Preclinical Development

Enzymatic Depletion of Tumor Hyaluronan Induces Antitumor Responses in Preclinical Animal Models

Curtis B. Thompson, H. Michael Shepard, Patrick M. O’Connor, Salam Kadhim, Ping Jiang, Ryan J. Osgood, Louis H. Bookbinder, Xiaoming Li, Barry J. Sugarman, Robert J. Connor, Sinisa Nadjsombati, and Gregory I. Frost

Abstract

Hyaluronan (HA) is a glycosaminoglycan polymer that often accumulates in malignancy. Megadalton complexes of HA with proteoglycans create a hydrated connective tissue matrix, which may play an important role in tumor stroma formation. Through its colloid osmotic effects, HA complexes contribute to tumor interstitial fluid pressure, limiting the effect of therapeutic molecules on malignant cells. The therapeutic potential of enzymatic remodeling of the tumor microenvironment through HA depletion was initially investigated using a recombinant human HA-degrading enzyme, rHuPH20, which removed HA-dependent tumor cell extracellular matrices in vitro. However, rHuPH20 showed a short serum half-life ($t_{1/2} < 3$ minutes), making depletion of tumor HA in vivo impractical. A pegylated variant of rHuPH20, PEGPH20, was therefore evaluated. Pegylation improved serum half-life ($t_{1/2} = 10.3$ hours), making it feasible to probe the effects of sustained HA depletion on tumor physiology. In high-HA prostate PC3 tumors, i.v. administration of PEGPH20 depleted tumor HA, decreased tumor interstitial fluid pressure by 84%, decreased water content by 7%, decompressed tumor vessels, and increased tumor vascular area >3-fold. Following repeat PEGPH20 administration, tumor growth was significantly inhibited (tumor growth inhibition, 70%). Furthermore, PEGPH20 enhanced both docetaxel and liposomal doxorubicin activity in PC3 tumors ($P < 0.05$) but did not significantly improve the activity of docetaxel in low-HA prostate DU145 tumors. The ability of PEGPH20 to enhance chemotherapy efficacy is likely due to increased drug perfusion combined with other tumor structural changes. These results support enzymatic remodeling of the tumor stroma with PEGPH20 to treat tumors characterized by the accumulation of HA. Mol Cancer Ther; 9(11); OF1–13. ©2010 AACR.

Introduction

Hyaluronan (HA) is a glycosaminoglycan polymer of repeating N-acetylglucosamine and glucuronic acid disaccharide subunits and is a significant component of the extracellular matrix (ECM; refs. 1, 2). HA functions as a core polymer to which multiple hydrophilic matrix proteoglycans are assembled, thereby providing a hydrated viscoelastic gel-like matrix within the insoluble collagenous fibers that are supportive for tumor growth as well as a means of retaining growth factors and cytokines within the tumor microenvironment. HA has been linked to a variety of biological processes involved with tumor progression, including epithelial-mesenchymal transition, and the p53 tumor suppressor pathway, via its receptors, RHAMM and CD44 (3, 4). In addition, accumulation of tumor HA could result in increased tumor water uptake and tumor interstitial fluid pressure (IFP), resulting in a compressed tumor vasculature, thereby resulting in diminished accessibility of tumor foci to systemically applied therapeutics (5).

Accumulation of HA has been reported in a wide variety of human tumors, including those of the prostate, colon, breast, stomach, ovary, and pancreas (6–12). Elevated HA (stromal and/or pericellular staining) in tumor biopsies has been correlated with local invasion, the presence of distal metastasis, higher tumor grade, and poorer overall survival (13). Previous work has suggested a role for HA in drug resistance and hyaluronidase in cancer therapy (14, 15). The abundance of HA in the ECM is regulated by the local-regional balance between HA synthase activity and degradation by membrane-associated and lysosomal hyaluronidases. PH20 is unique as a membrane-bound, neutral active enzyme with high specific activity toward HA in vitro and in vivo, which is capable of degrading matrix-associated megadalton HA polymers to small tetrasaccharide and hexasaccharide products (16–19). Given the rapid endogenous turnover of HA, accumulation in tumors could involve disequilibrium between the enzymes that synthesize HA and the enzymes that catabolize HA or receptors for cellular uptake, which subsequently mediate intracellular metabolism (20–23).
Based on the hypothesis that HA may act as a barrier to efficient drug delivery, pilot clinical studies with a bovine hyaluronidase preparation investigated whether this enzyme could increase anticancer drug action in combination therapies (24). However, despite early signs of clinical activity, systemic administration of the bovine hyaluronidase elicited allergic reactions which, combined with a poor plasma half-life, limited clinical efficacy to local-regional chemotherapy in bladder carcinoma (25).

In the present study, a polyethylene-glycol (PEG) modified form of the recombinant human hyaluronidase PH20 (rHuPH20) was characterized for its potential systemic application in human malignancies. The antitumor activity of this pegylated rHuPH20 (PEGPH20), as a single agent and when combined with chemotherapy, was investigated using both in vitro and in vivo models. These studies showed that multisite pegylation dramatically increased the in vitro half-life of the rHuPH20 enzyme and enabled it to deplete HA from the ECM surrounding tumor cells. PEGPH20-mediated depletion of HA from xenografts, which accumulate this glycosaminoglycan, was shown to diminish tumor IFP and water content, resulting in decomposition of tumor vasculature and increased tumor vascular perfusion. In addition, single agent PEGPH20 induced an antitumor response. PEGPH20 also enhanced the activity of coadministered chemotherapies (docetaxel and liposomal doxorubicin), resulting in decompression of tumor vasculature and was shown to diminish tumor IFP and water content, possibly as a result of increased accumulation of chemotherapeutic agents.

Materials and Methods

rHuPH20 and PEGPH20

Highly purified Chinese hamster ovary–derived recombinant human PH20 (specific activity, >90,000 units/mg containing <1 μg residual hamster protein/mg protein, <0.05 endotoxin unit/mg protein, <1 ng DNA/mg protein) was provided by Halozyme Therapeutics, Inc. PEGylated rHuPH20 was generated by conjugating N-hydroxy succinimidyl1 ester of methoxypoly(ethylene glycol)-butanoic acid (MBA30K or PEG) to recombinant human hyaluronidase (rHuPH20) at a 10:1 molar ratio (PEG/rHuPH20). Following conjugation and purification, the pegylated rHuPH20 enzyme retained at least 25% of the initial specific activity (≈30,000 units/mg versus 100,000 units/mg; final PEGPH20 specific activity, >25,000 units/mg).

Cell lines and culture conditions

The human hormone refractory prostate cancer (HRPC) cell lines PC3 and DU145 and the mouse syngeneic 4T1 breast cancer cell line were obtained from the American Type Culture Collection and grown in the appropriate American Type Culture Collection recommended culture medium containing 10% fetal bovine serum at 37°C in a humidified incubator supplied with 5% CO2/95% air. Authentic Mat LyLu rat prostate carcinoma cells, created and originally obtained from Dr. J. Isaacs (John Hopkins School of Medicine), were generously donated by McGill University Health Centre. The cells were maintained in tissue culture as an adherent monolayer in DMEM with 10% fetal bovine serum at 37°C in a humidified incubator supplied with 5% CO2/95% air.

Hyaluronidase-sensitive particle exclusion assay

To visualize the HA pericellular matrices across different cell lines in vitro, particle exclusion assays were used as previously described (26), with some modifications. Briefly, cells were plated for 24 hours and then treated with culture cell media alone or media containing 1,000 units/mL rHuPH20 at 37°C for 1 hour, followed by incubation with 0.5 mg/mL of bovine aggrecan (Sigma-Aldrich) at 37°C for 1 hour. Subsequently, media were removed and replaced with 107/mL suspension of 2% gluteraldehyde-fixed mouse RBC in PBS (pH 7.4). Cells were imaged with a phase-contrast microscope coupled with a camera scanner and imaging program (Diagnostic Instruments, Inc.). In time course experiments, PC3 cells were incubated in vitro immediately with bovine aggrecan (t = 0) or incubated with 1,000 units/mL of rHuPH20 for 2, 8, 16, 24, and 48 hours before addition of bovine aggrecan and processed as described above.

Measurement of relative HA pericellular matrix expression

Particle exclusion assay micrographs of PC3 cells were analyzed to determine the duration of HA pericellular matrix removal following rHuPH20 treatment. The relative HA pericellular matrix value at each time point was derived by dividing the area of both the HA pericellular matrix and the cell by the cell area alone using Image-Pro Analyzer software (Media Cybernetics). Values are plotted relative to control untreated PC3 cells (shown as 100%) and represent the mean ± SEM of 25 independent measurements at each time point (five independent cells from five separate imaging fields).

Evaluation of rHuPH20 and PEGPH20 pharmacokinetics following a single i.v. dose

Male, 4- to 6-week old CD-1(ICR) mice, weighing 22 to 36 g, were used for the rHuPH20 and PEGPH20 pharmacokinetic studies. Animals were dosed i.v. via the tail vein with either 0.433 mg/kg (~43,700 units/kg) rHuPH20 or 3.75 mg/kg (~125,000 units/kg) PEGPH20. Levels of hyaluronidase activity in plasma were determined using a modified version of a 96-well plate-based enzymatic assay (27). As this assay quantifies rHuPH20 or PEGPH20 based on enzymatic activity in plasma, a higher dose of PEGPH20 was used to offset the ~75% reduction in specific activity associated with pegylation of rHuPH20 to ensure that a quantifiable signal would be observed for subsequent pharmacokinetic analysis. Plasma hyaluronidase activity versus time data was analyzed using WinNonlin Professional (Pharsight Corporation); pharmacokinetic parameters were derived using noncompartmental and compartmental analysis.
**Prostate cancer xenograft models**

Male, 6- to 8-week-old nu/nu (Ncr) athymic nude mice, handled in accordance with approved Institutional Animal Care and Use Committee protocols, were used for xenograft studies. For inoculations, PC3, DU145, or Mat LyLu cells were harvested, washed 2× with HBSS, and resuspended in HBSS. Mice were then inoculated with 0.05 mL i.m. injection of 1 × 10^6 PC3 or 5 × 10^6 DU145 cultured tumor cells or with a 0.04 mL i.m. injection of 2 × 10^5 Mat LyLu cultured tumor cells, pretiliously in the left hind leg (adjacent to the tibia peristemeum).

**Mouse syngeneic 4T1 breast cancer model**

Female, 6- to 8-week-old BALB/c mice, handled in accordance with approved Institutional Animal Care and Use Committee protocols, were used for the orthotopic growth of 4T1-GFP breast carcinomas. For inoculations, cells were harvested at a subconfluent growth stage of 80% to 90% confluence, washed 2× with sterile HBSS, counted, and diluted with HBSS to 1 × 10^7 cells/mL. Animals were then inoculated with 50 μL of 5 × 10^7 4T1 cells orthotopically by s.c. injection in the right mammary fat pad.

**Tumor HA and PEGPH20 immunohistochemistry**

Tumor HA staining with a biotinylated HA-binding probe (HABP; 5 μg/mL, Seikagaku Kogyo) was done as described (9). Specificity of the staining was confirmed by digesting a subset of sections with rHuPH20 (1,000 units/mL in PBS) before addition of HABP. For PEGPH20 staining, fixed sections were incubated with rabbit anti-human PH20 antibody (2 μg/mL, Halozyme Therapeutics, Inc.), washed with 0.05% Tween 20 in PBS (PBS-T), and then incubated in horse radish peroxidase–labeled goat anti-rabbit IgG (1:200) for 30 minutes at room temperature (RT). After washing with PBS-T, color development was with 0.05% 3,3′-diaminobenzidine (Sigma Chemical Co.) and 0.03% hydrogen peroxide in PBS for 5 minutes at RT. Specificity of staining was confirmed by incubating a subset of sections with nonspecific rabbit IgG (2 μg/mL). Micrographs were captured using a ZEISS microscope coupled with the Spot imaging program (Diagnostic Instruments, Inc.).

**Tumor IFP measurements**

Tumor IFP was measured as described previously (28). In brief, a microtipped catheter pressure transducer (SPR-671, Millar Instruments, Inc.) was connected to a data acquisition unit (PowerLab 4/30, ADInstruments, Inc.) and a portable laptop computer for continuous IFP measurements (mm Hg). The system was calibrated to 0 mm Hg and considered stable when ambient pressure measurements did not deviate by more than ±1.0 mm Hg over 15 to 20 minutes. For probe placement, the pressure catheter was inserted into the inner bore of a 20-G needle, the needle was introduced into the central core area of the tumor, and the needle was withdrawn while the pressure catheter was simultaneously held in position. For PEGPH20 dose-response measurements, IFP was recorded for 20 to 30 minutes to establish a baseline before dosing, with subsequent postdose recording continued for ~2 hours. For PEGPH20 single-dose time course experiments, tumor IFP was measured for a minimum of 20 minutes at the appropriate time point.

**PC3 tumor microvessel area**

Tumor microvessels were visualized by staining endothelial cells for CD31 as described (29). In brief, frozen sections were cut and fixed in cold acetone for 2 minutes, air dried, and rinsed in PBS. Endogenous peroxidase was blocked with 3% H2O2 for 10 minutes, and nonspecific binding was blocked with 2% normal rat serum in PBS for 30 minutes, treated with biotin/avidin block (Vector Labs) for 5 minutes each, and incubated with rat antihuman CD31 (10 μg/mL, Pharmingen) for 1 hour at RT. The sections were then incubated in biotinylated goat anti-rat IgG (10 μg/mL, Becton Dickinson) for 30 minutes at RT, rinsed with PBS-T, and incubated with streptavidin–horseradish peroxidase (Becton Dickinson) for 30 minutes at RT. After washing with PBS-T, color was developed with 0.05% diaminobenzidine (Sigma Chemical Co.) and 0.03% H2O2 in PBS at RT for 5 minutes. The slides were then counterstained with Gill’s hematoxylin for 2 minutes, washed, dehydrated, and mounted. Rat IgG2a κ was used as a negative staining control. Micrographs were taken, and microvessel area was quantified using Image-Pro Analyzer software (Media Cybernetics).

**Tumor tissue water content**

Tumor pieces harvested at termination were blotted, weighed, dried in a lyophilizer, and then reweighed. Tumor water weight was calculated from tissue wet weight minus tissue dry weight, and percentage of water content was calculated from (H2O weight/tissue wet weight) × 100%.

**Tumor vascular area**

Changes in tumor vascular area were determined using a Vevo 770 high-resolution ultrasound (Vevo 770, VisualSonics Inc.) in contrast mode, coupled with hyper-echoic MicroMarker microbubbles (MB). MBs administered i.v. are primarily confined to the vascular space, unable to escape capillaries, and can therefore be used to visualize the vasculature “space” or vascular area of a defined region of interest (e.g., a tumor). In brief, animals were anesthetized, and two dimensional cross sections of tumors selected for signal acquisition. After recording baseline frames, a bolus of MBs (50 μL at 2 × 10^8 MBs/mL) were infused i.v. and additional frames were recorded. The relative vascular area before treatment was calculated as the ultrasound signal intensity after MB injection minus the ultrasound signal intensity before MB injection for a specific tumor region of interest. Following this initial measurement (t = 0), animals were dosed with vehicle or PEGPH20. Images were obtained at the appropriate time points (t = 2, 8, 24, 48, 72, 96, or 120 hours postdose) pre- and post-MB. The relative
vascular area was calculated as the change in relative vascular area between two time points.

**Doxorubicin content in PC3 tumor xenografts**

A direct fluorimetric assay of tumor homogenate was used to estimate tumor liposomal doxorubicin content by spectrofluorimetric readings at excitation of 485 nm and emission of 530 nm. Tumor tissue was homogenized at 0.2 g/mL in 1% Triton X-100 in PBS via FastPrep-24 Instrument (MP Biomedicals). Because doxorubicin and its metabolites cannot be differentiated fluorimetrically, doxorubicin equivalents were determined. For normalization, homogenates were assayed for DNA content by PicoGreen assay (Invitrogen) to yield nanograms of liposomal doxorubicin per microgram of DNA.

**Results**

**Human recombinant PH20 removes HA-rich pericellular matrices from cancer cell lines *in vitro***

Initial studies investigated the effects of rHuPH20 *in vitro* on human prostate cancer PC3 and DU145 cells.

**Figure 1.** Human recombinant PH20 hyaluronidase removes HA pericellular matrices from *in vitro* cultured PC3 and DU145 prostate cancer cell lines. A, representative image of PH20-sensitive HA pericellular matrices in HA<sup>high</sup> PC3 and HA<sup>low</sup> DU145 tumor cells (magnification, 100×). Arrows indicate the HA-mediated RBC exclusion zone (or halo) surrounding PC3 and DU145 cells. Cells were treated with 1,000 units/mL rHuPH20 at 37°C for 1 h and then processed as described in Materials and Methods. B, time course of HA pericellular matrix reformation in PC3 cells following treatment with 3,000 units/mL rHuPH20 at 37°C for 1 h. Values are plotted relative to control untreated PC3 cells (shown as 100%) and represent the mean ± SEM of 25 independent measurements at each time point (five independent cells from five separate imaging fields).
Cancer cells capable of generating HA-dependent pericellular matrices are readily identified in vitro by hyaluronidase-sensitive exclusion of fixed RBC from the ECM immediately surrounding the cell surface (26, 30). As shown in Fig. 1A, human PC3 prostate cancer cells expressed a HA<sup>high</sup> pericellular matrix as measured by RBC exclusion (Fig. 1A, top left) that was removed following treatment with the rHuPH20 enzyme (Fig. 1A, bottom left), whereas human DU145 prostate cancer cells expressed a HA<sup>low</sup> pericellular matrix (Fig. 1A, top right) that was similarly removed following rHuPH20 treatment (Fig. 1A, bottom right). In addition to PC3 and DU145 cells, a variety of cancer cell lines were surveyed for the formation of stable pericellular matrices (data not shown). In all cases, incubation of cell lines with rHuPH20 effectively removed these HA-dependent pericellular matrices, confirming the role of HA in sustaining pericellular matrix in tissue culture. A time course experiment using PC3 cells revealed that the pericellular matrix was completely reconstituted within 24 hours after removal of rHuPH20 from the culture media, consistent with the rapid endogenous turnover of HA and subsequent stromal remodeling of HA-overexpressing tumor xenografts in nude mice. Pegylation of rHuPH20 extends in vitro half-life following i.v. administration to mice

I.v. administration of rHuPH20 in mice showed it to have a very short serum residence time (t<sub>1/2</sub> = 2.3 minutes; Fig. 2). Coupled with the observation that HA-associated matrix resynthesis is rapid (Fig. 1B), it was essential to create a derivative of the rHuPH20 with an extended serum residence time to achieve sustained tumor HA depletion. This was achieved by pegylating the human recombinant hyaluronidase PH20 (rHuPH20). The PEG-modified rHuPH20 (PEGPH20) exhibited a ~270-fold increased in vivo half-life in mice (t<sub>1/2</sub> = 10.3 hours) compared with rHuPH20 (Fig. 2). PEGPH20 was therefore used for subsequent in vivo studies to analyze the effect of HA removal and subsequent stromal remodeling of HA-overexpressing tumor xenografts in nude mice.

**PEGPH20 depletes tumor HA and reduces IFP in a dose- and time-dependent manner in HA<sup>high</sup> tumor xenografts**

To assess the ability of PEGPH20 to remove HA from tumor pericellular matrices in vivo, a pair of hormone refractory prostate tumor cell lines varying in pericellular matrix production (PC3, HA<sup>high</sup>; DU145, HA<sup>low</sup>) were used. Administration of a single i.v. bolus dose of PEGPH20 (15 mg/kg) effectively removed HA from the tumor ECM within 2 hours postadministration compared with tumor-bearing mice treated with vehicle alone (Fig. 3A, right), and was concomitant with the appearance of PEGPH20 in the tumor (Fig. 3B).

The mechanisms leading to elevated IFP in solid tumors are not fully understood. Increased cellular density, fenestrated vasculature, decreased lymphatic function, and modified ECM composition may contribute to this effect (31). The association of HA with elevated tumor IFP in the literature is inconsistent (14, 32). We hypothesized that enzymatic digestion of the HA component of the tumor ECM by PEGPH20 and the subsequent disassembly of the HA-dependent matrix would result in the movement of HA fragments down a concentration gradient into the systemic circulation and/or lymph, thereby reducing water content, tumor IFP, and decompressing tumor blood vessels. To test this hypothesis, the effects of PEGPH20 on tumor IFP in mice bearing PC3 (HA<sup>high</sup>) and DU145 (HA<sup>low</sup>) hormone refractory prostate tumors were compared. As illustrated in Fig. 3A, the mean baseline tumor IFP of PC3 and DU145 tumors (400–500 mm Hg in size) was ~40 and 34 mm Hg, respectively. Relative to mean baseline measurements, a PEGPH20 dose of 15 mg/kg significantly reduced tumor IFP in PC3 tumor–bearing animals (84% decrease, P < 0.002), with only modest IFP reductions in the HA<sup>low</sup> DU145 tumor-bearing animals (15% decrease, P = 0.04). Consistent with the selectivity of PEGPH20 for IFP reduction in HA<sup>high</sup> tumor tissue, there was no IFP change in non–tumor-bearing contralateral limbs of mice (data not shown). These results suggest that tumor IFP can be specifically modulated following degradation of HA in tumors that accumulate this glycosaminoglycan and that HA-independent mechanisms exist to support increased IFP as found in HA-negative tumors.

Additional studies characterized the dose dependence of PEGPH20 in the reduction of tumor IFP in PC3 xenografts, which correlated with depletion of HA from the tumor stroma (Fig. 3C). In parallel, tumor HA staining was markedly reduced within 2 hours of i.v. administration of PEGPH20 (Fig. 3C, inset). Assessment of IFP changes in PC3 tumor–bearing mice over 96 hours revealed that...
Figure 3. Impact of PEGPH20 on tumor-associated HA and tumor IFP in prostate cancer xenografts growing in nude mice. A, tumor IFP measurements in HA<sup>high</sup> PC3 tumors compared with HA<sup>low</sup> DU145 tumors growing in mice before and 2 h after i.v. administration of vehicle or PEGPH20 (15 mg/kg; n ≥ 6). Columns, mean; bars, SD. *, statistically significant decrease in PEGPH20-treated tumor IFP at 120 min, relative to baseline (P < 0.05). †, statistically significant decrease in PEGPH20-treated tumor IFP at 120 min relative to control treated tumor IFP at 120 min (P < 0.05). Adjacent (right) to each IFP plot are representative images of PEGPH20-sensitive HA pericellular matrix expression analyzed 2 h following treatment with vehicle (top) or PEGPH20 (bottom; arrow denotes weak HA signal in HA<sup>low</sup> DU145 vehicle tumor sample). The presence of HA in tumor xenografts was detected using biotinylated HABP, as described in Materials and Methods. B, assessment of PEGPH20 and HA levels in PC3 xenografts harvested at 2, 8, 24, 72, and 96 h following i.v. administration of PEGPH20 (15 mg/kg). PEGPH20 and HA levels were assessed using either an antibody to rHuPH20 or a HABP, as described in Materials and Methods. PEGPH20-mediated HA removal was sustained for >72 h, with a gradual return of HA signal by ∼10 d (not shown). The tissue areas selected for representation are slightly different between the anti-PH20 and HABP sections. Bar, 50 μm (for area comparison).
PEGPH20 (15 mg/kg) reduced the absolute tumor IFP by 84% within 2 hours of a single i.v. dose with a gradual return to pretreatment baseline IFP levels by ∼72 hours (Fig. 3D). The return of IFP before the repletion of tumor HA suggests that additional, not well-characterized, mechanisms are contributing to elevated IFP.

**PEGPH20 induces a time-dependent increase in microvessel area and vascular area and a decrease in water content of PC3 tumors growing in mice**

Tumor IFP has previously been implicated as a mechanism preventing efficacy of cancer therapeutics (24). Given the elevated IFP in these tumors, we hypothesized that tumor vessel compression might occur, resulting in hypoperfused regions of the tumor. To investigate whether the reduction in tumor IFP by PEGPH20 was associated with changes in tumor blood vessel perfusion, CD31-positive microvessel area was measured over a 96-hour period following administration of a single i.v. bolus dose of PEGPH20 (15 mg/kg) to PC3 tumor-bearing mice. PEGPH20 induced a significant (∼4-fold) increase in microvessel luminal area by 8 to 24 hours postadministration, consistent with a decrease in vascular compression (Fig. 4A and inset). By 72 hours, tumor microvessel luminal area returned to pretreatment levels consistent with the return of elevated tumor IFP. Similar results were found using an orthogonal method, whereby tumor vascular perfusion was measured in vivo with high-resolution ultrasound imaging coupled with hyperechoic MBs. This latter approach confirmed that PEGPH20 treatment resulted in a 3-fold increase in relative vascular area 8 hours after PEGPH20 administration to PC3 tumor-bearing mice (Fig. 4B and inset; Supplementary Fig. S1). Vessel area gradually returned to baseline by 96 hours, consistent with a model of decompression of vasculature, rather than angiogenesis.

Given the innate ability of HA to absorb water and potentially contribute to tumor edema or IFP, additional

---

**Figure 3. Continued.** C, dose-dependent effect of PEGPH20 (0, 0.015, 0.15, 1.5, 4.5, 10, and 15 mg/kg) on tumor IFP in HA<sup>high</sup> PC3 tumors over a 2-hour period following i.v. administration. Inset shows tumor HA expression 2 hours after i.v. administration of vehicle or PEGPH20 (0.15, 1.5, 10, and 15 mg/kg; n ≥ 7). D, time-dependent effects of PEGPH20 (15 mg/kg) on IFP in HA<sup>high</sup> PC3 tumors. PEGPH20 reduced the absolute tumor IFP by 84% within 2 hours of a single i.v. dose with a gradual return to pretreatment baseline IFP levels by ∼72 hours (n ≥ 7). *, statistically significant difference between vehicle- and PEGPH20-treated animals, at the same time point (P < 0.05). †, statistically significant difference between the mean absolute IFP for all controls and each specified time point for PEGPH20-treated animals (P < 0.05). Columns, mean; bars, SD.
Figure 4. Impact of PEGPH20 on microvascular area and tumor water content in HAhigh PC3 tumors. A, tumor microvessel area (measured via CD31 staining as described in Materials and Methods) increased at 8 and 24 h (\(P < 0.05; n = 3\)) following PEGPH20 administration. Mean ± SEM. Inset shows representative CD31 microvessel staining in PC3 tumor tissue obtained from mice 24 h posttest article administration. B, relative vascular area, determined by a high-resolution ultrasound in contrast mode coupled with hyperechoic MBs, increased >3-fold by 8 h, and remained elevated for up to 96 h (\(P < 0.05; n ≥ 8\)) following i.v. administration of PEGPH20. Hyperechoic MBs can be used to visualize the vasculature “space” or vascular area of a defined region of interest (e.g., a tumor). Vascular area measurements are shown normalized to pretreatment (\(t = 0\)) values, defined as 1.0 (or 100%). Inset shows representative transverse two-dimensional ultrasound images of peritibial PC3 tumors, taken in contrast mode, indicating increased echogenicity (vascular area) following PEGPH20 treatment, relative to vehicle. C, tumor water content was significantly reduced by PEGPH20 relative to vehicle control at all time points measured (\(P < 0.05; n ≥ 7\)). Columns, mean; bars, SD.
measurements were made to assess whether PEGPH20 also reduced tumor water content. As illustrated in Fig. 4C, PC3 xenograft tumor water content was significantly reduced by ∼7% in response to PEGPH20 treatment at 2 hours and remained reduced up to 120 hours following i.v. administration. Although it is unclear why tumor water remained depressed beyond 96 hours, histologic analysis of tumors revealed no apparent increase in tumor necrosis following PEGPH20 administration (data not shown).

These results are consistent with the conclusion that PEGPH20-induced depolymerization of tumor HA causes a reduction in tumor water content, presumably as the enzymatically liberated HA catabolites and their associated water molecules are cleared from the interstitial space by expanded local vascular flow. These events paralleled the appearance of PEGPH20 within the tumor, as measured by immunohistochemistry (Fig. 3B). In contrast to the effects of PEGPH20 on local blood vessels and tumor water content in PC3 tumors at 2 and 24 hours, PEGPH20 showed minimal or no significant effect on relative vascular area or water content of HAlow DU145 tumors at the same time points (data not shown).

**Antitumor activity of PEGPH20 and its combination with chemotherapy**

The ability of PEGPH20 to rapidly remodel the stroma of HAhigh tumor xenografts led us to inquire whether the changes described would alter tumor growth. We hypothesized that depletion of high molecular weight HA and its associated water (Fig. 4C) could influence tumor growth, perhaps through collapsing the hydrated tumor matrix or depleting soluble factors important for tumor progression. For these experiments, three diverse HAhigh tumors (human prostate PC3, murine mammary 4T1, and rat prostate Mat LyLu) were evaluated for response to PEGPH20 as a single agent in a repeat dose schedule (PEGPH20 was dosed every 2 or 3 days to ensure complete HA removal). The results of these studies are shown together with both pretreatment and posttreatment HA staining (Fig. 5A and insets). All three tumor models showed significant tumor growth inhibition (TGI), varying from ∼70% inhibition of tumor growth for PC3, ∼44% for 4T1, and ∼34% for Mat LyLu at study termination. In all studies, TGI was calculated when vehicle controls reached 2,500 mm3 or at study termination. These results support the hypothesis that depletion of HA in HAhigh tumor xenografts may affect the tumor microenvironment resulting in diminished support of tumor growth or expansion. Following these results, we further explored whether PEGPH20 remodeling of the tumor stroma, with its associated reduction in tumor IFP, vascular decompression, and increased perfusion (Figs. 3B–D and 4A and B), could lead to enhanced efficacy of chemotherapy, perhaps by improved drug perfusion of HA-depleted tumor tissue. To focus on the effect of HA depletion and minimize differences between tumor types, two hormone-resistant prostate adenocarcinomas were initially compared for response to PEGPH20 in combination with docetaxel (PC3, HAhigh; and DU145, HAlow). In separate studies, docetaxel or liposomal doxorubicin were each dosed weekly (2× docetaxel; 3× liposomal doxorubicin) at subtherapeutic levels to evaluate PEGPH20 benefit on combinatorial treatment. The results showed that PEGPH20 enhanced the effect of docetaxel, inducing initial tumor regression and sustained growth inhibition in the PC3 (HAhigh) tumor xenograft (Fig. 5B, left), but not in the DU145 (HAlow) tumor model (Fig. 5B, middle). PEGPH20 also increased efficacy of liposomal doxorubicin in the PC3 model, with transient tumor regression during the treatment period, followed by sustained inhibition of tumor growth (Fig. 5B, right). The liposomal doxorubicin model was also used to assess whether increased tumor drug accumulation may, at least in part, explain the chemotherapy-enhancing effect of PEGPH20. To test this possibility, we used the fluorescent properties of liposomal doxorubicin. When normalized for DNA content, an approximate 4-fold increase in liposomal doxorubicin was observed in PC3 xenograft tumors when PEGPH20 was administered 3 hours in advance of liposomal doxorubicin (Fig. 5C). The results show that PEGPH20 can enhance the efficacy of chemotherapy, and that this effect may be the result of HA depletion by PEGPH20, subsequent stromal remodeling, and increased tumor accumulation of chemotherapy through increased vascular perfusion. Of note, in experiments with PC3 xenografts where satellite mice were evaluated for metastatic lesions, no histologic evidence of increased metastases was observed in any organs examined (liver, kidney, heart, spleen, or lung) following PEGPH20 administration compared with placebo control.

**PEGPH20 tolerability in mice**

PEGPH20, as a single agent and in combination with docetaxel or liposomal doxorubicin, was well tolerated without any overt adverse effects as assessed by cage side observations, body weight, complete blood counts, and histopathology, although a transient body weight loss of ∼10% is often observed following the initial PEGPH20 administration.

**Discussion**

The correlation of tumor HA with local invasion, lymph node and distant metastasis, higher-grade tumors, and poorer overall survival (13) supported initial clinical attempts to reverse the effects of HA overexpression in cancer patients using a bovine-derived hyaluronidase (24). Although these studies suggested that hyaluronidase might provide a therapeutic benefit to cancer patients, the bovine hyaluronidase created allergic reactions and had a short *in vivo* half-life, largely limiting its utility to local regional therapy.

The pharmacologic and pharmacodynamic actions of PEGPH20 were examined in several *in vitro* and *in vivo* models to characterize its activity. Initial experiments showed that HA accumulation occurred *in vitro* for many cell types, including HRPC PC3 cells, and that this matrix
Figure 5. PEGPH20 and combinations with chemotherapy in xenograft and syngeneic tumor models. Animal models were generated as described in Materials and Methods. A, PEGPH20 has high antitumor activity in HA<sup>high</sup> xenograft tumors. PEGPH20 inhibited tumor growth in PC3, 4T1, and Mat LyLu HA<sup>high</sup> tumors by 70% (P < 0.001), 44% (P < 0.04), and 34% (P < 0.02), respectively. PC3-bearing mice were treated with PEGPH20 (i.v., 4.5 mg/kg) every 3 d for 24 d (nine doses), whereas 4T1 and Mat LyLu tumor-bearing mice were treated with PEGPH20 (i.v., 4.5 mg/kg) every 2 d for 11 d (six doses). TGI for the respective tumor model was calculated as [1 - (Tn - T0)/(Cn - C0)] × 100%, wherein Tn is average tumor volume in the treatment group at day n after the last dose of PEGPH20, T0 is average tumor volume in the treatment group at day 0 before treatment, Cn is average tumor volume in the control group at day n after the last dose of vehicle, and C0 is average tumor volume in the control group at day 0 before treatment. Insets of each panel show HA staining before and 24 h after treatment with PEGPH20 in vivo, taken from satellite mice. Note: studies were terminated and TGI was calculated at the first data collection point where control tumors are ≥2,500 mm<sup>3</sup>. B, PEGPH20 enhances the antitumor activity of chemotherapeutics in HA<sup>high</sup> tumors. PEGPH20 enhanced the antitumor activity of docetaxel and liposomal doxorubicin in HA<sup>high</sup> PC3 tumor–bearing mice but did not enhance the antitumor activity of docetaxel in HA<sup>low</sup> DU145 mice. In docetaxel combinatorial studies, PC3 or DU145 tumor–bearing mice received either docetaxel (10 mg/kg) and PEGPH20 (15 mg/kg) on days 0 and 7 and PEGPH20 (15 mg/kg) alone on days 3 and 10, docetaxel alone (10 mg/kg) on days 0 and 7, or PEGPH20 (15 mg/kg) alone on days 0, 3, 7, and 10. Control vehicle was administered on days 0, 3, 7, and 10. PEGPH20 increased docetaxel tumor growth inhibition by ∼38% (P < 0.05) [62% TGI (P < 0.05) to 100% TGI (P < 0.001), docetaxel versus control and docetaxel plus PEGPH20 versus control, respectively]. In liposomal doxorubicin combinatorial studies, PC3 tumor–bearing mice received either liposomal doxorubicin (6 mg/kg) and PEGPH20 (15 mg/kg) on days 0, 7, and 14 and additional PEGPH20 (15 mg/kg) on days 3, 5, 10, 12; liposomal doxorubicin alone (6 mg/kg) on days 0, 7, and 14; or PEGPH20 (15 mg/kg) alone on days 0, 3, 5, 7, 10, 12, and 14. Control vehicle was administered alone on days 0, 3, 5, 7, 10, 12, and 14. PEGPH20 increased liposomal doxorubicin tumor growth inhibition by ∼42% (P < 0.05) [56% TGI (P < 0.05) to 98% TGI (P < 0.04), liposomal doxorubicin alone versus control and liposomal doxorubicin plus PEGPH20 versus control, respectively]. Note: in all studies, tumor growth inhibition was calculated either when vehicle controls were ≥2,500 mm<sup>3</sup> or at study termination. C, PEGPH20 significantly enhances the accumulation of liposomal doxorubicin in PC3 tumor xenografts. Tumor liposomal doxorubicin accumulation increased ∼3-fold (P < 0.05) when mice were predosed with PEGPH20 before liposomal doxorubicin administration. PC3 peritibial tumor xenografts, as described in Materials and Methods, were either administered liposomal doxorubicin alone (10 mg/kg) or administered PEGPH20 (4.5 mg/kg) 3 h before liposomal doxorubicin. Tumors were excised 5 h postliposomal doxorubicin administration. Columns, mean; bars, SEM.
could be transiently depleted in vitro by the administration of rHuPH20 hyaluronidase (Fig. 1A and B). The transient nature of rHuPH20 HA depletion in vitro, with reconstitution occurring in ~24 hours (Fig. 1B), coupled with its short half-life in vivo (Fig. 2), suggested that a therapeutic version of rHuPH20 would require a more sustained activity in vivo. For this reason, a pegylated variant of rHuPH20 was generated and tested for in vitro and in vivo activity. The extended half-life of PEGPH20 (~270-fold increase; Fig. 2) compared with rHuPH20 is not limited to mice and was found in both rats and primates. The modifications embodied in PEGPH20 therefore enabled it for in vivo testing as a potential tumor stromal remodeling therapeutic for cancers characterized by the accumulation of HA.

Following i.v. administration, PEGPH20 rapidly depleted interstitial HA from tumor xenografts (Fig. 3A, right), which correlated with lowered tumor IFP, specifically in the PC3 (HA<sup>high</sup>) prostate cancer, but not when applied to the DU145 (HA<sup>low</sup>) prostate cancer (Fig. 3A). This was consistent with expectations from earlier work with animal-derived hyaluronidase (14), which showed a dose-dependent correlation between decreased IFP in osteosarcoma xenografts. However, in studies presented here, complete removal of tumor-associated HA is clearly shown (Fig. 3C and inset). It is possible that prior studies did not detect changes in HA staining after hyaluronidase injection due to the rapid clearance of enzyme and regeneration of substrate. Finally, PEGPH20-mediated reduction of tumor IFP was maximized at ~2 hours post-administration (15 mg/kg, i.v.) and recovered slowly to baseline at ~72 hours. Because tumor-associated IFP is believed to create a barrier to therapeutics, potentially due to tumor vascular compression (33–35), we examined whether PEGPH20 could reverse these effects in the HA<sup>high</sup> PC3 xenograft model. For this purpose, three components of stromal remodeling were evaluated. First, decreased IFP is anticipated to normalize vascular perfusion. PEGPH20 administration was shown to increase microvessel area from 8 to 24 hours as measured by CD31 immunostaining (Fig. 4A and inset), along with a coordinate increase in vascular area as measured by ultrasound imaging of hyperechoic MBs (Fig. 4B and inset). However, the ultrasound evaluation of the vascular area suggested a longer duration of this effect (up to 4 days). PEGPH20-mediated loss in tumor water content occurred at 2 hours and persisted to 4 days, similar to the duration of increased tumor vascular area. These results show that PEGPH20 treatment of HA<sup>high</sup> tumors may lead to multiple changes in the tumor microenvironment, including depletion of tumor HA, decreased tumor water content, diminished IFP, and increased vascular perfusion. Current work is focused on expanding these observations, including examining whether PEGPH20-mediated stromal remodeling may result in depletion of soluble growth factors, cytokines, and chemokines, which are needed for tumor progression, in part because these soluble factors protect incipient tumors from immune cell-mediated cytotoxicity (36). To test whether the ability of PEGPH20 to induce stromal remodeling could affect tumor growth, three diverse tumor types were tested for sensitivity in xenograft models. In these studies, single agent PEGPH20 treatment suppressed the growth of the HA<sup>high</sup> tumor types: human prostate PC3, murine mammary 4T1, and rat prostate Mat LyLu (Fig. 5A). The observed tumor growth inhibition may be the result of collapsed tumor architecture and associated soluble factors, both of which are needed for sustained tumor expansion and progression.

Elevated tumor IFP is often observed in solid tumors, as it was in this study for the HRPC tumor xenografts, PC3 and DU145 (Fig. 3A). Although the mechanisms that determine increased tumor IFP are poorly understood and complex, they appear to involve compressed or abnormal blood vessels, poorly developed or absent lymphatics, and alterations in ECM components such as elevated HA, collagen, and other proteoglycans (31, 37, 38). As the DU145 tumors (HA<sup>low</sup>) were capable of generating elevated tumor IFP, elevated HA is clearly not required for IFP. Regardless of the mechanism(s), elevated tumor IFP is correlated with poor prognosis (31). Reducing tumor IFP could increase response to chemotherapeutic agents by increasing drug penetration or by augmenting radiation treatment by normalizing tumor oxygen tension. This is supported by studies showing that compounds that decrease tumor IFP in human or mouse models increase chemotherapeutic uptake into tumors and the efficacy of radiation treatments. Examples include vascular endothelial growth factor antagonists, tumor necrosis factor α, and PGE<sub>1</sub> (33, 39, 40). Recently, Hofmann et al. (38) proposed increasing drug delivery to tumors by increasing plasma colloid osmotic pressure, thereby trafficking water from the tumor interstitium to reduce tumor IFP. Alternatively, Brekken and colleagues (14) showed that intratumoral injections of a bovine hyaluronidase depolymerized HA in osteosarcoma tumor xenografts in mice and reduced tumor IFP. Brekken et al. (34) also reported that periodic lowering of tumor IFP might increase the in vivo delivery of monoclonal antibodies to solid tumors. Muckenschnabel and colleagues (15) independently showed that peritumoral hyaluronidase administration increased the intratumoral concentration of s.c. coadministered vinblastine. These and other studies (35) have collectively suggested a potential therapeutic role for hyaluronidase in reducing HA-mediated tumor IFP, which may in turn increase delivery of antitumor drugs in tumors. In the current work, we have shown that systemic administration of PEGPH20, critical for targeting disseminated neoplasms, potentiated the antitumor activity of both docetaxel and liposomal doxorubicin against PC3, HA<sup>high</sup> tumor xenografts, but had minimal effects on the antitumor activity of docetaxel on the HA<sup>low</sup> HRPC DU145 tumor xenografts in nude mice (Fig. 5B).

1 Unpublished observations.
The role of HA as a viscous barrier to diffusion of macromolecules is complex (37), and although it is generally accepted that hyaluronidase increases hydraulic conductivity, in some circumstances hyaluronidase may reduce diffusion, inhibiting the diffusive transport of macromolecules such as FITC-IgG, particularly if the HA is replaced by collagen fibrils (41). The effect of compacting collagen density through depletion of HA from the tumor matrix could, in theory, compete with the improved vascular perfusion of the tumor by decreased tumor IFP.

The effects of HA degradation on tumor transport may also be dependent on the size and molecular characteristics of the molecule being measured. Hyaluronidase has been shown to increase the uptake and delivery of liposomal doxorubicin in osteosarcoma xenografts by increasing tumor transcapillary pressure gradients (35) but reduce the interstitial diffusion of immunoglobulins (42). Taken together, this work shows that PEGPH20 has antitumor activity as a single agent but potentiates the antitumor activity of chemotherapy in tumor xenografts, both in a HA-dependent fashion (Fig. 5A and B). These experiments showed that pretreatment with PEGPH20 enhanced the accumulation of liposomal doxorubicin in the PC3 xenograft model (Fig. 5C). These findings support future investigations of PEGPH20 combinations with chemotherapy in patients that exhibit tumor HA accumulation. Recently, Olive et al. (43) reported that inhibition of hedgehog signaling enhanced the delivery of gemcitabine in a mouse model of pancreatic cancer that was associated with depletion of tumor-associated stromal elements and increased tumor perfusion. It may be useful in the future to evaluate combinations of PEGPH20 with other stromal remodeling agents for use in the treatment of pancreatic cancers and other stromal-rich malignancies.

The potential of PEGPH20 to diminish peritumoral edema may, by itself, be a useful adjunctive treatment for cancer. For example, most brain tumors and metastases to the brain create abnormally permeable tumor vasculature with resultant cerebral edema in humans and mouse models (44, 45). The resultant increased IFP could contribute to hypoxia, reduced tumor penetration of chemotherapy, and resistance to radiation therapy. PEGPH20 is currently being evaluated in preclinical models of cerebral metastases and glioblastomas to directly test this hypothesis.

Endogenous lysosomal hyaluronidase expression has been implicated as a tumor promoter (46), with local digestion of HA generating HA fragments that may stimulate endothelial cell proliferation, adhesion, and capillary formation (47). Conversely, a reduction of tumor HA levels in preclinical studies, using exogenous hyaluronidase, has been shown to suppress tumor growth (48). In peritibial PC3 xenograft and syngeneic mouse mammary 4T1 models, no increase in distal metastases were observed following PEGPH20 treatment, similar to that reported previously for intratumoral injection of rHuPH20 in tumor xenografts (49). Additional studies are under way to evaluate the effect of PEGPH20 on the metastatic behavior of different malignant cells in vivo.

Tumor stroma, including cellular, protein, and carbohydrate components, plays an important role in malignant progression (36). Several cancer therapeutics that perturb stromal-malignant cell interactions are in use or development (43, 50, 51). PEGPH20 represents a novel approach to targeting the stroma via degradation of HA, a significant glycosaminoglycan component of many tumors. In this work, we have shown that PEGPH20 potently removed HA from the ECM surrounding tumor cells in vitro and in vivo, while concomitantly reducing tumor IFP, decompressing local tumor blood vessels, and reducing tumor tissue water content in HAhigh DU145 tumors. As these effects were greatly reduced in HAlow DU145 tumors, we conclude that they are likely HA dependent. Moreover, PEGPH20 potentiated the antitumor activity of both docetaxel and liposomal doxorubicin in HAhigh PC3 tumors. Based on the results described here, PEGPH20 may warrant investigation as an agent for the treatment of cancer through its ability to enzymatically remodel tumor stroma.

Disclosure of Potential Conflicts of Interest

The authors of this article are or were employees and/or shareholders of Haloxyze Therapeutics, Inc.

Acknowledgments

We thank Rebecca Symons, Xiaokun Xiao, and Jennifer Anderson for assistance with histology; Celine Denues for formulation development of the PEGPH20 compound; Dr. Ellen Cheung for assistance with pharmacokinetic analysis; Marie Printa for tumor doxorubicin assay development; Gerard Fu for assistance with in vivo xenograft experiments; and Scott Patton for assistance with manuscript preparation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 05/20/2010; revised 08/26/2010; accepted 09/14/2010; published OnlineFirst 10/26/2010.

References


40.Underhill CB, Toole BP. Transformation-dependent loss of the extracellular matrix in serous ovar-
Enzymatic Depletion of Tumor Hyaluronan Induces Antitumor Responses in Preclinical Animal Models


Mol Cancer Ther  Published OnlineFirst October 26, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-0470

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2010/10/26/1535-7163.MCT-10-0470.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.