Improving the pharmacokinetics/pharmacodynamics of prolactin, GH, and their antagonists by fusion to a synthetic albumin-binding peptide

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Abstract

To prolong the circulation half-life of human prolactin (hPRL), human GH (hGH), and their competitive antagonists, hPRL-G129R and hGH-G120R, we examined the effects of fusing a serum albumin-binding peptide (SA20) to their amino- or carboxyl-terminus. Fusion of the SA20 peptide to the amino-terminus of the ligands was less detrimental upon their ability to induce or inhibit signal transduction and cell proliferation *in vitro* than fusion to the carboxyl-terminus. Pharmacokinetic (PK) studies in mice revealed that the half-life of SA20-hPRL and SA20-hGH was prolonged and their clearance was reduced in comparison with hPRL and hGH. Pharmacodynamic (PD) studies in 8-week-old female mice

revealed that lobuloalveolar development in mammary glands was greater in all three groups (daily, every 2 days, or every third day over a 12-day period) of mice treated with SA20-hPRL (4 mg/kg) compared with hPRL (3.59 mg/kg). Similarly, daily administration (i.p.) of SA20-hGH (8 mg/kg) or hGH (7.15 mg/kg) to 23-day-old female mice over a 40-day period revealed the superiority of SA20-hGH over hGH as measured by weight gain, body length, and lobuloalveolar development in the mammary glands. These findings indicate that SA20 modification of hPRL, hGH, and their respective antagonists improves their PK/PD properties.

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Introduction

Human prolactin (hPRL) and GH are pituitary hormones that regulate the survival, proliferation, and differentiation of cells in a variety of tissues and endocrine glands. The effects of hPRL are mediated via the PRL receptor (PRLR; Goffin *et al.* 1994) and human GH (hGH) via the GH receptor (GHR; Ultsch *et al.* 1994) and PRLR (Cunningham & Wells 1991, Somers *et al.* 1994). Introduction of a single amino acid substitution into GH and PRL results in analogs that behave as competitive hormone antagonists (Chen *et al.* 1990, 1994, 1999, Fuh *et al.* 1992, 1993, Fuh & Wells 1995, Goffin *et al.* 1996, Ramamoorthy *et al.* 2001). Owing to their relatively low molecular weight and protein nature, these hormones and hormone antagonists are rapidly removed from circulation by renal clearance and proteolytic degradation within responsive tissues. For these reasons, recombinant hGH requires daily s.c. administration to maintain physiologically relevant plasma concentrations (Kastrup *et al.* 1983, MacGillivray *et al.* 1996).

To the best of our knowledge, there have been no attempts to enhance the pharmacokinetics (PK) of hPRL or its antagonist, hPRL-G129R. In contrast, this has been achieved for hGH by extending its absorption half-life

using sustained release formulations (Johnson *et al.* 1996, Tracy 1998, Brodbeck *et al.* 1999, Reiter *et al.* 2001, Cook *et al.* 2002, Jostel *et al.* 2005, Tae *et al.* 2005) and by extending its elimination half-life using polyethylene glycol (PEG) modification to reduce renal clearance (Clark *et al.* 1996, Cox *et al.* 2007, Webster *et al.* 2008). PEGylation has also been used to extend the circulation half-life of hGH antagonists (Thorner *et al.* 1999, Trainer *et al.* 2000, Muller *et al.* 2001, Ross *et al.* 2001). Unfortunately, the PEGylation process not only results in heterogeneous products that require additional purification but also decreases the accessibility of these ligands for receptor binding, thus reducing their efficacy (Clark *et al.* 1996, Ross *et al.* 2001).

Another strategy to enhance the PKs of hGH has been to fuse it with a large carrier protein such as an immunoglobulin's Fc fragment or serum albumin. Serum albumin is a macromolecular carrier protein that reversibly binds a number of low molecular weight molecules and transports them throughout the body. In humans, it has a long circulation half-life (19 days) because its high molecular weight prevents its removal, and the removal of bound molecules, by the kidney. Fusion of hGH to serum albumin was found to reduce its renal clearance and result in

an extended circulation half-life (Osborn *et al.* 2002). The drawbacks of fusing a ligand with a large carrier protein are that it is difficult to produce and may sterically hinder the ability of the ligand to interact with its receptor.

Recently, alternative approaches for extending the serum half-life of rapidly cleared proteins have been developed, which utilize serum albumin-binding peptides (Dennis *et al.* 2002, Holt *et al.* 2008). SA20 is one of many synthetic peptides discovered using phage display that binds to serum albumin in an apparently noncompetitive manner (Dennis *et al.* 2002). The 20 amino acid disulfide-constrained peptide (QRLIEDICLPRWGCLWEDDF) is primarily composed of lipophilic residues (Leu, Ile, Cys, Trp, Phe) and anionic residues (Glu, Asp), which contribute to increased serum albumin binding (Martinez-Gomez *et al.* 2006). The purpose of this investigation was to determine whether the PKs of hPRL, hGH, and their antagonists, hPRL-G129R and hGH-G120R, could be enhanced by fusing a small serum albumin-binding peptide, SA20, to their amino- or carboxyl-terminus. The rationale being that a low molecular weight serum albumin-binding peptide (2.2 kDa) would afford the same benefits as fusion to serum albumin (66.3 kDa) without the complications associated with its preparation, purification, and ultimately its potency since the interaction with serum albumin would be reversible in nature.

We report that the fusion of SA20 to the amino-terminus of hPRL resulted in minimal deleterious effects on its ability to induce signaling via the janus-activated kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) signaling cascade; it induced proliferation of Nb2 cells in a manner similar to that of unmodified hPRL *in vitro*; it increased its mean residence time (MRT) by reducing glomerulus filtration; and it enhanced its efficacy *in vivo* as measured by lobuloalveolar development within the mammary glands of mice. Fusion of SA20 to the amino-terminus of hGH reduced the potency of signaling via the STAT5 and Ras/Raf/MEK/mitogen-activated protein kinases (MAPKs) signaling cascades only threefold yet markedly enhanced its efficacy *in vivo*. Fusion of SA20 to the amino-terminus of hPRL-G129R and hGH-G120R reduced their potency as antagonists about fivefold and tenfold *in vitro* and likewise are expected to improve their efficacy *in vivo*.

Materials and Methods

Cell lines and reagents

T-47D human breast ductal carcinoma cells and IM-9 Epstein-Barr virus-transformed human B-lymphoblastoid cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Nb2-11 rat pre-T lymphoma cells (Nb2) were kindly provided by Dr Amea Walker and are now commercially available from Sigma-Aldrich. All reagents were purchased from Invitrogen unless otherwise stated. T-47D cells were maintained in RPMI

medium 1640 supplemented with 10% (v/v) fetal bovine serum (FBS) and 10 µg/ml gentamicin. IM-9 cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10 µg/ml gentamicin. Nb2 cells were maintained in RPMI medium 1640 supplemented with 10% (v/v) FBS, 10% (v/v) horse serum, 10 µg/ml gentamicin, and 0.1 mM 2-mercaptoethanol. The cell lines were incubated at 37 °C with humidity and 5% CO₂.

Construction of plasmids for expression of albumin-binding fusion protein

Plasmids encoding the cDNAs for hPRL, hGH, hPRL-G129R, and hGH-G120R were used as DNA templates for the PCR. PCR was performed using the Advantage-HF Kit (BD Biosciences, Palo Alto, CA, USA). All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Oligonucleotide primers for PCR were designed to incorporate restriction endonuclease cleavage sites upstream and downstream of the gene of interest. The PCR products were ligated with pCR2.1 T/A cloning vector and transformed into *Escherichia coli* TOP10 cells obtained from Invitrogen. Positive clones were identified by plating the cells on Luria-Bertani agar plates containing 100 µg/ml ampicillin and 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen), restriction endonuclease digested, and the DNA fragments were separated by electrophoresis. The NdeI-hPRL-BamHI, NdeI-hPRL-G129R-BamHI, NdeI-hGH-BamHI, NdeI-hGH-G120R-BamHI and BamHI I-hPRL-stop-XhoI, BamHI I-hPRL-G129R-stop-XhoI, BamHI I-hGH-stop-XhoI, and BamHI I-hGH-G120R-stop-XhoI DNA fragments were purified from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen). Double-stranded DNA encoding the serum albumin-binding peptide, SA20, was prepared by annealing five oligonucleotides together that had been treated with T4 polynucleotide kinase. The oligonucleotides were designed to create cohesive ends ready for ligation with restriction digested DNA utilizing optimal codons for expression in *E. coli*. The DNA fragments were ligated with NdeI and XhoI cut pET22b(+) (Novagen, Madison, WI, USA) to create pET22b-SA20-hPRL, pET22b-hPRL-SA20, pET22b-SA20-hPRL-G129R, pET22b-hPRL-G129R-SA20, pET22b-SA20-hGH, pET22b-hGH-SA20, pET22b-SA20-hGH-G120R, and pET22b-hGH-G120R-SA20. All restriction and modifying enzymes were purchased from New England BioLabs (Beverly, MA, USA).

Production and purification of albumin-binding fusion proteins

To define the nomenclature, SA20-hPRL is hPRL fused at its amino-terminus to the carboxyl-terminus of a serum albumin-binding peptide and has a molecular mass of ~25.7 kDa. In contrast, hPRL-SA20 is hPRL fused at its

carboxyl-terminus to the amino-terminus of a serum albumin-binding peptide and has a molecular mass of ~ 25.8 kDa. In addition to hPRL, SA20 derivatives of hPRL-G129R, hGH, and hGH-G120R were produced in *E. coli*, isolated from inclusion bodies, refolded, and purified as previously described (Langenheim *et al.* 2006). The purified proteins were separated by reducing SDS-PAGE and nonreducing SDS-PAGE gels and stained with SYPRO Orange (Molecular Probes, Eugene, OR, USA) to confirm their concentration and purity.

STAT5 and Erk1/2 phosphorylation assays

T-47D cells were trypsinized and resuspended in RPMI medium 1640 supplemented with 10% (v/v) charcoal/dextran-treated FBS (CSS; HyClone, Logan, UT, USA) and 10 $\mu\text{g}/\text{ml}$ gentamicin. Approximately 1×10^6 cells were seeded per well in 12-well tissue culture plates. The cells were grown overnight and depleted for 1 h in RPMI medium 1640 supplemented with 0.5% (v/v) CSS prior to treatment. IM-9 cells were harvested by centrifugation and resuspended in RPMI containing 0.5% CSS. The volume was adjusted with medium to yield a final concentration of 1×10^6 cells per ml. The cells were aliquoted (1 ml) into Eppendorf tubes and depleted for 1 h prior to treatment. T-47D and IM-9 cells were treated for 30 min at 37 °C with the indicated concentrations of hPRL, hPRL-G129R, hGH, hGH-G120R, or their albumin-binding SA20 derivatives. The cells were washed with ice cold PBS, lysed, clarified, and ~ 80 μg of the supernatants were used for western blotting as previously described (Langenheim *et al.* 2006). Blots were initially performed with a 1:1000 dilution of mouse anti-phospho STAT5A/B antibody (Upstate Biotechnology, Inc., Lake Placid, NY, USA) and 1:1333 dilution of mouse anti-phospho extracellular signal-regulated kinase (ERK, sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by a 1:2000 dilution of goat anti-mouse conjugated HRP (Bio-Rad). Blots were stripped and reprobed with a 1:1333 dilution of rabbit anti-STAT5 polyclonal antibody (sc-835), 1:1000 dilution of rabbit anti-ERK1 (sc-93), and 1:1000 dilution of rabbit anti-ERK2 (sc-154) from Santa Cruz Biotechnology followed by a 1:2000 dilution of goat anti-rabbit conjugated HRP (Bio-Rad).

Nb2 cell proliferation assay

Rat Nb2 cells were harvested by centrifugation and resuspended to a final concentration of 5.5×10^5 cells per ml in RPMI 1640 supplemented with 10% low lactogen horse serum (Cat. 2921154; MP Biomedicals, Irvine, CA, USA), 10 $\mu\text{g}/\text{ml}$ gentamicin, and 0.1 mM 2-mercaptoethanol. After depletion overnight, the cells were counted, harvested by centrifugation, and reseeded in flat-bottom 96-well plates at a density of 20 000 cells per well in 150 μl of medium. Treatments were prepared in culture medium and added to each well in 50 μl volumes. The cells

were incubated for 72 h, and the relative number of cells was determined by incubating the cells for 1–4 h with 40 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS) solution (CellTiter 96 AQueous non-radioactive cell proliferation kit; Promega) per well. The reduction in MTS by living cells was determined by measuring the absorbance at 492 nm using a GENios Pro microplate reader (Tecan, Durham, NC, USA). All assays were performed at least thrice in quadruplicate.

Pharmacokinetic studies in mice

Female FVB/N mice (Charles River Laboratories, Wilmington, MA, USA) 8 weeks of age and weighing 22–25 g were used for all PK studies. Mice were acclimated to a 12 h light:12 h darkness cycle for at least 1 week prior to the start of the studies, and provided LabDiet Prolab RMH 3000 feed (PMI Nutrition International, St Louis, MO, USA) and water *ad libitum*. All animal studies were approved by the Institutional Animal Care and Use Committee. For PK studies, groups of mice ($n=12$) were administered a single i.p. injection of hPRL (3.59 mg/kg), SA20-hPRL (4 mg/kg), hGH (3.57 mg/kg), or SA20-hGH (4 mg/kg) using 27 gage needles with a 0.4 mm bore and 13 mm length. Blood drippings (10–50 μl) from six mice were collected via tail sectioning in heparinized glass capillary tubes for each time point, which were determined empirically. The samples were centrifuged, and the plasma was harvested and stored at -80 °C until assayed.

Concentrations of hPRL and SA20-hPRL were measured using a commercial ELISA kit (Active Prolactin ELISA; DSL, Webster, TX, USA) determined to be insensitive to mouse PRL. Concentrations of hGH and SA20-hGH were measured using a commercial ELISA kit (Active GH ELISA; DSL) determined to be insensitive to mouse GH. hPRL and SA20-hPRL standards (2–200 ng/ml) and hGH and SA20-hGH standards (2.5–40 ng/ml) were prepared in the zero calibrator buffer provided with the kits and used to prepare standard curves. Dilutions of the plasma samples were determined empirically and were made in the zero calibrator buffers. Plasma concentrations were extrapolated from the standard curves prepared for each protein.

The plasma concentration (C_p) versus time (t) profiles were plotted, and noncompartmental methods were used to estimate basic PK parameters. The maximum plasma concentration (C_{max}) and the time at which maximum concentration occurred (T_{max}) were obtained by inspection of the C_p - t curve. The terminal elimination rate constant (k) was obtained from the terminal slope of the log-linear C_p versus t plots using the last three or four data points within the linear portion of their elimination phases. The terminal half-life ($t_{1/2}$) was calculated as $\ln 2/k$. The area under the serum concentration–time curve (AUC_{0-t}) and area under the first moment of serum concentration–time curve (AUMC_{0-t}) were calculated by numerical integration using the trapezoidal

rule. AUC_{∞} was estimated by dividing the observed concentration at the last time point ($C_{p \text{ last}}$) by k . $AUMC_{\infty}$ was determined as $C_{p \text{ last}} \times t_{\text{last}}/k + C_{p \text{ last}}/k^2$. The % AUC extrapolated (AUC_{extrap}) was a function of $(AUC_{0-\infty} - AUC_{0-t}) \times 100/AUC_{0-\infty}$ and is an indicator of completeness of the ADME profile. The MRT was calculated by dividing the $AUMC_{0-\infty}$ by $AUC_{0-\infty}$. The plasma clearance (CL) was calculated by dividing the dose by $AUC_{0-\infty}$. The volume of distribution at steady state (V_{dss}) was obtained from the product of CL and MRT. CL and V_{dss} values were normalized to animal weight. All calculations were made using Microsoft Excel.

Pharmacodynamic studies

Starting at 60 days of age, mice ($n=6$) were injected i.p. with hPRL, SA20-hPRL, or PBS once daily for 12 consecutive days (days 1–12), every other day (days 1, 3, 5, 7, 9, and 11), or every third day (1, 4, 7 and 10). Individual body weights of the mice were determined using an analytical balance, and the dose of hPRL administered was equimolar with the amount of hPRL in a 4 mg/kg dose of SA20-hPRL. Blood samples were not collected throughout the study because we did not want to stress the mice and potentially compromise the study. Mice were killed by cervical dislocation on day 13, and the right abdominal mammary glands were harvested to prepare whole mounts to assess mammary gland morphology as previously described (Ip & Asch 2000). The whole mounts were photographed under a dissecting microscope to obtain high resolution images of the glands and scored for alveolar development on a subjective scale of 1–8 (Hovey *et al.* 2005). The scoring system used was: 1) mature ducts with few side branches and no alveolar buds; 2) ducts with side branches and no alveolar buds; 3) ducts with few alveolar buds; 4) ducts with end buds and few alveoli; 5) ducts with limited alveolar development; 6) ducts with moderate alveolar development; 7) ducts with extensive alveolar development; and 8) ducts with alveolar development equivalent to that in glands from a midpregnant female mouse. The glands were also subjectively scored for lactiferous ductal dilation on a scale of 1–4. The left abdominal mammary glands were fixed in 4% PBS-buffered formaldehyde, paraffin embedded, and used to prepare hematoxylin and eosin (H&E)-stained slides. The slides were photographed under a light microscope and the images examined for alveolar-like structures and the presence of secretions within the alveoli.

Pharmacodynamic (PD) studies were also carried out for hGH and SA20-hGH. Starting at 23 days of age, groups of mice ($n=6$) were injected i.p. with hGH, SA20-hGH, or PBS daily for 40 consecutive days. The dose of hGH was equimolar with the amount in a 8 mg/kg dose of SA20-hGH. Individual body weights of the mice were determined daily until 44 days of age and three times a week thereafter. Mice were killed by cervical dislocation on day 63. Tail lengths were measured from anus to tail tip.

The right and left abdominal mammary glands were harvested, whole mounts and H&E-stained slides were prepared, and alveolar development and ductal dilation were scored as mentioned above.

Statistical analyses

Data were analyzed using Prism 4 for Windows software (GraphPad Software, La Jolla, CA, USA). Differences between independent variables were compared overtime and between treatment groups using two-way ANOVA followed by Bonferroni's multiple comparison test for each treatment. Differences between treatment groups at a single point in time were evaluated using one-way ANOVA followed by Bonferroni's multiple comparison test. Differences were considered significant when the P values were <0.05 .

Results

Construction, expression, and purification of serum albumin-binding derivatives of hPRL, hPRL-G129R, hGH, and hGH-G120R

SA20 derivatives of hPRL, hPRL-G129R, hGH, and hGH-G120R were recombinantly engineered by gene fusion. The cDNA encoding the mature form of these ligands were ligated with DNA encoding the SA20 peptide and cloned into an *E. coli* expression vector. Expression of each construct in *E. coli* resulted in a protein with the SA20 moiety attached to the amino- or carboxyl-terminus of hPRL, hPRL-G129R, hGH, or hGH-G120R via a Gly-Ser or Gly-Gly-Gly-Ser peptide linker respectively. The fusion proteins were isolated from inclusion bodies, refolded, and purified by anion exchange chromatography. The purified proteins migrated in accordance with their predicted molecular masses and exhibited $>90\%$ purity under reducing conditions using SDS-PAGE (Fig. 1A). The proteins were predominantly monomeric under nonreducing conditions using SDS-PAGE (Fig. 1B); however, a small portion of the proteins were dimeric.

Serum albumin-binding derivatives of hPRL and hGH induce signal transduction

Dose-response experiments were performed with hPRL, hGH, and their derivatives with SA20 fused to their amino- or carboxyl-terminus to determine their potency at inducing signal transduction. Treatment of PRLR-expressing T-47D cells with various concentrations of hPRL (Fig. 2A) or hGH (Fig. 2B) resulted in the phosphorylation of STAT5 in a dose-dependent manner. The potency of SA20-hPRL appeared to be equivalent to that of hPRL; in contrast, the potency of hPRL-SA20 was reduced, requiring approximately threefold higher molar concentration to achieve the same degree of

Figure 1 Reducing SDS-PAGE and nonreducing SDS-PAGE analysis of purified hPRL and hGH derivatives. hPRL, hPRL-G129R (G129R), hGH, hGH-G120R (G120R), and their derivatives formed by fusing SA20 to their amino-terminus were separated by reducing SDS-PAGE (A) or nonreducing SDS-PAGE (B) on 12% polyacrylamide gels and stained with SYPRO Orange to confirm their size and purity.

STAT5 phosphorylation observed for hPRL (Fig. 2A). The potency of SA20-hGH at activating STAT5 and Erk1/2 was reduced approximately threefold in comparison with hGH; whereas, the potency of hGH-SA20 was reduced approximately tenfold (Fig. 2B). To determine whether the SA20 derivatives of hGH also retain the ability to bind to GHRs and activate signaling, we examined the phosphorylation of STAT5 in IM-9 cells. Treatment with various concentrations of hGH resulted in the phosphorylation of STAT5 in a dose-dependent manner (Fig. 2C). The potency of SA20-hGH appeared to be approximately tenfold less than that of hGH, and hGH-SA20 appeared to be far less potent (Fig. 2C).

Serum albumin-binding derivatives of hPRL-G129R and hGH-G120R competitively inhibit hPRL and hGH-induced signal transduction

To determine whether the SA20 derivatives of hPRL-G129R and hGH-G120R retain the ability to antagonize PRLR-mediated signal transduction, the phosphorylation of signaling molecules associated with the JAK2/STAT5 and Ras/Raf/MEK/MAPK signaling cascades were examined

in T-47D cells. Treatment with various concentrations of hPRL-G129R (Fig. 2D) or hGH-G120R (Fig. 2E) resulted in a dose-dependent inhibition of hPRL and hGH-induced STAT5 phosphorylation respectively. The potency of SA20-hPRL-G129R was fivefold less than that of hPRL-G129R; in contrast, the potency of hPRL-G129R-SA20 was reduced more than tenfold (Fig. 2D). The potency of SA20-hGH-G120R at inhibiting STAT5 and Erk1/2 phosphorylation was reduced approximately tenfold in comparison with hGH; whereas, the potency of hGH-G120R-SA20 was reduced even more so (Fig. 2E).

Serum albumin-binding derivatives of hPRL induce proliferation of rat Nb2 cells in a dose-dependent manner

The biological activity of SA20-hPRL and hPRL-SA20 relative to hPRL and of SA20-hGH and hGH-SA20 relative to hGH were evaluated by measuring their ability to induce proliferation of rat Nb2 cells, which proliferate in response to lactogenic hormones such as hPRL and hGH. The relative number of viable cells was determined after 72 h treatment by colorimetrically measuring the reduction of MTS by living cells. Treatment of the cells with increasing doses of SA20-hPRL or hPRL-SA20 (Fig. 3A) stimulated cell proliferation in a dose-dependent manner as did SA20-hGH and hGH-SA20 (Fig. 3B). SA20-hPRL and SA20-hGH were not as potent as hPRL and hGH, but were more potent than hPRL-SA20 and hGH-SA20 at stimulating cell proliferation. Proliferation induced by these ligands correlated well with their ability to activate signal transduction in T-47D cells, consistent with the bioactivity of SA20-hPRL and SA20-hGH being superior to that of hPRL-SA20 and hGH-SA20.

Bioanalysis

Before performing PK studies, it was necessary to confirm that SA20 derivatives of hPRL and hGH could be detected using commercial immunoassays. The ELISA kits tested showed specificity for hPRL or hGH with no cross-reactivity with mouse PRL and GH respectively. The ELISA kits recognized the SA20 derivatives of hPRL and hGH equally well with sufficient sensitivity (data not shown). Examination of the stability of hPRL, SA20-hPRL, or hPRL-SA20 in plasma revealed negligible metabolism indicating that plasma decay is not a major player in clearance (data not shown).

Pharmacokinetics of hPRL, SA20-hPRL, hGH, and SA20-hGH

To examine PK behavior, female FVB/N mice were i.p. administered a single dose of hPRL (3.59 mg/kg), SA20-hPRL (4 mg/kg), hGH (3.57 mg/kg), or SA20-hGH (4 mg/kg). The plasma concentrations (C_p) were monitored over time (t), and a linear plot of C_p versus t was used to visually emphasize the initial absorption, distribution, metabolism, and elimination phases (Fig. 4, insets);

Figure 2 Dose-dependent activation of signaling induced by hPRL, hGH, and their SA20 derivatives and dose-dependent inhibition of signaling by hPRL-G129R, hGH-G120R, and their SA20 derivatives. T-47D and IM-9 cells were serum starved as described in the Materials and Methods. For PRLR-mediated agonism, T-47D cells were treated with increasing doses of hPRL, SA20-hPRL, or hPRL-SA20 (A) or increasing doses of hGH, SA20-hGH, or hGH-SA20 (B), and the response of the signaling molecules was examined after 30 min. For GHR-mediated agonism, IM-9 cells were treated with increasing doses of hGH, SA20-hGH, or hGH-SA20 (C), and the response of the signaling molecules was examined after 30 min. For PRLR antagonism, T-47D cells were treated with 5 nM hPRL and increasing doses of hPRL-G129R (G129R), SA20-hPRL-G129R (SA20-G129R) or hPRL-G129R-SA20 (G129R-SA20) (D) or 5 nM hGH, and increasing doses of hGH-G120R, SA20-hGH-G120R or hGH-G120R-SA20 (E), and the response of the signaling molecules was examined after 30 min. Cell lysates were harvested, and 80 µg of lysate from each treatment was separated by reducing SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed for activated Erk1/2 (p-ERK1/2), activated STAT5 (p-STAT5), and total STAT5 (STAT5) to confirm equal loading. Data shown are representative of three experiments.

Figure 3 The effects of hPRL, hGH, and their SA20 derivatives on Nb2 cell proliferation. Rat Nb2 cells were treated with various doses of hPRL, SA20-hPRL or hPRL-SA20 (A) or treated with various doses of hGH, SA20-hGH or hGH-SA20 (B) for 72 h as described in the Materials and Methods. Cell proliferation was assessed colorimetrically by measuring the reduction of MTS. Each data point represents the mean $\pm$ s.d. of at least three independent experiments performed in quadruplicate.

a semi-logarithmic plot of C_p versus t (Fig. 4) was used to emphasize the terminal elimination phase and to derive PK parameters. The PK parameters were determined using noncompartmental methods and are summarized in Table 1.

Absorption of hPRL and SA20-hPRL into the bloodstream differed, reaching their maximal observed concentration after $\sim 43.2 \pm 6$ min and 1 h 42 ± 13.2 min respectively. Absorption of hGH and SA20-hGH into the bloodstream also differed, reaching their maximal observed concentration after $\sim 40.2 \pm 12$ min and 1 h 49 ± 7.2 min respectively. The observed maximum concentration achieved by SA20-hPRL was only slightly higher than that of hPRL (Fig. 4A and Table 1); in contrast, the observed maximum concentration achieved by SA20-hGH was significantly higher than that of hGH (Fig. 4B and Table 1), indicating that the SA20 derivatives of hPRL and hGH were absorbed into the blood stream more slowly. The area under the plasma concentration–time curve of SA20-hPRL was increased approximately fivefold in comparison with hPRL,

and SA20-hGH was increased ~ 66 -fold in comparison with hGH, with the difference reflecting alterations in the distribution and terminal elimination phase of SA20-hPRL (Fig. 4A). The differences also reflected alterations in the absorption of SA20-hGH (Fig. 4B).

The elimination patterns for SA20-hPRL and SA20-hGH had two distinct phases (α and β). Elimination in the second phase occurred according to first-order kinetics, i.e. a linear relationship was observed between the semi-log plot of drug concentration versus time (Fig. 4). This β -phase was used to determine the elimination half-life of the proteins. The terminal elimination half-life of SA20-hPRL was increased ~ 5 –7-fold in comparison with hPRL, and SA20-hGH was increased approximately fourfold in comparison with hGH (Table 1). This extension in terminal elimination half-life is reflected by a reduction in the clearance of SA20-hPRL by approximately fivefold and SA20-hGH by ~ 68 -fold (Table 1). The reduction in clearance from 210.27 ± 12.26

Figure 4 Mean plasma concentration–time profiles of hPRL, SA20-hPRL, hGH, and SA20-hGH after i.p. administration to mice. A single dose of hPRL (3.59 mg/kg) and SA20-hPRL (4 mg/kg) (A), or hGH (3.57 mg/kg) and SA20-hGH (4 mg/kg) were i.p. administered to female FVB/N mice, and their plasma concentrations determined as described in Materials and Methods. Semi-log plot of plasma concentration versus time profiles for hPRL and SA20-hPRL (A), or hGH and SA20-hGH (B). Insets in A and B show the plasma concentration versus time profile. Points, time point from individual mice; line, mean of six mice per time point.

Table 1 Pharmacokinetic parameters estimated for human prolactin (hPRL), SA20-hPRL, human GH (hGH), and SA20-hGH after a single treatment of female FVB/N mice. Plasma clearance profiles of the proteins were derived as described in the Materials and Methods. Values represent mean ± s.e.m. (n=6)

	hPRL	SA20-hPRL	hGH	SA20-hGH
Compound				
Route of administration	i.p.	i.p.	i.p.	i.p.
Dose (mg/kg)	3·59	4	3·57	4
C _{max} (ng/ml)	14 556 ± 1260	17 501 ± 1339	15 020 ± 4675	186 526 ± 28 714
T _{max} (h)	0·72 ± 0·10	1·67 ± 0·22	0·67 ± 0·20	1·83 ± 0·68
AUC _{0-∞} (ng h/ml)	19 078 ± 1134	94 060 ± 5490	18 617 ± 4426	1 221 657 ± 154 051
AUC _{extrap} (%)	0·09 ± 0·05	0·04 ± 0·02	0·00 ± 0·00	0·00 ± 0·00
t _{1/2 term} (h)	0·84 ± 0·13	4·78 ± 0·49	0·49 ± 0·02	1·94 ± 0·12
CL/W (ml/h per kg)	210·27 ± 12·26	42·65 ± 2·59	203·05 ± 58·71	2·97 ± 0·43
V _{dss} /W (ml/kg)	263·01 ± 15·18	191·33 ± 17·44	233·14 ± 83·63	14·80 ± 2·38

C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; AUC, area under the concentration–time curve; AUC_{extrap}, percentage of total AUC extrapolated; t_{1/2 term}, terminal elimination half-life; CL, clearance; V_{dss}, volume of distribution at steady state.

to 42·65 ± 2·59 ml/h per kg by addition of the serum albumin-binding peptide to the amino-terminus of hPRL, and reduction from 203·05 ± 58·71 to 2·97 ± 0·43 ml/h per kg by the addition of the serum albumin-binding peptide to the amino-terminus of hGH is consistent with an ‘effective’ increase in molecular size. Distribution studies using iodinated hPRL and SA20-hPRL or hGH and SA20-hGH were not performed; however, the volume of distribution at steady state for hPRL and hGH was observed to be much larger than the plasma volume; in contrast, the volume of distribution of SA20-hPRL and SA20-hGH was reduced 1·4- and 15·7-fold respectively (Table 1).

Pharmacodynamics of hPRL and SA20-hPRL

We examined the biological activity of SA20-hPRL on mammary gland alveolar development in comparison with hPRL and PBS controls. Groups of FVB/N female mice (n=6) were i.p. administered 4 mg/kg SA20-hPRL, an equimolar amount of hPRL, or PBS daily, every other day, or every third day over a period of 12 days. No adverse side effects with respect to weight loss, behavioral changes, respiration, or condition of fur or eyes were observed in the mice after administration of hPRL or its serum albumin-binding derivative. Both hPRL and SA20-hPRL increased alveolar development in the mammary glands in a dosing-dependent manner (Fig. 5A), the more often they were treated, the greater the extent of alveolar development. The groups receiving SA20-hPRL consistently had more alveolar development than the groups receiving hPRL, and secretions consistent with milk proteins were observed (Fig. 5C). Mice receiving SA20-hPRL every other day exhibited more alveolar development than mice receiving hPRL daily (Fig. 5A and D). Significant alterations were also observed in the dilation of the lactiferous ducts in the group of mice receiving SA20-hPRL daily (Fig. 5B and E).

Pharmacodynamics of hGH and SA20-hGH

Female FVB/N mice (n=6) were i.p. administered 8 mg/kg SA20-hGH, an equimolar amount hGH, or PBS in a volume of 200 µl for 40 consecutive days. No adverse side effects were observed in the mice after administration of hGH or SA20-hGH. Significant increases in mammary gland alveolar development were observed in the mice treated with hGH or SA20-hGH in comparison with PBS (Fig. 6A and D); however, the alterations were not statistically different between hGH and SA20-hGH using the subjective scoring system (Fig. 6D). Mammary gland ductal dilation was significantly increased in the mice treated with hGH or SA20-hGH in comparison with PBS (Fig. 6B and E), likely attributable to the secretion of milk proteins (Fig. 6C). The ductal dilation was significantly increased in the mice treated with SA20-hGH compared with hGH (Fig. 6E), likely attributable to more alveoli secreting milk proteins (Fig. 6C). Extremely significant changes were also observed in the length of the tails (Fig. 6F) and overall body weight (Fig. 6G) of mice treated with hGH or SA20-hGH in comparison with PBS. In comparison with mice receiving PBS, a significant weight gain was observed in the mice after the sixth and seventh day of treatment with SA20-hGH and hGH respectively (Fig. 6G). A significant difference in the body weights of mice treated with SA20-hGH in comparison with hGH was observed after 20 days of treatment (Fig. 6G).

Discussion

Numerous therapeutic indications have been proposed for recombinant hPRL including the support and sustainment of lactation (Page-Wilson *et al.* 2007); enhancement of the lymphopoietic portion of the immune system when under dysregulation (Richards & Murphy 2000, Sun *et al.* 2004); recovery from myelosuppression caused by chemotherapy

Figure 5 Anatomical and histological analysis of the fourth inguinal mammary gland isolated from mature nulliparous mice treated with PBS, hPRL, or SA20-hPRL. Groups of 8-week-old nulliparous FVB/N female mice ($n=6$) were treated (i.p.) with hPRL (3.59 mg/kg), SA20-hPRL (4 mg/kg), or PBS everyday, every other day, or every third day over a period of 12 days. Representative whole mount stainings of the mammary gland distal (A) or proximal (B) to the nipple used to score for alveolar development (D) and ductal dilation (E) respectively as described in Materials and Methods. (C) Representative H&E staining of paraffin sections of the mammary glands at 40 $\times$ and 400 $\times$ resolution. Data are means $\pm$ S.E.M. ($n=6$ glands per treatment). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared with PBS.

(Woody *et al.* 1999a, Zhang *et al.* 2005a), bone marrow transplant (Woody *et al.* 1999b, Sun *et al.* 2003), burn injury (Dugan *et al.* 2002), or immunosuppressive disease (Richards *et al.* 1998); adoptive immunotherapy for cancer (Oberholtzer *et al.* 1996, Majumder *et al.* 2002, Zhang *et al.* 2005b) or pathogen-induced infection (Meli *et al.* 1996); and use as a B-cell adjuvant *in vivo* (Richards *et al.* 1998, Zhang *et al.* 2007). Likewise, studies indicate that hGH may support and

sustain lactation in women suffering from lactation insufficiencies (Gunn *et al.* 1996, Milsom *et al.* 1998); it may have similar effects on multilineage human hematopoiesis (Hanley *et al.* 2005); and it may be useful for recovering from myelosuppression (Chen *et al.* 2003, Carlo-Stella *et al.* 2004); in addition to its current indications for GH deficiency and other growth disorders (Hardin *et al.* 2007).

PRLR antagonists have also been sought for their potential anticancer activity, especially in breast cancer (Fuh & Wells 1995, Reynolds *et al.* 1997, Chen *et al.* 1999), but may also have utility for the treatment of immune diseases such as rheumatoid arthritis (Figuerola *et al.* 1997), systemic lupus erythematosus (McMurray *et al.* 1995, Peeva *et al.* 2004), and Reiter's syndrome (Bravo *et al.* 1992). The PRLR antagonist, hPRL-G129R, is a rationally designed antagonist (Goffin *et al.* 1996, Chen *et al.* 1999) based on homology modeling and on prior knowledge of bGH-G119R (Chen *et al.* 1990, 1991a,b,c, 1995) and hGH-G120R (Fuh *et al.* 1992, 1993, Chen *et al.* 1994, Fuh & Wells 1995). Carcinogen-treated and syngeneic murine models of mammary carcinoma (Zhang *et al.* 2002, Beck *et al.* 2003, Tomblyn *et al.* 2005), xenografts of established breast cancer cell lines in athymic nude mice (Chen *et al.* 2002), and clonogenic survival assays of breast cancer cell lines and clinical tumors (Howell *et al.* 2008) predict that hPRL-G129R will have clinical utility. *In vitro* studies also suggest that hPRL-G129R will enhance the effectiveness of conventional breast cancer chemotherapies (Chen *et al.* 1999, Howell *et al.* 2008, Scotti *et al.* 2008).

To improve the therapeutic efficacy of hPRL-G129R, we have fused it to a bacterial toxin to enhance tumor cytotoxicity (Langenheim & Chen 2005), interleukin 2 to stimulate an antitumor immune response (Zhang *et al.* 2002), and endostatin to prevent tumor angiogenesis from occurring (Beck *et al.* 2003). The enhanced antitumor efficacy observed for the latter two fusion proteins may not only reflect their dual functionality but also an alteration in their PKs. The larger size of these fusion proteins reduces their renal clearance and extends their circulation half-life (Zhang *et al.* 2002, Beck *et al.* 2003).

In this study, we examined whether the fusion of a small serum albumin-binding peptide (SA20) to PRL receptor agonists (hPRL, hGH) and antagonists (hPRL-G129R, hGH-G120R) would afford the same benefits as fusion to serum albumin. Serum albumin naturally extends the circulation half-life of bound molecules by preventing their renal clearance and enhances their efficacy by distributing them throughout the body. The use of a low molecular weight serum albumin-binding peptide (2.2 kDa) as opposed to direct fusion to serum albumin (66.3 kDa) may reduce the complications associated its preparation, purification, and ultimately its potency since the interaction with serum albumin would be reversible in nature.

Production and purification of the fusion proteins with SA20 fused to the amino- or carboxyl-terminus of PRLR agonists or antagonists required no alterations to our standard

Figure 6 Anatomical and histological analysis of the fourth inguinal mammary gland and measurements of tail length and body weight in mature nulliparous mice treated with PBS, hGH, or SA20-hGH. Groups of nulliparous FVB/N female mice ($n=6$) were treated (i.p.) with hGH (7.15 mg/kg), SA20-hGH (8 mg/kg), or PBS daily beginning at 24 days of age and ending at 63 days of age. Representative whole mount stainings of the mammary gland distal (A) and proximal (B) to the nipple used to score for alveolar development (D) and ductal dilatation (E) respectively as described in Materials and Methods. Data are means $\pm$ S.E.M.; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared with PBS. (C) Representative H&E staining of paraffin sections of the mammary glands at 40 $\times$ and 400 $\times$ resolution. (F) Tail length measurements at the end of the study. Points, mean tail length of five mice; bars, S.E.M.; ***, indicate a statistically significant difference ($P\leq 0.001$) after 39 days of treatment compared with PBS controls. (G) Body weight measurements throughout the study. Points, mean weight of six mice per time point; bars, S.E.M.; ***, represents a statistically significant difference ($P<0.001$) occurring after 5 days of hGH or 4 days of SA20-hGH treatment compared with PBS; **, represents a statistically significant difference ($P<0.01$) between hGH and SA20-hGH treatment occurring after 28 days (51 days of age).

production and purification protocols, allowing high yields of the correctly refolded proteins to be recovered. SA20 derivatives exhibited similar purity to their unmodified counterparts and migrated in accordance with their predicted molecular mass on reducing SDS-PAGE gels (Fig. 1A). All of the proteins migrated predominantly as monomers on non-reducing, non-denaturing SDS-PAGE gels (Fig. 1B). Consistent with the SA20 moiety being highly anionic, the SA20 derivatives exhibited increased mobility on native PAGE gels in comparison with their unmodified counterparts (data not shown), so much so that the loss in mobility due to the addition of SA20 was offset by the gain in mobility from its anionic charge.

SA20-hPRL and SA20-hGH were more potent activators of signal transduction than hPRL-SA20 and hGH-SA20 (Fig. 2A–C). Likewise, SA20-hPRL-G129R and SA20-hGH-G120R were more potent antagonists of signal transduction than hPRL-G129R-SA20 and hGH-G120R-SA20 (Fig. 2D and E). The bioactivity of SA20-hPRL was about threefold less than hPRL in the Nb2 assay; whereas, the bioactivity of hPRL-SA20 was about 30-fold less (Fig. 3A). The bioactivity of hGH was affected even less by the serum albumin-binding peptide, with the SA20-hGH similarly retaining more bioactivity than hGH-SA20 (Fig. 3B). Relative to unmodified hPRL, the reduction in bioactivity for SA20-hPRL was similar to that reported for serum albumin fused to hGH *in vitro* (Rosen & Haseltine 2006). In contrast, hGH modified by PEGylation retains little bioactivity *in vitro* (Jacobs *et al.* 2006), indicating that the strategy of serum albumin binding can have less of an effect on the ligand/receptor interactions than the strategy of PEGylation.

Although the fusion of a small peptide to the carboxyl-terminus of these ligands was detrimental to their activities *in vitro*, consistent with the observations of others (Goffin *et al.* 1993), a more favorable PK profile could compensate for their reduced potency, so we examined the PKs of both SA20-hPRL and hPRL-SA20 in mice. The PK behavior of SA20-hPRL and hPRL-SA20 was very similar, indicating that SA20 retains its ability to interact with serum albumin when fused to the amino- or carboxyl-terminus. SA20-hPRL was absorbed slightly slower than hPRL-SA20; its area under the plasma concentration–time curve was greater, and its duration during the first 24 h appeared more favorable than hPRL-SA20 (data not shown). For these reasons, we focused our efforts on examining the PKs and PDs of SA20-hPRL and SA20-hGH in comparison with unmodified hPRL and hGH respectively.

i.p. administered hPRL and hGH were rapidly cleared and exhibited a large volume of distribution at steady-state consistent with hPRL and hGH being widely distributed and/or metabolized (Table 1). SA20-hPRL and SA20-hGH were absorbed slower than hPRL and hGH, and remained in circulation nearly six times longer ($t_{1/2}=4\text{ h }46\text{ min}\pm 29\text{ min}$ versus $50\pm 6\text{ min}$) and four times longer ($t_{1/2}=1\text{ h }56\text{ min}\pm 7\text{ min}$ versus $29\pm 1\text{ min}$) respectively. Clearance of

SA20-hPRL was reduced about fivefold, and its volume of distribution at steady state was reduced only 25%, indicating that binding to serum albumin reduces clearance while having only a modest effect on tissue distribution and/or metabolism. In contrast, clearance of SA20-hGH was reduced about 66-fold, and its volume of distribution at steady state was reduced 94%, suggesting that a majority of it was retained in the circulatory system. These favorable PK alterations afforded by serum albumin binding led us to believe that it should improve efficacy of hPRL and hGH, and possibly reduce the frequency of dosing.

Morphological alterations in the mammary glands of virgin female mice were examined after being administered hPRL or SA20-hPRL every third day, every second day, or daily over a 12-day period. Only modest alveolar development was observed in the mice receiving hPRL daily (Fig. 5A); in contrast, alveolar development was more extensive in mice receiving SA20-hPRL daily, resulting in ductal dilation (Fig. 5B and E) and secretions consistent with milk proteins (Fig. 5A and C). No morphological changes were observed in mice receiving hPRL every other day; whereas, mice receiving SA20-hPRL every other day exhibited morphological alterations similar to those observed in mice receiving hPRL daily (Fig. 5A and D). Since the Nb2 bioassay revealed that SA20-hPRL had at least a threefold lower potency than unmodified hPRL (Fig. 3A), the enhanced alveolar development *in vivo* indicates that the favorable PK profile of SA20-hPRL compensates for its reduced *in vitro* potency and contributes to an *in vivo* PD response that is of greater magnitude and longer duration than the response to hPRL.

Even though signaling via the PRLR (Fig. 2B) and GHR (Fig. 2C) were reduced more than threefold and tenfold *in vitro* respectively the *in vivo* PD response to daily injections of SA20-hGH was greater in magnitude than the response to hGH using alveolar development (Fig. 6A and D), ductal dilation (Fig. 6B and E), and milk protein production (Fig. 6C) as lactogenic biomarkers and gains in body size (Fig. 6F) and body weight (Fig. 6G) as somatogenic biomarkers. These results suggest that the enhanced PDs of SA20-hGH is related to its extended circulating half-life and reduced clearance.

In summary, genetic fusion of a serum albumin-binding peptide to the amino-terminus of hPRL, hPRL-G129R, hGH, and hGH-G120R appears to be an economically feasible alternative to the strategies of fusion to a large carrier protein or modification with PEG to reduce clearance and enhance half-life. This strategy required no alterations to the standard procedures for producing and purifying the proteins; it had minimal deleterious effects upon signal transduction and bioactivity *in vitro*; it resulted in favorable tissue distribution of hPRL and hGH; and it is amendable to further modifications such as sustained release formulations to further enhance the effectiveness of these proteins by reducing the frequency of injections.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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