18
Heart	1.68±1.79	1.39	2.94±1.57	0.37	7.75±3.05	0.34
Lung	2.72±2.19	2.25	4.33±2.41	0.55	23.3±6.31	1.01
Liver	2.73±1.79	2.26	9.37±4.36	1.19	30.7±7.72	1.34
Spleen	2.06±1.52	1.70	3.65±1.96	0.46	22.9±4.73	1.00
Kidney	3.16±2.25	2.61	6.22±2.75	0.79	38.0±8.29	1.65
Bladder	40.7±3.44	33.64	215±9.41	27.28	285±11.0	12.4

Data based on Bailer estimate

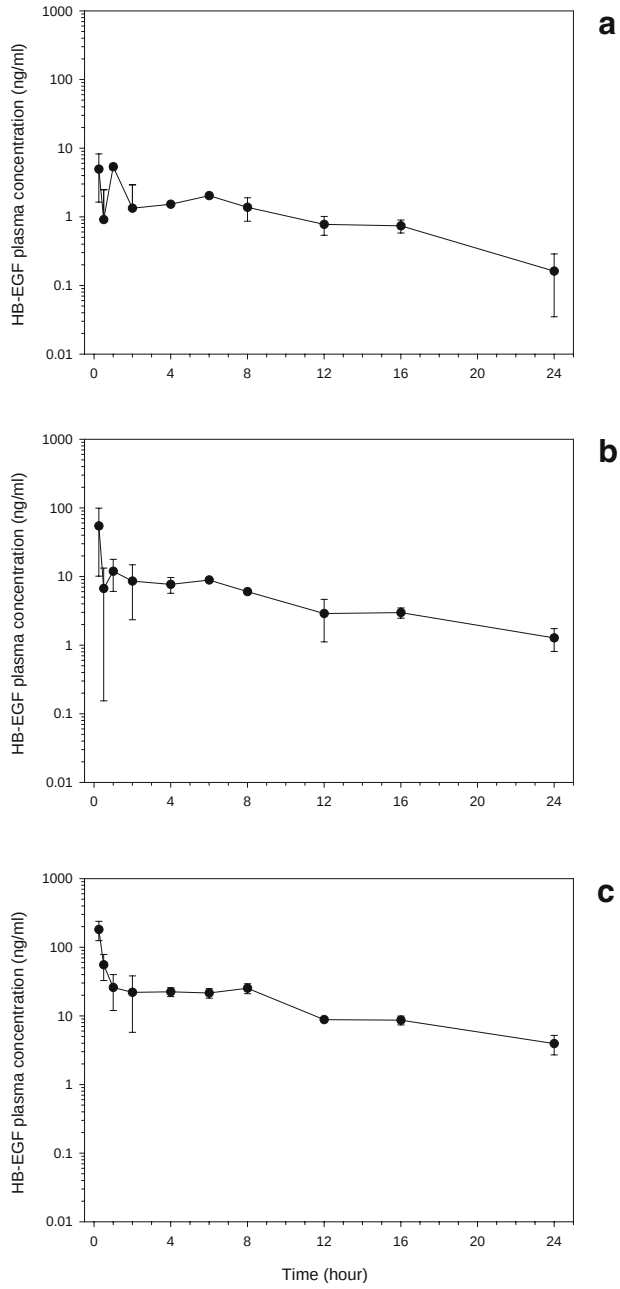

Fig. 2. Mean plasma concentration *versus* time plot for rhHB-EGF obtained following intravenous (IV) administration of 3 (**a**), 10 (**b**), and 30 (**c**) μg/kg. *Solid circles* represent the mean concentration at each time and the *error bars* represent the standard deviation (s.d.) of the mean.

with a mean of zero and a variance of Ω^2 which describes the deviation of P_i from P.

Residual variability [^{125}I] rhHB-EGF equivalent pharmacokinetics was modeled by a proportional error model:

$$C_{obs} = C_{pred}*(1 + \varepsilon_1) \quad (2)$$

where C_{obs} is the observed concentration, C_{pred} is the model-predicted concentration, and ε_1 is the proportional error component. Model appropriateness was evaluated by the value of the objective function, the Akaike Information Criteria (AIC) and visual inspection of the diagnostic plots.

The area under the concentration-time curve at the steady state (AUCss; and 95% CI) of the [^{125}I] rhHB-EGF equivalents in plasma and tissue was obtained using the Bailer Estimation method (18). Statistical comparison of the difference between AUC values was performed using the variance and confidence interval of the AUC means.

Western Blot Analysis

Rat urine specimens were concentrated by freeze-drying and resuspending in phosphate buffered saline. Protein concentration of the urine specimens was measured using a Folin reagent-based protein assay kit (Bio-Rad). The gel was loaded with 20 μl samples at a protein load of 85 μg per well. Proteins in the samples were then separated by one dimensional polyacrylamide gel electrophoresis using 4–12% NuPAGE Novex Bis-Tris polyacrylamide gels (In-Vitrogen) according to the manufacturer's instructions, and transferred to nitrocellulose membranes according to the NuPAGE manufacturer's protocol for Western transfer (at 30 V constant voltage for 1 h). Following protein transfer, the nitrocellulose membranes were blocked with 5% nonfat dry milk in TBS-T buffer (Tris-buffered saline, pH 7.4, with 0.1% Tween 20), and incubated overnight at 4°C in 5% blocking buffer containing monoclonal antibodies raised against human HB-EGF (R&D Systems). The membranes were subsequently washed with TBS-T, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz) for 1 h at room temperature, and developed with ECL chemiluminescence Reagent (Amersham Biosciences). Relative quantification of rhHB-EGF was achieved by relative densitometry with a Personal Densitometer SI (Molecular Dynamics) and using Image Quant 5.0 (Molecular Dynamics).

RESULTS AND DISCUSSION

Plasma [^{125}I] rhHB-EGF equivalent concentration *versus* time profiles following IB doses of 3, 10, and 30 μg/ml on treatment Day 7 are depicted in Fig. 1. The concentrations increased rapidly with multiple peaks being observed throughout the sampling period following each of the three doses. Because the plasma concentrations observed following IB administration were highly variable and had no clear elimination phase, these data were not amenable to pharmacokinetic modeling using NONMEM. An attempt was made to fit the data to one and two compartmental models with extravascular absorption, however attempts at fitting several parameters including clearance failed. Similar concentration *versus* time profiles were obtained following 28 days of IB dosing, which were also not amenable to pharmacokinetic modeling using NONMEM (see below for a discussion of AUCss for the 7 *vs* 28 day data).

The relatively low plasma concentrations of [^{125}I] rhHB-EGF equivalents following IB administration at all doses administered (3, 10, and 30 μg/kg) may be due to the fact that the urinary bladder is not an absorptive organ, but a barrier to the external environment. Additionally, the study design may also have contributed since the occlusive water insoluble material was removed from urethral orifice within 1 h after drug administration, to protect the rats from discomfort, thus allowing them to void. The concentration of native HB-EGF, as well as the concentrations of heparin sulfate proteoglycans and specific cell membrane receptors for rhHB-EGF, may

Table III. Area Under the Curve (AUCss; ng h^{-1} ml^{-1}) for Plasma and Tissue Levels of [^{125}I] rhHB-EGF Equivalents After IV Administration at 3, 10, and 30 μg/kg Once Daily for 7 Days

Plasma/Tissue	3 μg/kg		10 μg/kg		30 μg/kg	
	AUCss±SD	Tissue/Plasma Ratio	AUCss±SD	Tissue/Plasma Ratio	AUCss±SD	Tissue/Plasma Ratio
Plasma	27.0±2.66		123.6±5.09		385±7.57	
Brain	2.19±0.78	0.08	7.20±1.82	0.06	23.6±2.55	0.06
Heart	9.36±1.88	0.35	35.6±3.09	0.29	121±4.34	0.32
Lung	30.7±2.60	1.13	128±7.02	1.03	365±10.3	0.95
Liver	44.3±3.20	1.64	183±6.79	1.48	517±9.18	1.34
Spleen	30.1±1.61	1.11	111±3.94	0.90	226±6.15	0.59
Kidney	53.0±5.03	1.96	159±8.46	1.29	472±7.47	1.22
Bladder	70.1±4.88	2.60	258±10.7	2.09	1102±25.8	2.86

Data based on Bailer estimate

also be able to influence the plasma concentration of labeled HB-EGF, but these were not determined for this study.

The AUCss of the [^{125}I] rhHB-EGF equivalents in plasma and tissue after bladder instillation are presented in Table I and Table II. The plasma AUCss on day 7 were calculated to be 4.28±2.29, 7.75±2.70, and 7.1±1.42 ng h^{-1} ml^{-1} at doses of 3, 10, and 30 μg/kg, respectively (Table I). These results showed a significantly higher AUCss of the plasma samples with increasing IB dose from 3 to 10 μg/kg (α=0.05) but no additional increase with the 30 μg/kg dose at 7 days. A similar trend was seen for heart, lung, liver, spleen, and kidney tissue. In the brain, however, no significant dose-dependent change in tissue concentration was found over the 10–30 μg/kg dose range following 7 days. The general lack of increase in exposure with a dose increase from 10 to 30 μg/kg might be explained by the fact that the bladder epithelium is not an absorptive surface; it is possible that absorption across this membrane is saturable and thus no increased systemic exposure was observed at the final two dose levels. However, a dose-dependent increase was obtained for plasma AUCss following 28 days of dosing (1.21±1.55, 7.88±3.73, 23.0±5.88 ng h^{-1} ml^{-1} at doses of 3, 10, and 30 μg/kg, respectively) with similar dose-dependent increases evident for the 10–30 μg/kg doses in all tissues (Table II). These data suggest that repeated intravesical instillations might result in increased absorption by day 28, possibly because of changes induced by the procedure itself in the bladder epithelium over that time.

Plasma [^{125}I] rhHB-EGF equivalent concentration *versus* time profiles following IV doses of 3, 10, and 30 μg/ml for 7 days are depicted in Fig. 2. Plasma concentrations of HB-EGF following IV administration were found to be significantly higher compared to the IB administration at every time point for all three doses. Maximum observed plasma HB-EGF concentrations after IV administration for 7 days were 5.36±0.527, 54.4±44.3, and 181±56.7 ng/ml, at doses of 3, 10, and 30 μg/kg, respectively. Similar results were again obtained following 28 days dosing.

Exposure parameters for all organs following IV administration are presented in Table III and Table IV. All concentration-time profiles appeared to be biphasic, and as such, the two-compartment model best fit the data following NONMEM analysis. The pharmacokinetic model parameters are presented in Table V. The relationship between CL and VC was investigated. As shown in Fig. 3, ETA of CL and VC obtained by NONMEM were plotted using S-PLUS 2000 Professional Release2 program (Mathsoft, Inc., USA). The result indicated no correlation between these parameters.

The plasma concentration *versus* time curve for all three doses fit with a two compartment model using NONMEM as presented in Fig. 4. Additional diagnostic plots (not shown) included, individual predicted concentrations (IPRED) *versus* observed concentrations (DV) of [^{125}I] rhHB-EGF equivalents after IV administration that showed the concentrations closely associated with, and randomly distributed around the line of unity. The relationship between the weighted residuals

Table IV. Area Under the Curve (AUCss; ng h^{-1} ml^{-1}) for Plasma and Tissue Levels of [^{125}I] rhHB-EGF Equivalents After IV Administration at 3, 10, and 30 μg/kg Once Daily for 28 Days

Plasma/Tissue	3 μg/kg		10 μg/kg		30 μg/kg	
	AUCss±SD	Tissue/Plasma Ratio	AUCss±SD	Tissue/Plasma Ratio	AUCss±SD	Tissue/Plasma Ratio
Plasma	34.2±3.40		149±6.60		401±520	
Brain	2.34±0.691	0.07	8.15±1.87	0.06	30.4±6.33	0.08
Heart	8.98±1.17	0.26	43.0±4.58	0.29	134±11.9	0.33
Lung	26.1±3.35	0.76	115±6.44	0.77	307±22.6	0.77
Liver	52.1±3.13	1.52	205±11.5	1.38	889±21.9	2.22
Spleen	29.9±2.47	0.85	186±12.7	1.25	726±17.2	1.81
Kidney	57.6±3.70	1.68	230±8.55	1.54	640±29.1	1.60
Bladder	122±14.2	3.57	242±12.7	1.62	1650±2160	4.12

Data based on Bailer estimate

Table V. Plasma Pharmacokinetic Parameters of [^{125}I] rhHB-EGF Equivalents After IV Administration at 3, 10, and 30 μg/kg

Parameter	Mean(95% CI)	Interanimal Variability (95% CI)
CL (ml/h)	69.7 (57.8, 81.6)	5.01
VC (ml)	29.8 (2.56, 57.0)	47.4
Q (ml/h)	204 (21.3, 387)	38.2
V2 (ml)	473 (348, 598)	13.9

Data based on NONMEM results, 7 day dosing
CL Clearance, *VC* central volume, *Q* inter-compartmental clearance, *V2* peripheral volume

(WRES) *versus* time plot revealed that all weighted residuals fell within three standard deviations with only three points falling outside the two standard deviation range. Also the weighted residual values were evenly distributed and without bias around the line of origin.

Since a primary objective of this study was to assess the distribution of drug at the site of action, the bladder epithelium, the concentration of [^{125}I] rhHB-EGF equivalents in this tissue was viewed as more relevant than the plasma concentrations. It is therefore important to note that the AUCss of [^{125}I] rhHB-EGF equivalents in the bladder following 7 days of IB administration increased in a dose related manner from 62.7±6.36 to 79.6±3.78, and 707±16.5 ng h^{-1} ml^{-1} at a doses of 3, 10, and 30 μg/kg, respectively. These results show an independence of dose on the AUCss of all tissues except bladder that may be caused by saturation of the drug absorption from the bladder over the shorter (7 day) dosing period. In comparison, following IV administration for 7 (as well as 28) days, the relationship between the dose and AUCss of drug in plasma and all tissues was found to be approximately dose proportional (Fig. 5). For example, the AUCss of drug in plasma increased from 27.0±2.66 to 123.6±5.09, and 385±7.57 ng h^{-1} ml^{-1} with increasing doses of 3, 10, and 30 μg/kg, respectively. The AUCss of drug in bladder also increased proportionally following IV administration from 70.1±4.88, to 258±10.7, and to 1102±25.8 ng h^{-1} ml^{-1}, with doses of 3, 10, and 30 μg/kg, respectively.

The tissue/plasma ratios of the [^{125}I] rhHB-EGF equivalents obtained from both the IB and IV administration at the doses of 3, 10, and 30 μg/kg for 7 days are shown in Table I and Table III. The ratio of brain and heart to plasma obtained after the IB administration was less than 1 at all doses and at both 7 and 28 day time periods, indicating a very low distribution of the drug into both tissues. Tissue/plasma ratios of the drug were highest (and greater than one for all three doses given by either IB or IV route) in liver, kidney and bladder tissue which are known to contain specific receptors for ligands belonging to the EGF family (including HB-EGF; 19,20). Again, similar results were found following 28 days of dosing, with brain and heart to plasma ratio less than one, and lung, liver, spleen, kidney, and bladder to plasma ratios generally higher than one. HB-EGF is also known to be produced by human bladder epithelial cells from normal controls and stimulates the proliferation of these cells (9,21). Unexpectedly, it was observed that the bladder tissue concentrations were significantly higher at both time-points following IV administration as compared to direct IB instillation and the total exposure of the bladder was higher following the IV route as well. One potential explanation for HB-EGF accumulation in the bladder tissues might be the abundance of HB-EGF receptors at this organ site.

A Western blot was performed to determine the presence and approximate concentration of intact rhHB-EGF in the

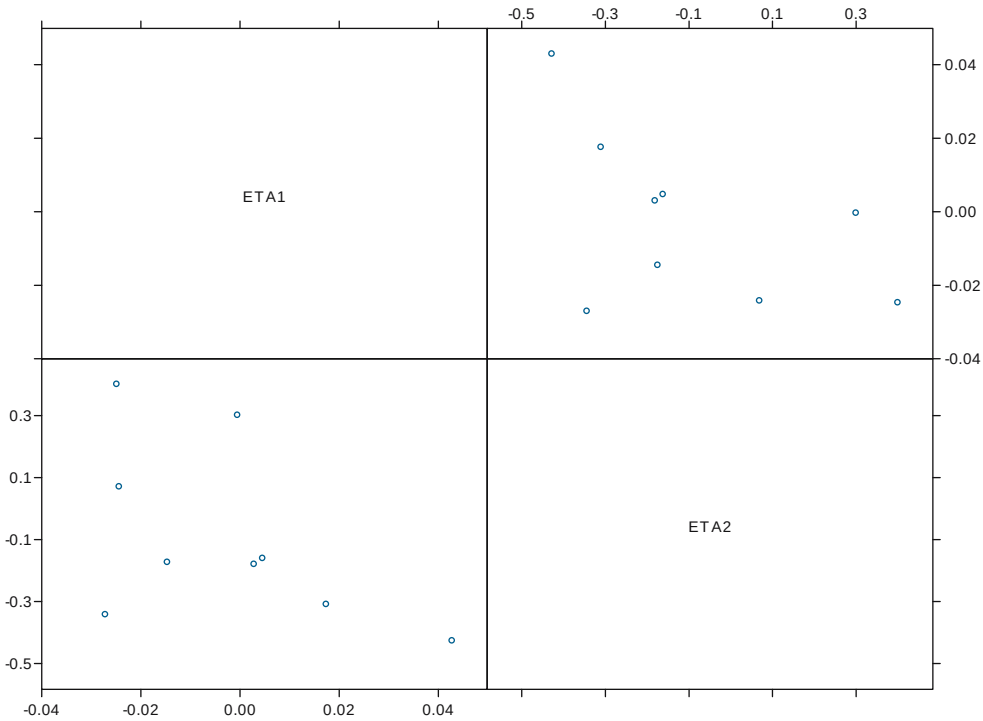

Fig. 3. Relationship between CL and VC estimated following fit of the data with NONMEM.

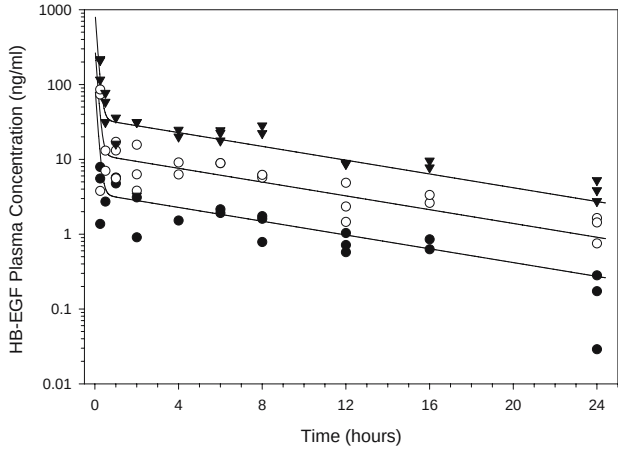

Fig. 4. Plasma concentration *versus* time plot of individual animals following intravenous (IV) administration of rhHB-EGF at 3, 10, and 30 μg/kg. *Solid triangles* represent the individual measured plasma concentrations at the 30 μg/kg dose, *open circles* represent individual measured concentrations at the 10 μg/kg dose, *solid circles* represent individual measured concentrations at the 3 μg/kg dose, and the *solid lines* represent the model predicted concentrations based on the two compartment model.

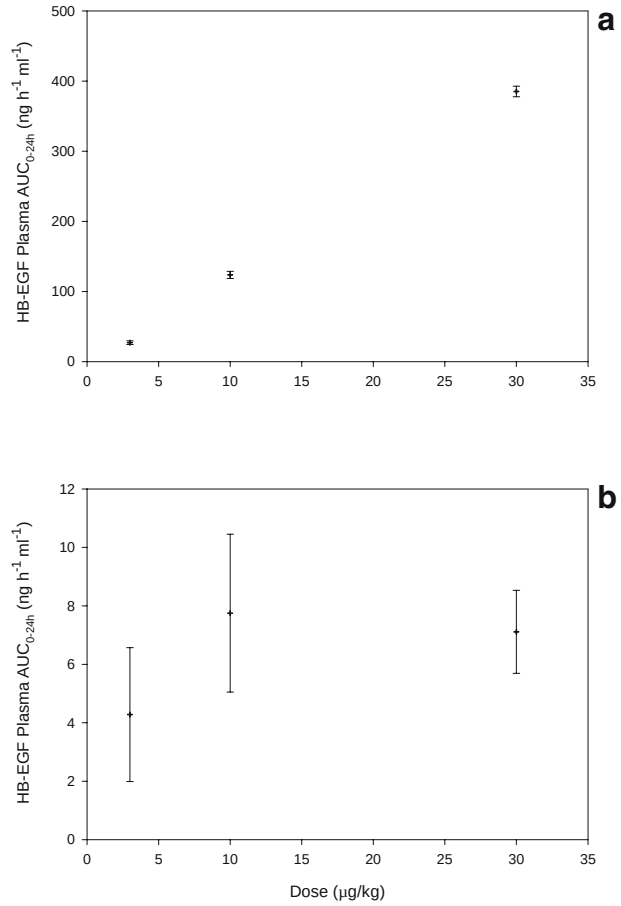

Fig. 5. Relationship between AUC and dose. Plasma rhHB-EGF $AUC_{0-24\ h}$ (ng h^{-1} ml^{-1}) *versus* Dose (mg/kg) obtained after administration by intravenous injection (**a**), and after administration via intrabladder instillation (**b**). Data are mean and standard deviation of the mean.

Fig. 6. Western blot for rhHB-EGF in the urine of rats administered 10 μg/kg rhHB-EGF via the IV route.

urine of animals dosed via the IV route, Fig. 6. The approximate concentrations based on the semi-quantitative Western blot analysis were 6 ng/ml for Rat #1, 61 ng/ml for Rat #2 and 48 ng/ml for Rat #3. The corresponding radioactivity calculated concentrations for the urine in these animals was 13, 38, and 42 ng/ml, respectively. This difference could result from the complex nature of the purification and Western blot analysis and the fact that the Western blot analysis was performed on previously frozen urine samples whereas the radioactivity was determined on fresh specimens.

Toxicity was assessed on a total of 96 female Sprague Dawley rats (six animals per dose level including vehicle control), dosed daily by the IB or IV route for 28 days, using doses up to 100 times the concentration normally present in human serum or urine (30 μg/kg). All animals were observed once daily for general appearance, behavior, signs of toxicity, body weight, neuromuscular coordination and mortality for the duration of the study. Additional analysis following animal sacrifice included gross tissue examination, and blood or serum parameters including complete blood counts, hematocrit, hemoglobin, serum creatinine, blood urea nitrogen, total protein, total globulin, total cholesterol, calcium, phosphorus, sodium, potassium, chloride, bicarbonate, CPK, and liver function tests (SGPT, SGOT, total bilirubin, and alkaline phosphatase). Blood chemistry and hematology data are presented in Table VI, VII, VIII, and IX.

There was no observed difference in animal appearance or behavior, weight, coordination, or mortality between animals receiving any dose of rhHB-EGF *vs.* controls that received vehicle alone. Histologic tissue examination also indicated no toxicity attributable to rhHB-EGF. In the urinary bladder, acute or subacute inflammation or lymphocytic infiltration involving one or more layers of the mucosa and submucosa were evident across all groups dosed via the IB route. Four of six animals treated with vehicle alone, one of six animals from group 2 (3 μg/kg), four of six animals from group 3 (10 μg/kg), and two of six animals from group 4 (30 μg/kg) were observed to have inflammatory infiltrates in the bladder tissue following IB dosing. In comparison, only one rat dosed with 3 μg/kg via IV administration had cellular inflammation of the bladder submucosa. All other animals dosed by IV administration including the vehicle controls, were free of urinary bladder pathological alterations. It was therefore thought that the urinary bladder responses in animals dosed via the IB route were associated with irritation and trauma from the injection procedure itself rather than from rhHB-EGF. Sporadic findings in the other organs following either IB or IV administration of rhHB-EGF or vehicle had no apparent relationship to agent or dose levels, were typical of spontaneous pathology reported in tissues and organs of Sprague

Table VI. Complete Blood Counts

Dose/Route	Hemoglobin (g/dl)	Hematocrit (%)	WBC ($\times 10^3/\mu l$)	RBC ($\times 10^6/\mu l$)	MCV	MCH	MCHC	Platelet Count ($\times 10^3/\mu l$)
0 μg/kg IB	13.7±1.38	36.3±4.56	10.3±4.16	5.99±0.46	60.3±7.53	22.9±1.53	38.3±2.90	839±297
3 μg/kg IB	13.7±0.61	35.3±1.55	10.5±3.22	6.14±0.30	57.2±3.06	22.3±0.72	39.0±1.10	937±56.9
10 μg/kg IB	13.7±0.65	34.2±1.75	12.5±2.33	6.26±0.38	55.2±0.75	21.8±0.60	39.6±0.60	982±36.63
30 μg/kg IB	13.5±1.20	34.2±3.33	12.3±2.85	6.08±0.71	56.3±2.07	22.3±1.05	39.6±0.90	885±115
0 μg/kg IV	14.3±0.88	35.0±1.10	16.5±1.67	6.40±0.19	55.7±1.75	22.4±1.24	40.1±1.90	500±547
3 μg/kg IV	13.6±0.48	34.3±1.36	14.8±2.45	6.14±0.22	55.7±1.51	22.2±1.05	40.0±2.26	167±408
10 μg/kg IV	13.2±0.64	34.0±1.77	11.8±3.27	6.16±0.30	5.0±0.89	21.5±1.18	39.0±1.89	334±516
30 μg/kg IV	13.4±1.25	34.2±1.22	12.6±1.34	6.04±0.21	56.7±1.97	22.3±2.22	39.5±3.96	167±408

Table VII. White Blood Cell Differential Counts

Dose/Route	Neutrophils ($\times 10^3/\mu l$)	Bands ($\times 10^3/\mu l$)	Lymphocytes ($\times 10^3/\mu l$)	Monocytes ($\times 10^3/\mu l$)	Eosinophils ($\times 10^3/\mu l$)	Basophils ($\times 10^3/\mu l$)
0 μg/kg IB	1619±1515	0.00±0.00	8576±3518	243±191	70.8±59.6	23.7±58.0
3 μg/kg IB	1208±669	0.00±0.00	9205±2788	399±408	82.7±45.2	55.7±61.2
10 μg/kg IB	1186±574	0.00±0.00	10798±1822	357±171	103±93.7	39.7±61.6
30 μg/kg IB	1159±424	0.00±0.00	10676±2501	296±236	60.5±66.3	143±293
0 μg/kg IV	2522±1154	0.00±0.00	13143±1817	557±483	187±41.0	107±88.7
3 μg/kg IV	2412±394	0.00±0.00	11843±2219	407±68.6	83.8±91.6	30.3±74.3
10 μg/kg IV	1615±560	0.00±0.00	9520±2945	562±397	106±58.0	47.2±73.3
30 μg/kg IV	1558±713	0.00±0.00	10376±1265	474±328	175±143	65.8±72.8

Table VIII. Serum Glucose, Electrolyte, and Renal Function Tests

Dose/Route	Glucose (mg/dl)	Urea Nitrogen (mg/dl)	Creatinine (mg/dl)	Phosphorus (mg/dl)	Sodium (mEq/l)	Potassium (mEq/l)	Chloride (mEq/l)	Calcium (mg/dl)
0 μg/kg IB	239±176	19.7±2.50	0.62±0.19	13.2±3.94	145±3.58	8.08±2.09	98.2±3.25	11.5±1.41
3 μg/kg IB	129±48.6	20.7±2.58	0.67±0.05	13.8±1.13	146±2.58	5.98±0.44	98.0±2.83	11.0±0.44
10 μg/kg IB	117± 33.6	19.5±3.15	0.53±0.08	13.3±2.40	148±6.05	6.58±0.84	98.5±3.45	11.4±1.57
30 μg/kg IB	190±66.4	19.3±2.73	0.58±0.75	13.3±1.20	143.7±3.50	6.80±0.40	98.7±1.97	11.4±0.44
0 μg/kg IV	195±67.7	20.0±3.52	0.77±0.12	15.4±1.99	149±5.96	6.47±0.68	98.0±4.34	12.7±0.95
3 μg/kg IV	219±58.8	20.8±3.19	0.80±0.14	18.5±1.36	148±1.60	7.82±1.12	96. 7±1.97	12.1±1.50
10 μg/kg IV	217±59.8	20.8±1.60	1.93±2.83	16.7±2.09	149±2.04	6.78±0.90	98.0±2.10	12.3±0.83
30 μg/kg IV	238±115	20.3±2.42	0.75±0.14	16.0±1.92	148±3.22	7.22±1.50	96.8±1.94	12.3±0.96

Table IX. Liver Function Tests And Serum Proteins

Dose/Route	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	CPK	Total Bilirubin (units/l)	Alkaline Phosphatase (units/l)	ALT (SGPT; units/l)	AST (SGOT; units/l)	Cholesterol (mg/dl)
0 μg/kg IB	6.48±0.62	3.87±0.15	2.62±0.59	604±378	0.13±0.05	94.7±65.5	46.5±6.92	132±40.7	96.2±10.5
3 μg/kg IB	6.60±0.22	3.85±0.10	2.75±0.16	530±246	0.12±0.04	183±57.9	58.8±23.5	104±27.8	104±9.18
10 μg/kg IB	6.48±0.32	3.80±0.13	2.68±0.34	739±371	0.17±0.05	150±36.4	48.5±9.07	134±56.6	91.5±15.4
30 μg/kg IB	6.43±0.34	3.83±0.24	2.60±0.23	1357±1240	0.13±0.05	144±15.7	63.7±12.4	157±78.8	92.7±6.86
0 μg/kg IV	7.50±0.48	3.83±0.22	3.67±0.58	523±194	0.15±0.06	199±40.2	90.0±31.1	167±156	124±15.7
3 μg/kg IV	7.22±0.56	3.85±0.40	3.37±0.46	1190±1118	0.22±0.08	196±24.9	78.2±12.8	134±30.6	112±7.37
10 μg/kg IV	6.30±2.70	3.93±0.23	3.52±0.29	725±772	0.22±0.10	190±27.8	70.2±16.2	120±40.0	110±7.78
30 μg/kg IV	7.28±0.45	3.88±0.13	3.40±0.40	1222±1193	0.17±0.12	183±62.6	71.8±9.66	148.3±54.5	108±22.3

Dawley rats of this age, and were therefore not considered to be related to the administration of rhHB-EGF.

Similarly, there were no significant differences in complete blood count, hematocrit, hemoglobin, serum creatinine, blood urea nitrogen, total protein, total globulin, total cholesterol, calcium, phosphorus, sodium, potassium, chloride, bicarbonate, CPK, and liver function tests (SGPT, SGOT, total bilirubin, and alkaline phosphatase) between animals receiving any of the 3 doses of rhHB-EGF *vs.* those that received venicle alone, whether given by the IB or IV route.

CONCLUSION

The pharmacokinetics, distribution, and toxicity of rhHB-EGF were investigated following administration to female Sprague Dawley rats. Because rhHB-EGF is a potential clinical therapeutic for the treatment of IC, and because IB dosing has the potential for better delivery to bladder tissue and less systemic toxicity than IV dosing, both the IB and IV routes were investigated. The pharmacokinetics of [^{125}I] rhHB-EGF equivalents following IV administration were best described by a two compartment open linear model. Exposure to rhHB-EGF in the plasma as measured by AUCss was found to be dose dependent and the increase approximated proportionality. The lack of toxicity associated with IV or IB dosing of rhHB-EGF for 28 days provides evidence that either route may be useful for rhHB-EGF delivery, and the finding that bladder concentrations of rhHB-EGF were higher at both day 7 and day 28 following the IV *versus* IB administration at all dose levels (3, 10, and 30 μg/kg) strongly suggests that the IV route (or some other extra-bladder route) could be utilized for delivering rhHB-EGF to bladder tissue. One such method yet to be explored that has greater ease of administration is the subcutaneous route of administration, as used for other growth factors including insulin, erythropoietin, and granulocyte colony stimulating factor.

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