Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin

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Erythropoietin (EPO), a member of the type 1 cytokine superfamily, plays a critical hormonal role regulating erythropoietic production as well as a paracrine/autocrine role in which locally produced EPO protects a wide variety of tissues from diverse injuries. Significantly, these functions are mediated by distinct receptors: hemato-topoiesis via the EPO receptor homodimer and tissue protection via a heterodimer composed of the EPO receptor and CD131, the β common receptor. In the present work, we have delimited tissue-protective domains within EPO to short peptide sequences. We demonstrate that helix B (amino acid residues 58–82) of EPO, which faces the aqueous medium when EPO is bound to the receptor homodimer, is both neuroprotective in vitro and tissue protective in vivo in a variety of models, including ischemic stroke, diabetes-induced retinal edema, and peripheral nerve trauma. Remarkably, an 11-aa peptide composed of adjacent amino acids forming the aqueous face of helix B is also tissue protective, as confirmed by its therapeutic benefit in models of ischemic stroke and renal ischemia–reperfusion. Further, this peptide simulating the aqueous surface of helix B also exhibits EPO’s trophic effects by accelerating wound healing and augmenting cognitive function in rodents. As anticipated, neither helix B nor the 11-aa peptide is erythropoietic in vitro or in vivo. Thus, the tissue-protective activities of EPO are mimicked by small, nonerythropoietic peptides that simulate a portion of EPO’s three-dimensional structure.

In its hormonal role, the cytokine erythropoietin (EPO) is released by the kidney into the circulation in response to hypoxia and binds to a preformed receptor homodimer (EPO)2 present on the cell membrane of erythrocytic progenitors. Subsequently, a molecular cascade begins with the phosphorylation of Janus tyrosine kinase 2 and ultimately results in inhibition of programmed cell death, fostering the survival and maturation of erythroid precursors to erythrocytes [reviewed by Fisher (1)]. However, over the last 15 years, it has been discovered that EPO is also synthesized locally by many tissues, especially in response to metabolic stress. This pool of EPO acts as a multifunctional protective molecule [reviewed by Brines and Cerami (2)]. In this paracrine/autocrine role, EPO inhibits apoptosis in a wide variety of cell types and activates multiple mechanisms to protect stressed tissues, e.g., reducing inflammation and local edema. EPO also plays crucial roles during development (3). Therefore, it is not surprising that in the adult organism, EPO mediates multiple trophic effects, leading to accelerated healing and tissue regeneration. Finally, EPO has been shown to enhance cognition in normal (4) as well as diseased (5) human subjects.

The molecular interaction of EPO with the erythropoietic receptor (EPO)2 has been studied intensively, such that the regions of EPO that interact with (EPO)2 have been identified (Fig. L4). These include portions of helices A and C (site 2), as well as helix D and the loop connecting helices A and B (site 1) (6–9). Chemical or mutational modifications of amino acid residues within these two regions of EPO abolish its binding to (EPO)2, and, therefore, these modified EPOs are not erythropoietic in vivo or in vitro. Remarkably, a number of these modified EPOs retain potent tissue-protective properties (10).

These observations suggest that an additional receptor for EPO mediates tissue protection. This receptor is pharmacologically distinct from that of erythropoiesis, because it exhibits a lower affinity for EPO and forms distinct molecular species in cross-linking experiments (11). In prior studies, we have provided evidence that the receptor that promotes tissue protection is a heteromer composed of EPO and CD131, the β common receptor (βcR) (12). CD131 also forms receptor complexes with the α receptor subunits specific for GM-CSF, IL-3, and IL-5 and has been termed the “common” receptor [reviewed by Murphy and Young (13)].

Results from experiments showing that chemical modification of lysine residues or amino acid substitutions made within sites 1 and/or 2 do not affect tissue protection suggest that other regions of EPO contain the recognition site for the tissue-protective receptor. Notably, in aqueous media, EPO’s tertiary structure is relatively well defined because of the interaction of the hydrophobic content of its four α-helices, constraining the molecule into a compact, relatively rigid, globular structure. When EPO is bound to the hematopoietic receptor (14), helix B and parts of the AB and CD loops face the aqueous medium, away from the homodimer binding sites [Protein Data Bank (PDB) ID code 1EER; Fig. 1]. These regions do not contain lysine and therefore are not modified by carbamylation of EPO, a procedure that produces a selectively tissue-protective compound (10).

In view of these observations, we hypothesized that tissue protection, as distinct from erythropoiesis, depends on a region within helix B and/or loop AB within the EPO molecule.


Conflict of interest statement: M.B., C.B., M.Y., Q.-w.X., T.C., and A.C. were employees of Warren Pharmaceuticals when this work was performed. Warren Pharmaceuticals is developing erythropoietin analogues and tissue-protective compounds for potential clinical uses.

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However, HBP possessed potent neuroprotective activity comparable to EPO (Fig. 2A) and to CEPO (15) in a rat motoneuron model in vitro. In this model, the neurotoxic effects of the glutamate receptor agonist kainic acid were blocked by either HBP (1.8 nM) or EPO (3.3 nM). Because HBP is small and does not contain features designed to resist proteolysis or decrease clearance, its plasma half-life is presumably very short. It was therefore of great interest to determine whether HBP exhibited protective properties in vivo.

HBP was protective in a rat model of middle cerebral artery occlusion that has previously shown large protective effects for EPO (16), asialo-EPO (17), and CEPO (10) (Fig. 2B). In this experiment, HBP administered as a single i.v. dose [1.5 nmol/kg of body weight (bw)] reduced infarct volume as determined by tetrazolium salt staining 24 h after reperfusion. HBP was also associated with an improved behavioral outcome (foot faults of the saline group 24.6 ± 2 versus 16.4 ± 2 in the single dose HBP group and 14.5 ± 0.9 in the four-dose HBP group; P < 0.05 between saline and treated groups). Notably, additional doses of HBP administered i.p. at 2-h intervals for three additional doses did not further improve the extent of neuroprotection.

Confirming that HBP was neuroprotective in vitro and in vivo, we then assessed whether HBP possessed other properties consistent with EPO’s nonerythropoietic activities. For example, EPO reduces injury-related local edema in a number of tissues (18–21), including the retina (22). Specifically, in models of diabetic retinopathy, hyperglycemia produces endothelial injury, leading to vascular leakage and retinal edema. Further, it is notable that in a small, retrospective study of diabetic patients with macular edema, EPO treatment was associated with an increase in visual acuity and a decrease in retinal exudates (23).

To determine whether HBP could inhibit diabetes-related retinal edema, rats were administered streptozotocin. After the confirmation of the diabetic state, HBP (1.5 nmol/kg of bw) or saline was administered i.p. 5 days each week. After 3 weeks of hyperglycemia, retinal leakage in the HBP group (as assessed by extravasation of Evans blue dye) was not different from animals without diabetes (Fig. 2C). In contrast, retinas from animals that received only saline exhibited significant edema.

The results of these experiments showed that a peptide fragment of EPO comprising the amino acid sequence corresponding to helix B exhibited neuroprotective effects similar to EPO and its nonerythropoietic derivatives in a variety of in vitro and in vivo models. Previous study has shown that peptides can

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**Fig. 2.** Helix B peptide (HBP) is tissue protective in vitro and in vivo. (A) HBP protects against kainic acid (KA)-induced motoneuron excitotoxic death in vitro. Mixed anterior horn cultures obtained from the ventral horn of the spinal cord of 14-day rat embryos were treated on the sixth day in vitro by incubation for 48 h with kainic acid (5 μM) alone or in cotreatment with EPO or HBP. Data are means and SEM; ***, P < 0.001 compared with kainic acid alone. (B) HBP is neuroprotective in a stroke model. A single dose of HBP administered i.v. immediately after a 1-h arterial occlusion significantly reduces infarct volume determined after 24 h. Additional doses of HBP do not improve protection. Data are means and SEM; **, P < 0.01 compared with saline. (C) HBP (n = 16) prevents the development of retinal edema in a rat model of diabetes (PBS; n = 14). Results of three experiments are shown. Evans blue extravasation in normal retinas (n = 12) was 6 ± 0.2 ng/mg of dry retina. Data are means and SEM; **, P < 0.01.
be synthetized to mimic the helical structure of a protein that interacts with its receptor to reproduce the biological activities of the full molecule (24). Upon further consideration, however, we reasoned that because helix B is amphipathic and of the 4-3 α-helix type, specific amino acid residues within the hydrophilic portion face the external, aqueous face (i.e., every fourth and third residue in the b and f position, respectively). Spatially (but not linearly) adjacent residues therein could constitute a recognition site for the tissue-protective receptor.

Data obtained from crystallographic studies of EPO bound to (EPOR)2 show that the aqueous face of helix B consists of amino acids QEQERAL (PDB ID code IEER; Fig. 1B). Thus, a peptide derived from surface-simulation analysis of EPO should possess the biological activities of helix B. To test this hypothesis, a peptide was synthesized to include these surface amino acids as well as the three residues within the proximal portion of the BC loop that are relatively constrained by the rigid structure of the associated helices. The resulting 11-mer helix B surface peptide (HBSP: QEQERALNSS), unrelated in primary sequence to EPO, was thus intended to mimic a particular feature of EPO’s three-dimensional structure, notwithstanding the possible steric constraints of spatially and not linearly adjacent residues bonded directly together. We subsequently assessed whether this peptide was a nonerythropoietic, tissue-protective molecule.

HBSP, which does not contain site 1 or 2, was, as predicted, not erythropoietic in vitro (UT7-EPO cells) or in vivo in the rat (data not shown). However, HBSP was highly active in reducing the degree of injury observed in a sciatic nerve crush injury model to a degree identical on a molar basis to EPO and chemical derivatives of EPO that are not erythropoietic (10, 17). In this model, the sciatic nerve was reversibly compressed by using a ligature for a duration of 1 min and single doses of HBP or HBSP (0.3 nmol/kg of bw) administered i.v. immediately after removal of the constriction. The tissue-protective potency of these molecules was assessed by the static sciatic index as we have previously reported for asialo-EPO (17) and CEPO (10) and were found to be equivalent (Fig. 3). In contrast, an equimolar amount of a 20-mer fragment of pigment epithelium-derived factor (amino acids 102–121), derived from a biologically active region of this molecule (25), was inactive.

Thus, a peptide designed to mimic the external, aqueous face of helix B resembled EPO sufficiently to activate tissue-protective pathways. In the past, successful surface-simulation synthesis has been reported for antigenic determinants in proteins (26), as well as the surface of the α-helix of HIV-1 virus (27). Although there have also been claims that peptides exhibiting enzymatic activity can be synthesized from surface simulation analysis of the catalytic site of an enzyme (28), these have not been substantiated (29). In retrospect, it seems very unlikely that a small peptide could effectively reproduce the complex three-dimensional structure required for enzymatic activity, because binding sites for proper orientation of the substrate to the catalytic site require a rigid, three-dimensional scaffold not structurally attainable by using a small peptide. In the case of receptor-mediated biological activity, however, binding and activation of a receptor can topologically be much simpler. Hence, a number of examples exist of small peptides that reproduce the biological activity of a larger protein. For example, the clinically useful parathyroid hormone fragment (1–34) possesses the same biological activity as the full 84-aa protein (30). Further, it should be noted that a 17-mer peptide derived from a portion of EPO’s AB loop (residues 30–47) has been reported to possess neurotrophic activity (31). It is currently unclear whether this peptide interacts with the tissue-protective receptor subtype or mediates its biological effects by a different mechanism.

With respect to the primary structure of HBSP, however, it is well known that N-terminal glutamine residues can undergo a spontaneous, irreversible cyclization (particularly at room temperature under acidic conditions) into pyroglutamate (32). In confirmation of this fact, amino acid analysis of production batches of HBSP revealed that ~90% of the product possessed a free N-terminal glutamine, whereas the remainder was cyclized. Thus, HBSP was actually a mixture of two peptides. To determine whether pyroglutamate HBSP (pHBSP) was biologically active, it was synthesized de novo (Fig. 1B).

pHBSP (which was nonerythropoietic; see Figs. S2–S5) was evaluated in a rodent model of renal ischemia–reperfusion injury. Specifically, mice were randomized into five experimental groups and administered either vehicle or various amounts of pHBSP as an i.p. bolus at 1 min, 6 h, and again at 12 h after reperfusion. Twenty-four hours later, plasma creatinine and

![Fig. 3](image-url) HBSP is equipotent to HBP and EPO in a sciatic nerve injury model. PEDF, pigment epithelium-derived factor. Compounds were administered at a dose of 0.3 nmol/kg of bw i.v. immediately after a 1-min compression of the sciatic nerve at the level of the mid-thigh. Data are means and SEM plotted as the negative of the static sciatic index (SSI). n = 6–8 for each group; *** P < 0.001 compared with PBS.

![Fig. 4](image-url) Pyroglutamate Helix B surface peptide improves renal ischemia–reperfusion injury. Plasma creatinine (A), urea (B), and aspartate aminotransferase (AST) (C) were measured from mice (n = 12 each group) as biochemical markers of renal dysfunction and injury subsequent to sham-operation or renal ischemia–reperfusion injury (bilateral renal pedicle occlusion for 30 min). PBS or pHBSP (8.0 nmol/kg of bw) was administered i.p. 1 min, 6 h, and 12 h into reperfusion. Data represent mean and SEM; *** P < 0.001 versus PBS.
urea were obtained to estimate renal function and aspartate aminotransferase to assess injury. The results show a dose-dependent renoprotective effect, with the lowest dose administered (0.08 nmol/kg of bw) ineffective (Fig. 4). The degree of protection observed was similar to previous observations of EPO in this model (33).

Results of administering pHBS in a stroke model as a single i.v. dose (1.5 nmol/kg of bw) upon reperfusion after 1 h of occlusion, followed by three additional injections at 2-h intervals, demonstrated a significant reduction in infarct volume at 24 h [225 ± 20 mm³ for pHBS (n = 8)] compared with 291 ± 23 mm³ for saline (n = 7); P < 0.05] and an improvement in neurological function (saline group feet faults 20.2 ± 0.8 versus 11.2 ± 1.1 in the pHBS group; P < 0.001). In contrast, a scrambled version of HBSP (LSEQARNQSEL; n = 6) was biologically inactive (20.1 ± 2.1 foot faults; P < 0.05 versus the pHBS group). This observation provides additional support that the surface structure of helix B is specific for tissue-protective activities of EPO.

As noted above, EPO has also been observed to mediate other biological activities in addition to purely tissue-protective effects (reviewed in refs. 2 and 34). Among these pleiotropic effects, EPO accelerates wound healing and modulates cognitive function. For example, EPO has been observed to promote incisional wound closure in rodent models by reducing ischemic and reperfusion injury, mobilizing endothelial progenitor cells, augmenting angiogenesis, and decreasing inflammation (35). To determine whether pHBS could also provide benefits in wound healing, we examined its effect in the healing of punch biopsy wounds.

In this experiment, 3.5-mm-diameter full-thickness skin wounds were placed at the corners of a 3-cm-wide square on the shaved and depilated scapular region of the rat. pHBS (24 nmol/kg of bw) or PBS was administered s.c. daily for 10 days. The area of open wound, measured in a blinded fashion from serial digital photographs, exhibited faster healing in animals receiving pHBS 1 h before the first object exposure did not show enhancement. Because pHBS was effective only when administered after training, this molecule likely acts by intensifying the consolidation phase of memory acquisition.

In summary, using a variety of in vitro and in vivo models, we have shown that helix B of EPO has tissue-protective activities representative of the full molecule. Further, a peptide constructed to mimic the external, aqueous surface of EPO without primary sequence similarity recapitulates EPO’s tissue-protective, neurotrophic, and reparative properties. Peptide doses that exhibited tissue protection were similar on a molar basis to those observed for EPO and are higher than those required for EPO-mediated erythropoiesis. For example, in the renal ischemia model, 0.08 nmol/kg of bw (equivalent to ~300 units/kg of bw of EPO) was ineffective, whereas a 10-fold higher dose elicited strong tissue protection.

Finally, pharmacokinetic studies confirm that pHBS possesses a plasma half-life of ~2 min in the rat and rabbit (see SI Materials and Methods, Figs. S6 and S7, and Tables S1 and S2). It is especially notable that, similar to asialo-EPO (17), an agent present within the circulation for only a short time after i.v. dosing elicits protective effects equivalent to EPO or CEPO with plasma half-lives of 4–6 h. Tissue-protective peptides may therefore be of use as pharmacological reagents to delineate aspects of timing in tissue protection and trophic effects, in addition to potentially being of therapeutic benefit in a wide variety of clinical scenarios.

Materials and Methods
The animal protocols followed in this study were approved by the respective Animal Use and Care Committees of each institution in accordance with the directives of the Guide for the Care and Use of Laboratory Animals of the National Research Council or the Home Office Guidance on the operation of animals (Scientific Procedures) Act 1986 published by Her Majesty’s Stationery Office or in compliance with national (D.L. n. 116, G.U., suppl. 40, Feb. 18, 1992) and international laws and policies (EU Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987).

Materials. Peptides were obtained from commercial manufacturers. The UT-7 EPO hematopoietic assay (10, 17), motoneuron excitotoxicity study (15, 43),
middle cerebral artery occlusion model (44), and sciatic nerve compression injury (10) were performed as previously reported.

Diabetic Retinal Edema. Fasting male Sprague–Dawley rats weighing ~250 g were administered streptozotocin (60 mg/kg of bw) i.p., and diabetes was confirmed by a fasting blood glucose of ~250 mg/dl 2 days later. Diabetic animals were administered HBP (1.5 ml/kg of bw) or saline i.p. 5 days a week, while a third group of normal animals received saline. After 3 weeks, animals were anesthetized by using isoflurane, and Evans blue solution (45 mg/ml in saline; 7.5 mg/ml solution) was injected via the tail vein. Three hours later, the animals were reanesthetized, a small blood sample was obtained to determine plasma concentration of Evans blue, and each rat was perfused with pH 7.4 citrate buffer at 120 mmHg for 2 min and, thereafter, both eyes were immediately removed. Under an operating microscope, the eyes were bisected along the equator and the retinas were removed. The retinas were dissected at 60°C overnight in a vacuum, weighed, crushed in 120 ml of formamide, and incubated at 70°C for 18 additional hours. The retinal formamide solution was filter- centrifuged at 15,000 × g for 30 min to remove retinal debris. Evans blue concentration was determined by a background-subtracted absorbance at wavelengths of 620 nm (maximum) and 740 nm (minimum). Fluid extravasation was calculated as Evans blue (µg)/retina dry weight (g). Data were analyzed by analysis of variance (ANOVA) followed by Dunnett’s post hoc test comparison.

Renal Ischemia–Reperfusion Model. Sixty male C57/BL6 mice (~25 g; Charles River Laboratories) were anesthetized with ketamine (150 mg/kg) and xylazine (15 mg/kg) i.p. Each animal was placed on a homeothermic blanket set at 37°C, and after a mid-line laparotomy, the renal pedicles were clamped for 30 min by using nontraumatic microvascular clamps. PHBSP was administered at the indicated dose via i.p. injection at 1 min, 6, and 12 h after reperfusion. Twenty-four hours later, mice were anesthetized and blood was obtained by cardiac puncture. Plasma urea and creatinine were used as indicators of renal dysfunction and aspartate aminotransferase was used as an indicator of renal injury. Data were analyzed by ANOVA followed by Dunnett’s post hoc test comparison.

Wound Healing. Methods were adapted from the protocol of Padgett et al. (45). Male Sprague–Dawley rats (~200 g) were fasted from the evening before the procedure. Under isoflurane anesthesia, a 5 × 5 cm region of skin was shaved on the dorsum in the subcapular region and washed with 0.9% saline solution. Four full-thickness wounds (3.5-mm diameter) were placed at the corners of a square of 3-cm sides by using a biopsy punch. The wound edge was then infiltrated with 1% lidocaine solution and lidocaine-saturated gel foam was attached with adhesive tape. Wound assessment was obtained by serial digital photographs that included a 3.5-mm diameter standard. Area was determined by using digital planimetry and the four measurements were averaged. Data were analyzed by using a repeated-measures analysis.

Novel Object Recognition in Rats. This model is based on the greater spontaneous exploration of a novel object, compared with a familiar object, shown by rodents (46). Male Wistar rats were assessed for cognitive ability in a test apparatus comprising an open-field arena placed in a sound-attenuated room under dim lighting.

After a 5-min habituation period, each rat was placed into the test arena in the presence of two identical plastic shapes, and the time spent actively exploring the objects during a 5-min test period (T1) was recorded. The rat was returned to its home cage between tests. After 24 h, each rat was again placed in the test arena for 5 min (T2) in the presence of one of the familiar objects and a novel object, and the time spent exploring each object was again recorded. A recognition index for each object, the ratio of the time spent exploring either the familiar object or the novel object over the total time spent exploring both objects (during retention session T2), was used to measure cognitive (memory) function.

Rats (n = 8–12/group) were treated with the test compounds before the test period (T1), after T1, or chronically for 5 days before T1, via the i.p. route. Groups consisted of those that received vehicle, galantamine (3 mg/kg of bw) administered 1 h before the first 5-min exposure to the two identical objects to be learned, PHBSP (24 mmol/kg of bw) administered 1 h before the first 5-min exposure to the two identical objects to be learned, or PHBSP (24 mmol/kg of bw) administered 1 h before the first 5-min exposure to the two identical objects to be learned, or PHBSP (24 mmol/kg of bw) administered 1 h before the first 5-min exposure to the two identical objects to be learned. After 12 h for 5 days before training and then 12 and 24 h after training (the last dose was administered 1 h before the novel object exposure). Data were analyzed by ANOVA followed by Dunnett’s post hoc test comparison.

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Supporting Information

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SI Materials and Methods

Hematopoietic Potency. Peptides were tested in vitro for hematopoietic potency by use of the EPO-responsive human erythroleukemic cell line UT-7 EPO as previously described in detail (1, 2). The assay was performed over 48 h, and proliferation was quantified by using WST-1 reduction (Roche; no. 1644807). As shown in supporting information (SI) Fig. S1, HBP over the range of 5 pM to 50 nM did not increase cell number, in contrast to the large hematopoietic effect of EPO.

Additional experiments were performed in which the rat or rabbit received repeated injections of pHBSP. Specifically, pHBSP was administered twice daily i.v. to Sprague–Dawley rats for 28 days. For this study, nine male and nine female rats were assigned to Groups 1–4 receiving 0, 60, 180, and 600 μg/kg per dose (0, 48, 143, and 477 nmol/kg, respectively) of pHBSP in PBS by bolus i.v. administration from days 1–28. Blood samples to assay for hematological variables were collected on day 29. There was no difference between any of the groups in hemoglobin concentration (Fig. S2).

Further, pHBSP was administered twice daily i.v. to New Zealand White rabbits for 28 days. For this study, six males and six females were assigned to Groups 1 and 4, and four male and four female rabbits were assigned to Groups 2 and 3 receiving 0 (Group 1), 30 (24 nmol/kg; Group 2), 90 (72 nmol/kg; Group 3), and 300 (240 nmol/kg; Group 4) μg/kg per dose pHBSP in PBS by bolus i.v. administration. Comparison of baseline versus day 29 hematological parameters showed no difference in hemoglobin concentration, hematocrit, or platelet count (Figs. S3–S5). Thus, studies carried out in two species confirm that pHBSP is not erythropoietic in vivo.

Pharmacokinetics of pHBSP. Rat. The pharmacokinetic behavior of pHBSP was determined after a single i.v. dose to male Sprague–Dawley rats ported with bilateral jugular vein cannulae. Two groups, each of three rats, received either 60 μg/kg (48 nmol/kg; Group 1) or 180 μg/kg (143 nmol/kg; Group 2) of pHBSP diluted in PBS. The dose was administered over a period of 10–12 sec into one of two ports, and samples were withdrawn from the other port predose and at 2, 4, 6, 8, 10, 12, 14, 16, and 18 min after administration of the dose. Plasma concentrations of pHBSP were determined by using a liquid chromatography–mass spectrometry assay.

Plasma pHBSP concentration as a function of time after dose is shown in Fig. S6. Individual and group mean pharmacokinetic parameters are shown in Table S1. The mean peak drug concentration (C_{max}) values were 254.67 (±53.59) and 1,103.67 (±194.53) ng/ml for Groups 1 and 2, respectively. Both C_{max} and area under the concentrations vs. time curve (area under the curve; AUC) increased with increasing dose in a slightly more than dose-proportional manner, although the variability between animals within each dose group was high. The mean t_{1/2} was 0.028 h for Group 1 and 0.047 h for Group 2.

Rabbit. The pharmacokinetic behavior of pHBSP was determined after a single i.v. dose to male New Zealand White rabbits. Two groups, each with three rabbits, received either 30 μg/kg (24 nmol/kg; Group 1) or 90 μg/kg (72 nmol/kg; Group 2) of pHBSP diluted in PBS. The dosing solution was administered over a period of 10–12 sec via ear vein. Blood samples were collected from the contralateral ear by venipuncture of a central auricular artery or a marginal vein. Samples were withdrawn predose and at 2, 4, 6, 8, 10, 12, 14, 16, and 18 min postdose. Plasma pHBSP concentration as a function of time after dose is shown in Fig. S7. Individual and group mean pharmacokinetic parameters are shown in Table S2.

The mean C_{max} values were 95.10 (±44.34) and 200.67 (±52.92) ng/ml for Groups 1 and 2, respectively. Both C_{max} and AUC increased with increasing dose in a less than dose-proportional manner. Similar to the observations in rats, variability in measured plasma drug concentrations in rabbits was high with near overlap between C_{max} and AUC values between animals in the two dose groups. The mean t_{1/2} was 0.028 h for Group 1 and 0.038 h for Group 2.

Fig. S1. Peptide HBP has no effect on cell number in the UT-7 EPO assay. In contrast, EPO promotes cell growth and is, therefore, hematopoietic.
pHBSP is not erythropoietic in vivo. Sprague–Dawley rats administered peptide twice daily i.v. at the indicated dosages did not exhibit changes in hemoglobin concentration over a 28-day period.
Fig. S3. Rabbits administered pHBSP twice daily i.v. did not exhibit changes in hemoglobin concentration over 28 days of administration.
Fig. S4. No change in the hematocrit was observed in rabbits administered pHBSP twice daily for 28 days.
Fig. S5. Platelet count did not change over 28 days after the administration of pHBSP twice daily to rabbits.
Fig. S6. Mean plasma levels of pHBSP in rats over time after a single dose at the indicated amount. The mean half-life in the rat was estimated to be ~2 min.
Fig. S7. Mean plasma levels in rabbits over time after a single dose of pHBS at the indicated amount. The mean half-life in the rabbit was estimated to be \(-2\) min.
Table S1. Pharmacokinetic parameters after a single i.v. bolus dose of pHBSP in rats

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<tr>
<td>6</td>
<td>0.318</td>
<td>180.00</td>
<td>0.033</td>
<td>1,100.00</td>
<td>96.37</td>
<td>96.75</td>
<td>0.034</td>
</tr>
<tr>
<td>Mean</td>
<td>0.30</td>
<td>180.00</td>
<td>0.033</td>
<td>1,103.67</td>
<td>103.52</td>
<td>104.06</td>
<td>0.047</td>
</tr>
<tr>
<td>SD</td>
<td>0.02</td>
<td></td>
<td></td>
<td>194.53</td>
<td>27.69</td>
<td>27.47</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Nominal time and dosage used for pharmacokinetic analysis. Median is calculated for \( T_{\text{max}} \), the time at which \( C_{\text{max}} \) (maximum observed plasma concentration) occurs. AUC LAST, area under the curve from time 0 to last measured concentration; AUC inf, area under the curve from time 0 to infinity.
Table S2. Pharmacokinetic parameters after a single i.v. bolus dose of pHBSP in rabbits

<table>
<thead>
<tr>
<th>Subject</th>
<th>Body wt, kg</th>
<th>Dose, μg/kg</th>
<th>$T_{\text{max}}$, h</th>
<th>$C_{\text{max}}$, ng/ml</th>
<th>AUC LAST, h·ng/ml</th>
<th>AUC inf, h·ng/ml</th>
<th>$t_{1/2}$, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.51</td>
<td>30.00</td>
<td>0.033</td>
<td>126.00</td>
<td>16.33</td>
<td>16.50</td>
<td>0.027</td>
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<tr>
<td>2</td>
<td>3.00</td>
<td>30.00</td>
<td>0.033</td>
<td>115.00</td>
<td>16.36</td>
<td>16.52</td>
<td>0.029</td>
</tr>
<tr>
<td>3</td>
<td>3.07</td>
<td>30.00</td>
<td>0.033</td>
<td>44.30</td>
<td>4.72</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Mean</td>
<td>3.19</td>
<td>30.00</td>
<td>0.033</td>
<td>95.10</td>
<td>12.47</td>
<td>16.51</td>
<td>0.028</td>
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<tr>
<td>SD</td>
<td>0.28</td>
<td>44.34</td>
<td>6.71</td>
<td>0.01</td>
<td>0.002</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>2.99</td>
<td>90.00</td>
<td>0.033</td>
<td>212.00</td>
<td>22.20</td>
<td>22.33</td>
<td>0.039</td>
</tr>
<tr>
<td>5</td>
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<td>90.00</td>
<td>0.033</td>
<td>247.00</td>
<td>30.64</td>
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<tr>
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<td>90.00</td>
<td>0.033</td>
<td>143.00</td>
<td>18.03</td>
<td>18.17</td>
<td>0.030</td>
</tr>
<tr>
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<td>90.00</td>
<td>0.033</td>
<td>200.67</td>
<td>23.63</td>
<td>23.76</td>
<td>0.038</td>
</tr>
<tr>
<td>SD</td>
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<td>52.92</td>
<td>6.43</td>
<td>6.42</td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nominal time and dosage used for pharmacokinetic analysis. Median is calculated for $T_{\text{max}}$.

*Not enough data available to calculate given parameter.