Expert Opinion

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Recombinant human hyaluronidase (rHuPH20): an enabling platform for subcutaneous drug and fluid administration

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The extracellular matrix is a significant barrier to the effective subcutaneous delivery of many drugs, limiting both pharmacokinetic parameters and injection volumes. The space outside adipocytes in the hypodermis is not a fluid, but rather a solid extracellular matrix of collageneous fibrils embedded within a glycosaminoglycan-rich viscoelastic gel that buffers convective forces. The extracellular matrix limits the volume of drug that can be injected at a single site, as well as the rate and amount that reach the vascular compartment. A fully human recombinant DNA-derived hyaluronidase enzyme (rHuPH20) has been developed to leverage the historical efficacy of animal testes extract-derived spreading factors to reversibly modify the hypodermis, in light of discovery of the human hyaluronidase gene family. The application of this technology to increase both injection volumes and bioavailability from subcutaneous injection may overcome some key limitations of this route of administration in multiple settings of care.

Keywords: hyaluronan, hyaluronic acid, hyaluronidase, Hylenex, rHuPH20

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1. Introduction

1.1 The extracellular matrix as a physiological barrier to drug delivery

Following subcutaneous injection, drugs must pass through the extracellular matrix (ECM) of the hypodermis and traverse capillaries or lymphatics in order to reach the vascular compartment [1]. The ECM controls the diffusion and bulk fluid flow of molecules and maintains specific tissue architecture. Within the hypodermis, the ECM exists as a network of fibrous proteins within a viscoelastic gel. Structural macromolecules such as collagen and elastin form the basic fibrous building blocks that support adipocytes and vascular structures. Whereas collagen exists essentially as a solid-phase in the ECM, glycosaminoglycans and proteoglycans form the hydrated viscoelastic gel-like substance in which the fibrous components are embedded. Glycosaminoglycans create a barrier to bulk fluid flow around the fibrous matrix by way of their viscosity and water of hydration [2]. Glycosaminoglycans are complex linear polysaccharides of the ECM characterized by repeating disaccharide structures of an *N*-substituted hexosamine and a uronic acid. These include hyaluronan, chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and keratan sulfate. Except for hyaluronan, all exist covalently bound to core proteins. The glycosaminoglycans with their core proteins are structurally referred to as proteoglycans.

Despite changes in salt and water intake, the interstitial fluid volume is normally tightly regulated by oncotic and hydrostatic gradients from the blood vascular system

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and by lymphatic flow [3,4]. The interstitial fluid volume of skin has been calculated at ~ 0.4 ml/g of tissue [4]. Albumin is capable of occupying ~ 50% of this volume [5]. Collagen comprises nearly 17% of the extracellular fluid volume of the hypodermis [4]. Although glycosaminoglycans are found at only 1% the concentration of collagen in the skin, they occupy a fluid exclusion volume 10-fold higher than that of collagen on a milliliter H_2O/mg basis [6]. Hyaluronan, the principal glycosaminoglycan of the hypodermis, is a mega-dalton molecule consisting of repeating disaccharide units of *N*-acetyl glucosamine and glucuronic acid. In contrast to collagen, which has a half-life approaching 15 years [7], hyaluronan is rapidly turned over in the body with a half-life of < 2 days in the skin [8,9].

Water-soluble low molecular weight compounds (< 1 kDa) are drawn into the vascular compartment by absorption across the capillaries from oncotic pressure gradients [10]. As low molecular weight compounds such as electrolytes or morphine sulfate are readily permeable to the endothelium, they are absorbed into the vascular compartment within minutes to hours following subcutaneous injection, provided that sufficient capillary flow and oncotic pressure differentials between plasma and the injected drug exist.

On the other hand, large molecule therapeutics such as recombinant biologics access the vascular compartment predominantly by way of bulk fluid flow through the draining lymphatics [1,11]. Large proteins such as monoclonal antibodies (150 kDa) may, therefore, take several days to reach maximal levels in plasma. For these biologics, significant amounts of the injected protein may not escape from the local tissue intact if the molecule has a short half-life in the extravascular compartment. One such example is Factor VIII (antihemophilic factor), which, due to its large molecular weight and sensitivity to inactivation in subcutaneous tissue, is not meaningfully bioavailable by local administration [12]. As a result, this molecule must be intravenously injected.

In the case of small-molecule injections, the osmolality and volume of the injected drug can limit the rate by which the material is absorbed or prevent their adequate dilution to a concentration that is not locally toxic. The balance of oncotic pressure in the subcutaneous space versus the vascular compartment is disrupted when the injected material is hyperosmolar. Extravasation of concentrated intravenous solutions in the interstitium can result in a form of compartment syndrome, whereby interstitial pressure may constrict vascular perfusion to the point of ischemia and necrosis.

1.2 Strategies to overcome the limitations of the extracellular matrix

Nearly all approaches to drug delivery require that the active agent traverse the ECM to some extent in order to enter and/or leave the vascular compartment. Although the epithelium is clearly the main challenge for oral, inhalable and transmucosal delivery technologies, drugs administered by these routes must still pass through the stromal ECM before reaching the capillary beds.

In the case of subcutaneous injections, the ECM of the hypodermis is the first barrier to entry into the vascular compartment. Although convenient for self administration compared with intravenous or intramuscular injections, subcutaneous injections are generally limited by the type and volume of fluid that can be administered. Unlike furred animals such as rodents that have loosely draped skin that can accommodate large volumes of subcutaneous fluid under the fascia without significant tissue distortion, humans and other furless animals have fibrous bands in the panniculus adiposus that reach into the deep fascia [13,14]. These anchors reduce the compliance of the tissue space to injected fluids, such that subcutaneous injections are generally limited to less than 2 ml.

To overcome these restrictions, a number of approaches have been taken to work within these volume limitations for subcutaneous injections. One approach is to increase the concentration of the active ingredient in the drug product formulation. In the case of proteins, viscosity, solubility and aggregation then may become major challenges for subcutaneous injections. High viscosity may become an obstacle for injection through small-gauge needles and introduce material with an oncotic pressure much higher than the interstitium.

Although many approaches have taken advantage of the ECM to retain a formulation, as in the case of depots, limitations on the volume of administration introduce significant challenges for injectables. Local subcutaneous injections are generally preferred over intravenous infusions for self administration, as they have a greater ease of administration and reduced risk of systemic infection. Intravenous infusions require skilled intervention and are, thus, typically performed in a physician's office or hospital. In contrast, local injections of chronic therapies can permit family members or patients themselves to administer the mediation outside the hospital setting and are generally looked upon as less costly than intravenous versions of such therapies due to ancillary infusion costs [15]. Subcutaneous administration increases the settings of care available for drug administration, some of which favor both patient convenience and pharmacoeconomics [16]. For patients receiving chronic therapy, self administration in the home setting may also improve health-related quality of life [17]. In some cases, systemic infusion reactions have also been reduced by converting to subcutaneous administration, although local irritation may occur [18].

For therapeutics such as monoclonal antibodies that require the administration of large volumes due to their limited solubility, local administration may necessitate more frequent dosing in small volumes, even when a therapeutic has a half-life and safety profile that would permit less frequent dosing. Additionally, drugs can become trapped in the matrix and become locally degraded, lowering bioavailability. Finally, the viscosity of the interstitium can cause retention of

high concentrations of the drug at the injection site. This may lead to adverse injection-site reactions, reduced bioavailability or increased immunogenicity. These barriers imposed by the ECM reduce the attractiveness of local administration.

For example, consider the conversion of a traditional 5 mg/kg monoclonal antibody intravenous dosing regimen to subcutaneous injection. With 60 – 70% bioavailability by subcutaneous administration, the reduced AUC from a 5 mg/kg intravenous dose every 4 weeks may require a 7.5 mg/kg subcutaneous dose to meet the intravenous pharmacokinetic profile. The 350-mg dose in the 70 kg patient becomes a 500 – 600 mg dose. Keeping the monoclonal antibody soluble in a 1 – 2 ml volume with low enough viscosity for administration becomes a significant challenge. Unable to concentrate the antibody to 250 – 500 mg/ml, the innovator may need to increase the dosing frequency with a lower dose, even if safety and efficacy support the more favorable dosing regimen. The risks of immunogenicity from subcutaneous administration may also increase when using either concentrated antibody solutions that can form aggregates or by increasing dosing frequency with lower doses. Thus, the innovator may need to choose between intravenous infusion or more frequent dosing that poses additional development risks.

Transiently degrading collagen or hyaluronan in the interstitium could permit the conversion of many drugs from intravenous to local administration. Collagen has a half-life approaching 15 years; thus removal of collagen would not be a likely target of modification for drug delivery purposes, as permanent tissue changes could result. Hyaluronan on the other hand is turned over rapidly in the body, with a half-life of 15 – 20 h, and may provide a more feasible target for modification [19].

1.3 Hyaluronidases: from spreading factors to the human genome

The existence of a spreading factor in extracts of rabbit testes was first described in 1928 by F Duran-Reynals [20]. He discovered that intradermal skin lesions in rabbits injected with live vaccinia virus were significantly larger when co-injected with extracts from rabbit testes compared with placebo or extracts from other organs [21]. Testes extracts were subsequently found to increase the dispersion of many types of injected materials, including bacterial toxins, xenogenic serum, hemoglobin and tracer dyes. Although the extracts increased the overall area of exposure for toxic agents, a reduction in the intensity of the reactions occurred presumably due to dilution of the effect [22]. Similar spreading activities could be extracted from the testes of other species, as well as from venoms and certain bacteria [23]. It appeared that a natural drug delivery system had evolved in multiple species in organs designed to penetrate the ECM.

The underlying mechanism behind the spreading activity remained unknown until 1939, when Duthie and Chain reported that a mucolytic activity co-fractionated with the spreading activity in testes extracts [24]. This enzyme activity was subsequently defined as a hyaluronidase, due to its ability to digest hyaluronan *in vitro* [25]. Based on this activity, fractionated extracts containing hyaluronidase activity from bovine testes were developed as pharmaceutical products. The first product derived from bovine testes, Wydase® (Wyeth), was approved in 1948. Subsequently, additional hyaluronidase products were approved, Alidase® (Searle) in 1949 and Hyazyme® (Abbott) in 1951. At one point, there were 10 hyaluronidase-containing products legally marketed with NDAs. However, the protein in these preparations responsible for the hyaluronidase activity was never identified.

In lieu of knowledge about their primary structure, hyaluronidases were classified by the type of digestion product they produced, their pH optima and the source from where they were extracted. Bacterial hyaluronate lyases (EC 4.2.99.1) degrade hyaluronan and, to various extents, chondroitin sulfate and dermatan sulfate. They are endo-β-*N*-acetylhexosaminidases that operate by a β elimination reaction, yielding primarily disaccharide end products [26]. Hyaluronidases (EC 3.2.1.36) from leeches are endo-β-glucuronidases that generate tetrasaccharide and hexasaccharide end products through hydrolysis of the β 1-3 linkage [27]. Mammalian-type hyaluronidases, (EC 3.2.1.35) are endo-β-*N*-acetylhexosaminidases, leaving tetrasaccharides and hexasaccharides as the major end products [28]. They reportedly have both hydrolytic and transglycosidase activities, and degrade hyaluronan and chondroitin sulfate. Mammalian hyaluronidases can be further subdivided into two groups: neutral active and acid active enzymes. Neutral active enzyme activity has been found in testes extracts, whereas acidic enzyme was found in organs such as the liver.

Numerous attempts were made to purify hyaluronidase to homogeneity from testicular extracts. However, the increasing specific activity from chromatographically fractionated preparations demonstrated that the enzyme was present in only trace amounts in the original marketed 'hyaluronidase' preparations [29,30]. Although commercial preparations contained enzyme with a specific activity of ~ 750 Units/mg, this material could be further purified to a specific activity of up to 50,000 Units/mg [31,101,102].

The structural identity of testicular hyaluronidase was finally elucidated through a rather circuitous path, > 60 years after Duran Reynals' original observations on the spread of vaccinia virus in the presence of rabbit testes extracts. The purification to homogeneity of a hyaluronidase from bee venom, and subsequent amino acid sequencing and cDNA cloning, revealed significant homology to the cDNA of a recently identified sperm surface antigen, PH20 [32,33]. The PH20 protein had been previously identified as a glycophosphoinositol lipid-anchored antigen on the plasma membrane of sperm without knowledge of its hyaluronidase activity. Ironically, expression of the PH20 enzyme in rabbit kidney cells using a recombinant vaccinia virus system

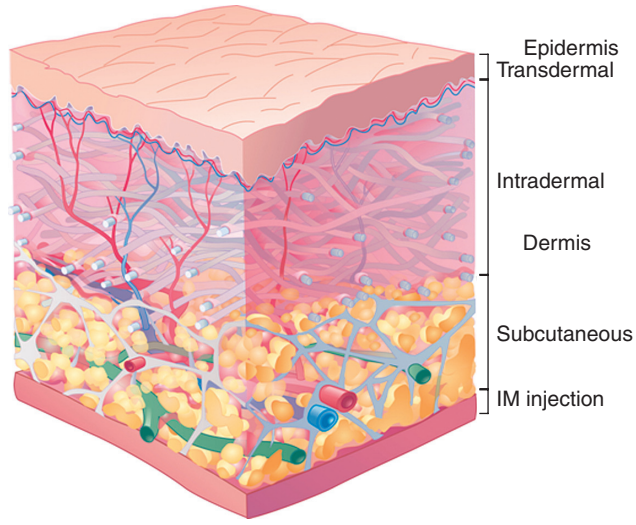

Figure 1. Cellular and extracellular barriers to drug administration in the skin. The epidermis (primarily the stratum corneum) represents the principal barrier to entry for transdermal delivery. The collagen-rich dermis is the initial barrier following intradermal injections, whereas the adipose-rich subcutaneous tissue is the principal barrier for subcutaneous injections. The muscle is the initial barrier to intramuscular injections. Absorption into the vascular compartment occurs through either capillaries (red) or lymphatic vessels (green) depending upon the size of the injected molecules.
IM: Intramuscular.

demonstrated the hyaluronidase activity associated with the cell membrane [34].

The biochemical purification, microsequencing and cloning of additional hyaluronidase family members from other tissue sources resulted in the identification of new hyaluronidase-like genes in the human genome (*HYAL1*, *HYAL2*, *HYAL3*, *HYAL4*) [35]. HYAL1 is the prototypical acid-active enzyme and was purified from human plasma [36]. Acid-active hyaluronidases, such as HYAL1, are soluble proteins but lack any catalytic activity towards hyaluronan at neutral pH and have an optimum pH of 4.0 *in vitro* with no catalytic activity *in vitro* at > pH 4.5. Some of the other hyaluronidase-like proteins have either little or no enzyme activity at all *in vitro*. These proteins possibly have non-catalytic functions or may not be active in cell-free systems.

2. The development of rHuPH20, an rDNA-derived human hyaluronidase enzyme

The discovery of the human PH20 gene and its subsequent identification as a hyaluronidase opened the possibility of producing a recombinant version of the testes-sourced hyaluronidase enzymes that had been used commercially as spreading factors for > 50 years. A purified form of the recombinant human hyaluronidase enzyme might allow production of a reliable source of authentic human enzyme that is free of any bovine material. However, controversy over the cellular location and identity of a lipid anchor of PH20 in different species and whether the soluble human protein retained enzyme activity at physiologic pH introduced several uncertainties [37,38].

2.1 Molecular engineering of a soluble human recombinant human PH20 enzyme

Alignment of the 553 amino acid bovine sequence with the 509 amino acid human PH20 sequence showed only weak homology, and multiple gaps existed from amino acid 470 to their respective carboxy termini (**Figure 2A**). The human PH20 enzyme was believed to be anchored to the plasma membrane using hydropathy plots, and the release of acid-active hyaluronidase activity from cells expressing full-length PH20 was achieved following phospholipase C treatment [34]. However, no clear glycophosphoinositol anchor attachment sites were predicted in several other species, including bovines (**Figure 2B**). Purification and amino acid sequencing of the bovine hyaluronidase protein identified a soluble form of bovine PH20 as a fragment of the full-length cDNA [39]. As previous attempts to produce the 382 amino acid bee venom enzyme in bacterial host systems showed only weak hyaluronidase activity, and human PH20 contained eight additional cysteine residues (12 total) with multiple *N*-linked glycosylation sites, expression in mammalian systems were considered for deletion mutagenesis [33].

Previous studies using deletion mutagenesis of the carboxy terminus of the human PH20 gene near the proposed glycophosphoinositol anchor site or truncated further to the equivalent carboxy terminus of the bee venom hyaluronidase domain have failed to produce an active secreted hyaluronidase in mammalian cells [40]. A series of deletion mutants of the human PH20 cDNA were produced containing HIS_6 epitope tags at the carboxyl terminus. Transfection of these deletion mutants into

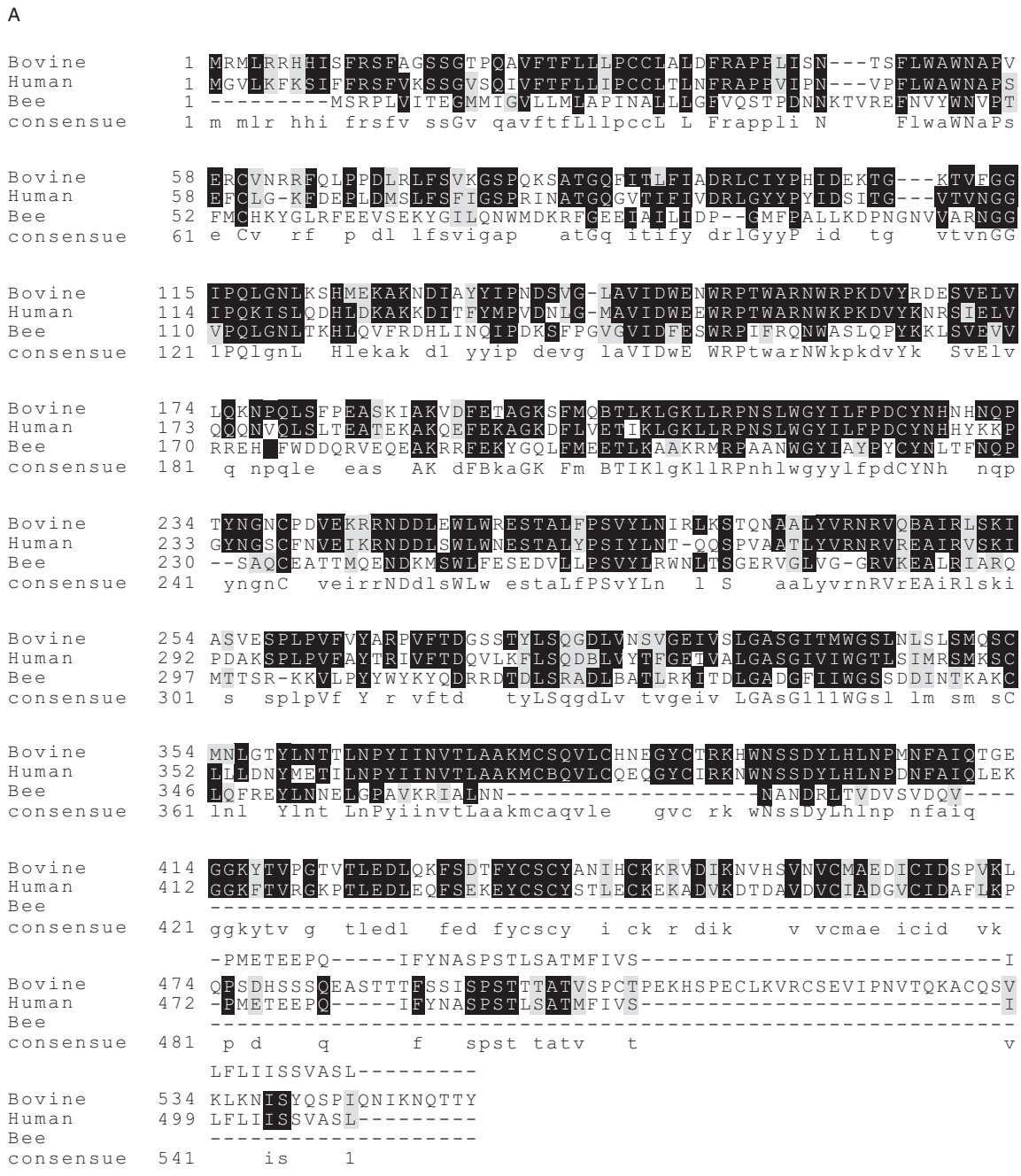

Figure 2. Structural diversity of the hyaluronidase family and development of a soluble recombinant human PH20 enzyme.
A. Alignment of human, bovine and bee venom hyaluronidase amino acid sequences reveals a lack of homology in the *C*-terminal domains. **B.** Hydropathy plots of human and bovine PH20 amino acid sequences reveal lack of a hydrophic glycophosphoinositol anchor attachment motif in bovine compared to human. **C.** Deletions in human PH20 gene within the carboxy terminus from amino acids 477 – 483 result in a secreted active enzyme at neutral pH.
rHuPH20: Human recombinant DNA-derived hyaluronidase enzyme.

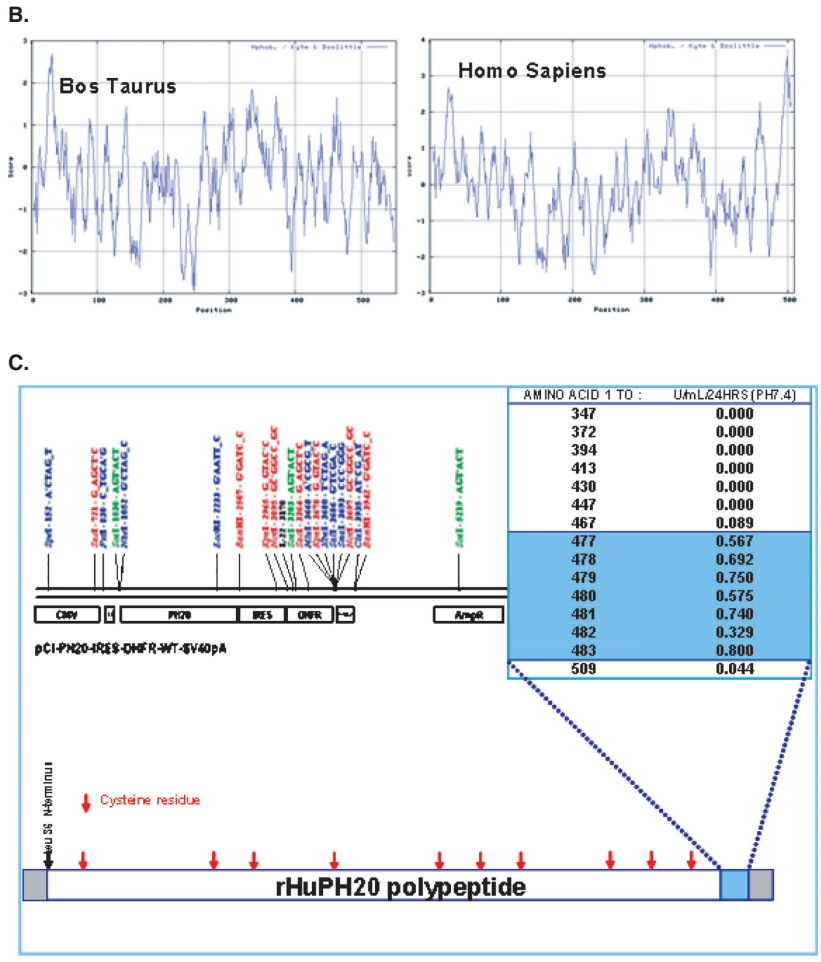

Figure 2. Structural diversity of the hyaluronidase family and development of a soluble recombinant human PH20 enzyme (continued). A. Alignment of human, bovine and bee venom hyaluronidase amino acid sequences reveals a lack of homology in the *C*-terminal domains. **B.** Hydropathy plots of human and bovine PH20 amino acid sequences reveal lack of a hydrophic glycophosphoinositol anchor attachment motif in bovine compared to human. **C.** Deletions in human PH20 gene within the carboxy terminus from amino acids 477 – 483 result in a secreted active enzyme at neutral pH.
rHuPH20: Human recombinant DNA-derived hyaluronidase enzyme.

Chinese Hamster Ovary (CHO) cells followed by screening for enzyme activity using a microtiter-based enzyme assay with biotinylated hyaluronan (**Figure 2C**) revealed that soluble hyaluronidase activity could be recovered in the conditioned medium from deletion mutants terminating after amino acids 477 – 483. Less than 10% activity was recovered when constructs terminated after amino acid 467 or when using the full-length PH20 cDNA [41].

2.2 Development of a manufacturing process for rHuPH20

To develop a recombinant human hyaluronidase for use as a pharmaceutical adjuvant for drug delivery, the soluble domain of the human PH20 hyaluronidase cDNA was cloned into a well-characterized mammalian cell line. A commercially acceptable expression vector containing the human PH20 cDNA encoding amino acids 1 – 482 (rHuPH20) followed by a cytomegalovirus-driven bicistronic vector with the dihydrofolate reductase (DHFR) selection marker was constructed to allow for positive selection in DHFR-deficient CHO cells previously adapted to grow in chemically defined media without any animal-derived materials. Transfectants were cloned in chemically defined media and selected with increasing concentrations of methotrexate to produce a high level rHuPH20-secreting cell line. A four-step column chromatographic downstream purification process was developed that resulted in a highly purified enzyme protein with a specific activity of over 100,000 USP Units/mg protein. The process resulted in a 447 amino acid 61 kDa glycoprotein with a properly processed amino terminus and 6 *N*-linked glycosylation sites.

Using a two-tiered banking system, DG44 clones expressing the rHuPH20 protein are first expanded from a working cell bank through a series of spinner flasks in chemically defined media and then run in a 100 l volume fed batch process in a

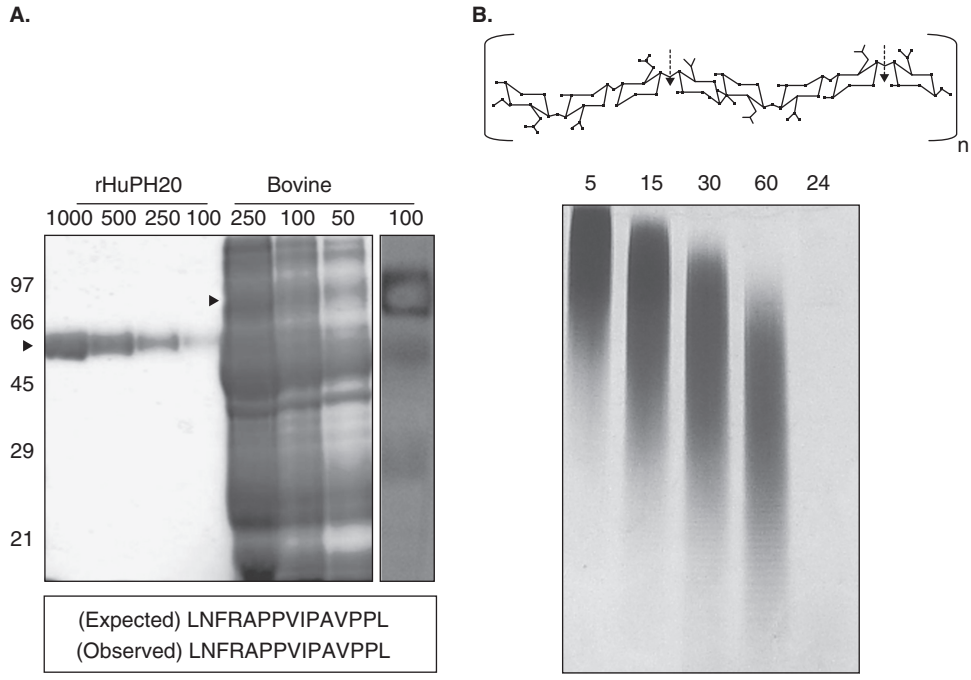

Figure 3. Characterization of purified rHuPH20. A. CHO-derived rHuPH20 1000, 500, 250, 100 Units per lane loaded, compared with 250, 100, 50 Units bovine testes hyaluronidase. Western blot analysis of bovine hyaluronidase preparations using anti-ram hyaluronidase monoclonal antibody reveals a single immunoreactive hyaluronidase band. *N*-terminal amino acid sequencing of purified rHuPH20 shows the predicted processed amino terminus. **B.** β 1 – 4 endoglycosidase-mediated depolymerization of high molecular weight hyaluronan by rHuPH20. Umbilical cord hyaluronan incubated with 1 Unit of rHuPH20 for the indicated times, followed by electrophoretic separation of hyaluronan digestion products, reveals the kinetics of rHuPH20-mediated hyaluronan depolymerization.

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CHO: Chinese Hamster Ovary; rHuPH20: Human recombinant DNA-derived hyaluronidase enzyme.

2-week fermentation cycle. The rHuPH20-containing supernatant is separated from the cells, diafiltered, chromatographed and viral filtered to produce a concentrated bulk drug substance suitable for production of drug product. The rHuPH20 protein can be purified to homogeneity, resulting in glycosylated enzyme with a specific activity 50 – 100-fold greater than commercially available animal hyaluronidase extracts used historically, measured on a Unit per milligram protein basis. Electrophoretic analysis of equivalent Units of the bovine-derived enzyme demonstrates that the majority of protein in the animal-derived USP reference standard preparation is in fact not PH20 hyaluronidase (**Figure 3A**). Only one minor band in the smear of proteins in the bovine preparation (~ 75kDa) crossreacts with a monoclonal antibody to bovine PH20 by western blot, confirming that bovine PH20 is only a minor component of such preparations, consistent with its low specific activity.

2.3 Pharmacology and safety assessment of rHuPH20

In addition to Duran Reynals' spreading factor, many biological activities have been identified in animal testes-extracted hyaluronidase preparations. Thus, the potential for confounding activities from contaminants present in animal-derived hyaluronidase preparations, such as proteases [42], anticoagulants [43], growth factors [44], vasopermeability factors [45], immunoglobulins [46] and other unidentified activities necessitated a comparative study of the spreading activity of purified rHuPH20 to bovine testes-derived hyaluronidase reference standard, calibrated by their ability to degrade hyaluronan *in vitro* by the USP enzyme assay.

The action of rHuPH20 towards glycosaminoglycans can be measured *in vitro* by a number of methods including reduction in turbidity, viscosity or the production of sugar reducing end units. As shown in **Figure 3B**, the digestion of hyaluronan with nanogram quantities of rHuPH20 can be visualized by simply resolving the digestion products on an acrylamide gel and staining for hyaluronan oligosaccharides with alcian blue. The USP method is based upon the reduction of turbidity of a hyaluronan-containing solution in acidified serum following enzyme treatment. This is measured against a qualified enzyme reference standard.

The ability of purified rHuPH20 to act as a bona fide spreading factor was initially tested using tracer dyes in the dermis of animals [47]. Intradermal injection of purified rHuPH20 in mice with trypan blue dye increased tracer dye area in a dose-dependent fashion relative to the carrier alone, thus confirming its spreading activity. The effect on dye dispersion relative to the control was significant when diluted down to as little as a 5 ng of rHuPH20 in a 50 µl volume.

Heat-inactivation of rHuPH20 destroyed both *in vitro* enzyme activity and by dye dispersion in animals.

Given that the original bovine PH20 preparations comprised < 1% PH20 by specific activity, it was conceivable that non-hyaluronidase contaminants in these preparations contributed to the spreading activity as well. To examine whether non-hyaluronidase contaminants present in the bovine testes extracts had any significant effect on drug dispersion, injection of equal unit doses of both rHuPH20 (100,000 USP Units/mg protein) and a crude commercial bovine hyaluronidase preparation (~ 700 U/mg protein) demonstrated that the spreading activity from 5 Units of bovine hyaluronidase was indistinguishable from that of 5 Units of rHuPH20 on a Unit per Unit basis when calibrated against the hyaluronan substrate *in vitro*. These studies show that the non-hyaluronidase impurities present in these crude extracts do not significantly contribute to the spreading activity.

Early studies with bovine hyaluronidase preparations revealed that hyaluronidase has little effect on dye dispersion in the absence of a pressure gradient [48,49]. Glycosaminoglycans in the skin normally buffer pressure gradients, limiting hydraulic conductivity and bulk fluid flow through tissues. This can be readily visualized in animal models by the infusion of fluid through the dermis in the presence and absence of hyaluronidase (**Figure 4A**) [50]. Infusions of albumin containing solutions in the dermis of rodents have been accelerated up to 20-fold under 20 – 40 cm H_20 pressure in the presence of rHuPH20 [47].

The pharmacologic action of rHuPH20 on the dispersion of molecules other than tracer dyes has also been examined. As collagen fibrils are the main structural components of the dermis, it would be expected that rHuPH20 would not facilitate dispersion of molecules larger than what could permeate through the collagen-based fibrous ECM. Increased dispersion with rHuPH20 should, therefore, be limited to particles small enough to flow through the 10 – 300 nm collagen fibrils of the ECM that remain [51]. Using fluorescent-labeled dextrans and latex particles of increasing diameter, it was found that rHuPH20 increased the dispersion of molecules in the dermis only up to 200 nm in diameter [47]. This was verified using other particles of known size, such as recombinant adenovirus expressing green fluorescent protein [47].

Knowledge of the clearance and metabolism of rHuPH20 is limited. In rats, an intravenous administration of rHuPH20 was rapidly cleared from the circulation following the injection of 86, 860 and 8600 Units/kg. Enzyme activity was detectable in the blood only at the 1 min time point and only in the 8600 Units/kg group. Based on the limit of detection of the assay, rHuPH20 demonstrated a half-life of < 1 min *in vivo*.

Intravenous administration of rHuPH20 in mice (50,000 Units/kg) established the clearance rate from the circulation but obtained limited data on the distribution and elimination in target organs. Enzyme activity has been measured in the plasma, liver, kidney and spleen following intravenous injection. rHuPH20 has been shown to be cleared from plasma with a half-life of ~ 30 s. Enzyme activity recovered in the liver and spleen is also rapidly inactivated. These data support a model of rapid clearance of rHuPH20 from the circulation with rapid inactivation in target organs similar to that reported with partially purified bovine hyaluronidase preparations [52].

The principal substrate of rHuPH20, hyaluronan, is rapidly turned over in the skin [53]. A 70 kg person contains on average ~ 15 g of hyaluronan, 30% of which is normally turned over each day; 85% is degraded locally and in the lymph, and the remainder is cleared by the sinusoidal liver endothelium and kidneys upon reaching the vascular compartment [8]. Hyaluronan internalization and degradation occurs by receptor-mediated endocytosis and by the intracellular action of lysosomal hyaluronidases and exoglycosidases [54,55].

Although the fate of hyaluronidase following subcutaneous injection has not been determined, its duration of action in the hypodermis has been found to be reversible within 24 – 48 h [56]. Furthermore, the measurement of hyaluronidase activity in tissue following local administration of as much as 200,000 U/kg in mice has revealed that ~ 25% of the initial activity remains 1 h after administration; this was nearly undetectable by 4 h post injection [57]. These findings suggest that hyaluronan synthesis is capable of restoring adequate amounts of the polymer in the ECM to restore the resistance to hydraulic conductivity consistent with its normal rate of turnover. Using a dermal dye dispersion assay in mice, the duration of the effects of rHuPH20 have been found to be comparable to a bovine hyaluronidase reference standard, being fully reversible within 24 h of administration [47]. These studies support the transient and reversible nature of rHuPH20 in this model, similar to the bovine enzyme.

The safety of rHuPH20 has been evaluated in rodents and non-human primates by intravenous, subcutaneous and periocular routes of administration. Doses from 38 – 12,000 Units/kg in rhesus primates and 10,500 Units/kg in rodents by intravenous administration have been generally well tolerated. Administration of 10,500 Units/kg in rats resulted in histopathological observations of slight renal tubule dilation and material consistent with hyaline casts.

In primates, rHuPH20 has been shown to be well tolerated at 45,000 Units/injection by subcutaneous injection, and 4,500 Units/injection by periocular administration. No remarkable test article-related inflammatory reactions or infiltrates to rHuPH20 were identified by clinical or histological evaluation of the injection site across a 3 log dose range relative to the carrier control in animals sampled from 24 h to 28 days post injection. No adverse local, electrocardiographic, hemodynamic, clinical or anatomic pathological changes have been noted throughout the 28-day observation period the study. No neutralizing antibodies to rHuPH20 were detected at day 21 or day 28 of the study.

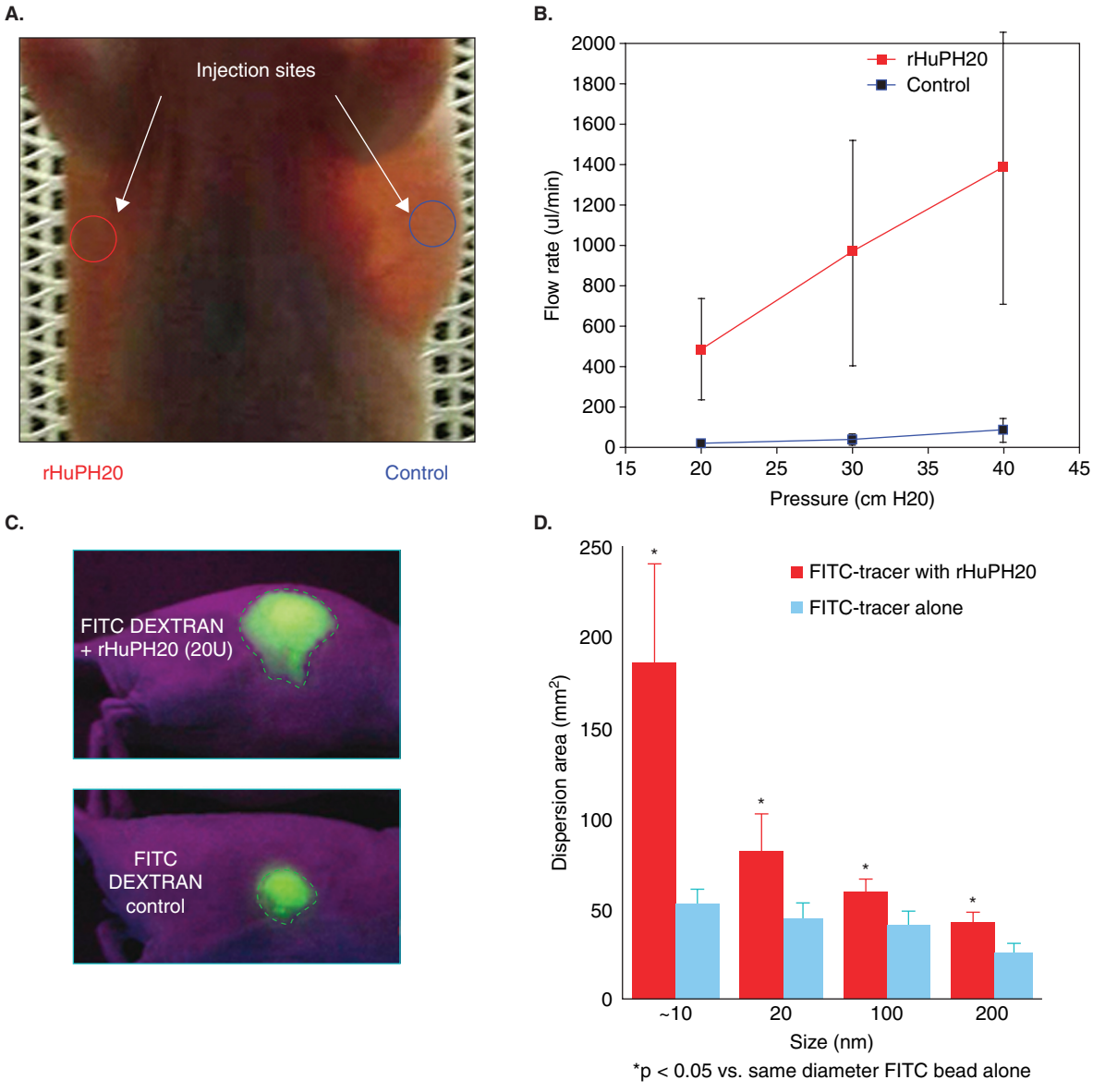

Figure 4. Pharmacological activity of rHuPH20 *in vivo*. A. Reduction of tissue distortion following injection of rHuPH20. An injection of rHuPH20 or carrier control followed by intradermal saline infusion at 300 µl/min for 2 min in both sites by infusion pump demonstrates the reduced tissue distortion with rHuPH20 compared with the carrier control. **B.** rHuPH20 increases infusion rates up to 20-fold in concentrated protein solutions. Human serum albumin (50 mg/ml) was infused at pressures from 20 – 40 cm H_2O with and without hyaluronidase. Flow rates were measured a 0.1 ml intervals at each pressure (n = 3 animals per group). **C.** Intradermal injection of FITC-labeled dextrans and latex beads of increasing size with and without rHuPH20, followed by measurement of dye area over time demonstrates the size constraints on the increase in dispersion in the presence of rHuPH20.

Figure 4A and 4B: This article was published in *J. Control. Release*, Volume 114, Issue 2, BOOKBINDER LH *et al.*, A enzyme for therapeutics, Pages 230-241, Copyright Elsevier (2006).

FITC: Fluorescein isothiocyanate; rHuPH20: Human recombinant DNA-derived hyaluronidase enzyme.

3. Development of Hylenex, recombinant (hyaluronidase human injection)

Hylenex, recombinant (hyaluronidase human injection) was approved by FDA in December 2005 as a new drug application under section 505(b)2. The 505(b)2 application allows for reliance on published literature or FDA's finding of safety and/or effectiveness of an approved drug product for which the applicant does not have a right of reference. The Drug Efficacy Study Implementation (DESI) review contracted by the FDA to the National Academy of Sciences-National Research Council (NAS/NRC) to review the efficacy claims of three hyaluronidase new drug applications in 1970 formed the basis of these findings of efficacy [58].

The many years of published clinical use from multiple animal-derived hyaluronidases served as the basis for the DESI review indications for Hylenex. Based upon this review, Hylenex is indicated as an adjuvant to increase the absorption and dispersion of other injected drugs, for hypodermoclysis, and as an adjunct in subcutaneous urography for improving resorption of radiopaque agents.

The rHuPH20 active pharmaceutical ingredient in Hylenex is a new chemical entity supported by its own chemistry, manufacturing, controls and toxicology. The only clinical study initially required for approval was a test for allergenicity in 100 healthy volunteers. Hylenex is supplied sterile as 150 USP units of non-preserved recombinant human hyaluronidase per milliliter in a single-use 2 ml glass vial.

3.1 Traditional hyaluronidase indications

The traditional indications for hyaluronidase were based upon the DESI review of the use of the animal-based hyaluronidase products since their first approval in 1948. Panels for the use of drugs in the correction of fluids and electrolyte balance, anesthesiology, anti-infective drugs, dentistry, diagnostic agents, ophthalmology, psychiatric drugs, rheumatic diseases and surgery evaluated claims for the three products based upon a review of the clinical literature with hyaluronidase. Indications were determined to be 'effective', 'probably effective', 'possibly effective' or 'ineffective' based upon the historical clinical data available. Indications determined to be effective were selected from subgroups of analysis in the DESI, and formed the basis for the Hylenex label as an adjuvant to increase the absorption and dispersion of other injected drugs, for hypodermoclysis, and as an adjunct in subcutaneous urography for improving resorption of radiopaque agents.

Based upon the DESI review of the original hyaluronidase NDA's, Hylenex is also contraindicated in patients with hypersensitivity to hyaluronidase enzyme or any other ingredients in the formulation. It should not be used to enhance the absorption and dispersion of dopamine and/or α-agonist drugs, and should be discontinued if sensitization occurs. The enzyme should not be applied directly to the cornea, and should not be injected around infected or acutely inflamed areas, nor used to reduce the swelling of bites or stings. Hyaluronidase should not be used for intravenous injections because the enzyme is rapidly inactivated. Furosemide, the benzodiazepines and phenytoin are reported to be incompatible with hyaluronidase. Hyaluronidase products have not been adequately studied to assess their effects on carcinogenesis, mutagenesis or impairment of fertility. Skin tests can also be performed to examine for sensitivity to hyaluronidase [57,59].

3.1.1 *Hypodermoclysis*

The first reported clinical use of hyaluronidase was for a procedure called hypodermoclysis [50]. Hypodermoclysis is a technique of administering fluids and electrolytes into the hypodermis of the skin in place of starting an intravenous line. A later study in 100 infants and children (8 – 30 lbs) infused with 150 – 200 ml of dextrose/saline into each thigh (with and without hyaluronidase) demonstrated flow rates 3.2 – 3.5-fold faster following the addition of hyaluronidase relative to control infusions [60]. A separate study with 45 matched subcutaneous infusions in children with varying ailments showed similar increases in flow rate with hyaluronidase [60]. By decreasing the viscosity of glycosaminoglycans in the interstitium, hyaluronidase increases the rate of infusion of hydration fluids under the skin.

Subcutaneous fluid administration using hypodermoclysis and hyaluronidase may have potential advantages when intravenous access is not feasible. However, the rate of absorption may be reduced in patients with low plasma protein levels. Absorption rates have also been noted to be faster with isotonic over hypertonic solutions. Although hyaluronidase effectively facilitates the infusion of relatively large volumes of fluids and electrolyte-containing solutions subcutaneously, risks for hypovolemia require caution when rapidly infusing electrolyte free solutions.

3.1.2 *Periocular blocks*

Although the use of hyaluronidase for periocular anesthesia is not specifically identified in the Hylenex indication, the historical use of hyaluronidase products in ocular anesthesia was found to be effective by the DESI review panel in ophthalmology. The first-reported use of hyaluronidase in retrobulbar anesthesia was in 1949, when Aktinson reported an increased onset of akinesia and decreased tissue distortion with the use of hyaluronidase [61]. The early applications of hyaluronidase with agents such as lidocaine, bupivacaine and adrenaline in retrobulbar blocks were adopted with little clinical data [62]. The use of hyaluronidase in periocular anesthesia has been studied in retrobulbar, peribulbar and subtenon's blocks, with most parameters evaluating time to akinesia or the need for supplemental blocks [63,64]. Additionally, the discontinuation of Wydase® (Wyeth) in 2001 was followed by increased reports of diplopia and ptosis following cataract surgery, presumably due to the removal of hyaluronidase from the procedure or attempts to compensate for its absence with more anesthetic [65,66].

3.1.3 *Extravasation*

The effects of perivascular infiltration of drugs due to slipped or misplaced peripheral intravenous lines can vary. The range of tissue damage varies from erythema to necrosis depending on the agent, concentration, dose and volume extravasated [67]. Most vesicants are concentration dependent, although the concentration used clinically for intravenous infusions may be far higher than what can be tolerated locally without causing necrosis. The use of hyaluronidase to prevent tissue injury following extravasation has not been adequately studied in well-controlled clinical settings. Its use appears to have relied more upon animal models of dermal tissue injury and limited case studies [68-70]. Its use has been reported for extravasations of solutions such as vinblastine, mannitol, calcium, certain antibiotics, contrast agents and total parenteral nutrition solutions [71-75]. Generally, these uses have reported the

injection of diluted hyaluronidase solutions into the infiltrated line or around the site of extravasation. The administration of hyaluronidase with extravasated ionotropes is not recommended.

3.1.4 *Absorption of radiopaque agents*

Hyaluronidase is indicated as an adjunct in subcutaneous urography. The use of hyaluronidase for the administration of contrast agents may have utility when venupuncture is not feasible. Although there is reasonably good evidence to support the absorption of contrast media following injection of hyaluronidase at the same site, data on the absorption of co-injected contrast agents are lacking [59,76]. Subcutaneous injection of hyaluronidase followed by the injection of contrast agent at the same site is therefore recommended.

4. Conclusion

The subcutaneous route of administration remains a prominent anatomical site for self-injected medications. Historically, animal testes-derived spreading agents have been used to modify the ECM in order to increase the volumes and types of medications that can be administered by this route. This is particularly useful when intravenous access is not available. Discovery of the human hyaluronidase gene family and molecular engineering of a purified soluble human rDNA-derived PH20 hyaluronidase enzyme enabled the commercialization of Hylenex, a 150-Unit standalone liquid drug product indicated for use in these traditional hyaluronidase indications.

Preclinical studies with rHuPH20 have demonstrated its versatility as an adjunct for drug delivery. By decreasing the viscosity of hyaluronan, co-injection with rHuPH20 reduces tissue distortion and disperses particles up to 200 nm in diameter without lasting histologic changes. Although a spreading factor was first described in venom and testes extracts over 70 years ago, and has been used clinically for over 50 years, its use has been largely limited to acute applications, perhaps due to the nature of the foreign proteins in the poorly characterized enzyme preparations. Animal-derived proteins that have been replaced with recombinant human versions may have decreased immunogenicity. The absence of animal-derived products in the manufacturing of rHuPH20 and the greatly increased purity may create a more reliable source than Duran Reynals' original preparations.

5. Expert opinion

Ongoing and future research is leveraging the pharmacology of rHuPH20 towards the delivery of co-formulated and co-injected drugs in multiple settings of care. The rapid action of hyaluronidase on the viscosity of hyaluronan in the ECM has been used successfully for many years to co-inject large volumes of other drugs. For example, local anesthetics compounded with hyaluronidase have been used in ophthalmic anesthesia to facilitate co-injection of 5 – 10 ml around the eye. Given its high potency, only microgram quantities of rHuPH20 should be needed. However, the exact concentration of rHuPH20 required will likely depend upon pH, tonicity and the desired rate of injection of the co-formulation such that optimization of each formulation may need to be determined experimentally. Alternatively, the progressive injection of enzyme followed by another drug product in a dual chamber syringe system could be developed when co-formulation is not feasible. The microgram quantities of enzyme required to elicit these effects is not anticipated to interfere with other water soluble molecules. Given the specificity of rHuPH20 towards the β 1 – 4 linkage in glycosaminoglycans, no activity toward *N*-linked glycans or the polypeptide backbone of other proteins would be anticipated in co-formulations with biologics. The rHuPH20 enzyme in liquid drug product formulations has demonstrated stability over 2 years at 2 – 8°C.

Many drugs administered intravenously or by suboptimal subcutaneous injections could potentially benefit from co-formulation or co-administration with rHuPH20. The application of rHuPH20 as a platform technology is particularly interesting for potentially altering the dosing regimens and bioavailability of biotechnology-derived proteins such as monoclonal antibodies. Compared with reformulation by concentration, crystallization, or the use of slow subcutaneous infusion devices, co-formulation/delivery with rHuPH20 represents a unique and in some ways complimentary delivery strategy.

Initial studies with purified rHuPH20 in animal models gave rise to several general findings worthy of further investigation [47]. First, molecules up to several hundred nanometers in diameter were readily dispersed by co-administration with rHuPH20. Second, rHuPH20 significantly increased the hydraulic conductivity of the co-injected solutions with noticeably reduced tissue distortion. Third, the pharmacokinetic profile of molecules co-injected with rHuPH20 resulted in a higher C_{max} and earlier T_{max} than when injected with carrier controls. Finally, the systemic bioavailability of larger protein molecules normally absorbed by the lymphatic route were significantly increased when co-injected with rHuPH20. These findings were supported by a favorable safety profile of the purified enzyme in several toxicologic models over a broad range of doses. However, applications with new molecules or formulations will require additional studies to support their combined use or co-formulation.

5.1 Route conversions and dosing frequencies with rHuPH20

If rHuPH20 is demonstrated to safely enable the administration of large volumes of fluids into the hypodermis with increased bioavailability for large molecules, several therapeutic categories may benefit. Unlike oral administration, subcutaneous administration of drugs eliminates the loss of bioavailability due to the first pass effect. Although the use of hyaluronidase to facilitate the infusion of large injection volumes of isotonic fluids has been examined by hypodermoclysis, maximal flow-rates under fixed pressures have not been clinically tested. Furthermore, the effects of rHuPH20 on the bioavailability of

subcutaneous injected large molecules need to be determined in relevant clinical settings. Clinical studies recently completed or underway with rHuPH20 have been designed to address such key questions.

For biologics such as monoclonal antibodies, overcoming the volume and bioavailability constraints of subcutaneous injections may permit intravenous dosing frequencies from the subcutaneous route. Considering again the 5 mg/kg intravenous antibody dose, if antibody bioavailability following subcutaneous injection increased from 65% alone to 95% when combined with rHuPH20, a 375-mg dose would generate a similar AUC to the 350 mg intravenous dose. Furthermore, if dosing volumes of 5 – 10 ml are proven feasible with microgram quantities of rHuPH20, considerable flexibility on antibody concentrations from 50 – 150 mg/ml could allow injection volumes from 2.5 – 7.5 ml. Additionally, the use of rHuPH20 to decrease the dosing frequency of biologics already injected subcutaneously by increasing bioavailability and dosing volume could allow for more favorable dosing regimens.

Although the immunologic implication of subcutaneous dosing of biologics with rHuPH20 is unknown, more effective absorption could also theoretically reduce the local degradation of protein aggregates in the skin and their subsequent antigen presentation.

For other therapies that are vesicants or irritants in a concentrated state, the ability to dilute such compounds below the concentrations that generate irritation may mimic the dilution effects described by Duran Reynals' or observed with extravasation. Certain antibiotics, cytotoxics and bisphosphonates that are poorly bioavailable by oral administration could, in theory, be developed for subcutaneous administration with rHuPH20 if sufficiently diluted by a combination of increased injection volume and the dispersive effects of rHuPH20. However, injecting drugs at concentrations that are well above that which induce necrosis could exacerbate their local side effects by increasing the area of irritation.

5.2 Modified pharmacokinetics following subcutaneous injection with rHuPH20

The application of rHuPH20 to increase the speed of absorption by subcutaneous injection when intravenous access is not available may also have significant utility. Similar to the effects of subcutaneously administered contrast agents, increasing the surface area of local capillaries exposed to the injected drug may increase the rate by which compounds are absorbed. Driving the pharmacokinetic profile from a traditional subcutaneous absorption curve towards an intravenous-like bolus profile may be valuable when peak blood levels and time to onset are critical to achieve the desired clinical response. For example, rapid onset could potentially benefit emergency medicines such as narcan or dextrose when intravenous access is not practical. Rapidly metabolized drugs that require sustained plasma levels would not benefit from this 'anti-depot' effect unless given by continuous subcutaneous infusion.

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