Differential selectivity of hyaluronidase inhibitors toward acidic and basic hyaluronidases

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Hyaluronidase (HAase), a class of enzymes which degrade hyaluronic acid (HA), are involved in the spread of infections/toxins, ovum fertilization, and cancer progression. Thus, HAase inhibitors may have use in disease treatments. We evaluated 21 HAase inhibitors against HYAL-1, testicular, honeybee, and Streptomyces HAases. Among these inhibitors, polymers of poly (styrene-4-sulfonate) (PSS) (i.e., molecular weight 1400–990,000 or PSS 1400–PSS 990,000) and O-sulfated HA (shHA) derivatives (shHA2.0, 2.5, and 2.75) were the most effective. HYAL-1 and bee HAases were the most sensitive, followed by testicular HAase; Streptomyces HAase was resistant to all inhibitors, except PSS 990,000 and VERSA-TL 502 (i.e. PSS 10^6 dalton). The length of the PSS polymer determined their potency (e.g., IC_{50} for HYAL-1, PSS 990,000: 0.0096 μM; PSS 210 no inhibition; IC_{50} for testicular HAase, PSS 990,000: 0.042 μM; PSS 210 no inhibition). The presence, but not the number, of sulfate groups on the shHA molecule determined its potency (e.g., IC_{50} for HYAL-1: shHA2.0, 0.019 μM; shHA2.75, 0.0083 μM). Other known HAase inhibitors, such as gossypol, sodiumurothiolamate, 1-tetradecane sulfonic acid, and glycerczillic acid, were not effective. Both PSS and shHA inhibited HAases by a mixed inhibition mechanism (i.e., competitive + uncompetitive) and were 5- to 17-fold better as uncompetitive inhibitors than as competitive inhibitors. These results demonstrate that HAase inhibitors show selectivity toward the different types of HAases, which could be exploited to inhibit specific HAases involved in a variety of pathophysiologic conditions.

Key words: HYAL1/hyaluronic acid/hyaluronidase inhibitors/hyaluronidase

Introduction

Extracellular matrix synthesis and its degradation not only regulate several normal physiologic functions but are also involved in disease processes, such as tumor metastasis, angiogenesis, and atherosclerosis. Inhibitors of extracellular matrix degrading enzymes (e.g., matrix-metalloproteinases) are useful in the treatment of gingivitis, dry eye syndrome, and cancer (Lokeshwar, 1999; Woessner, 1999; Dursun et al., 2002). Inhibitors of hyaluronidase (HAase) may be useful as contraceptives, because these inhibit the acrosomal reaction initiated by testicular HAase (Anderson et al., 2000, 2002; Zaneveld et al., 2002).

Hyaluronic acid (HA) is a glycosaminoglycan made up of repeating disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine. In addition to maintaining the tissues hydrated and osmotically balanced, HA regulates cell adhesion, migration, and proliferation (Laurent and Fraser, 1992; Delpech et al., 1997; Tammi et al., 2002). Concentrations of HA are elevated in cancer tissues, where it most likely promotes tumor metastasis (Delpech et al., 1997; Setala et al., 1999; Auvinen et al., 2000; Lokeshwar et al., 2000, 2001; Pirinen et al., 2001; Toole et al., 2002; Posey et al., 2003). Elevated urinary HA levels serve as an accurate marker for detecting bladder cancer regardless of the tumor grade (Lokeshwar et al., 2000, 2002b). The high molecular mass HA (>10^6 daltons) is anti-angiogenic, whereas small fragments of HA (3–25 disaccharide units) are angiogenic (Rooney et al., 1995; Trochon et al., 1997; Slevin et al., 1998; Lokeshwar and Selzer, 2000).

HAase cleaves internal β-N-acetyl-D-glucosaminidic linkages in the HA polymer. Exhaustive digestion of HA by HAase yields tetrasaccharides, whereas limited digestion yields angiogenic HA fragments (Rodent et al., 1989).

In humans, six HAase genes cluster into two tightly linked triplets on chromosomes 3p21.3 (HYAL-1, HYAL-2, and HYAL-3) and 7q31.3 (HYAL-4, HYALP1, and PH20) (Csoka et al., 2001). PH20, that is, the testicular HAase, is present on the sperm cell surface and is required for penetration through the follicle cell layer (Cherr et al., 2001; Vines et al., 2001). It has a broad pH spectrum (pH 3.2–9.0) (Vines et al., 2001; Franzmann et al., 2003). HYAL-1 type HAase is present in human serum and urine (Csoka et al., 1997; Frost et al., 1997). However, we have shown that HYAL-1 is the major tumor-derived HAase expressed in prostate, bladder, and head and neck cancer cells (Lokeshwar et al., 1999; 2001; Franzmann et al., 2003). Elevated HYAL-1 levels serve as an accurate marker for detecting intermediate- and high-grade bladder cancer and as a prognostic indicator for predicting prostate cancer progression (Lokeshwar et al., 2000; Posey et al., 2003). We have recently shown that expression of...
HYAL-1 in tissues is regulated by alternative mRNA splicing (Lokeshwar et al., 2002a).

Historically, HAases were considered “spreading factors,” because they are crucial for the spread of bacterial infections and toxins present in various venoms (Tu and Hendon, 1983; Henrisatt, 1991; Gmachl and Kreil, 1993; Kreil, 1995; Henrisatt and Baircho, 1996; Stern, 2004). Determination of the crystal structure of honeybee (bee) venom HAase has helped in the understanding of HA catalysis by mammalian HAases, since these HAases share ~30% sequence identity with bee HAase (Markovic-Housley et al., 2000). Many pathogenic streptococci such as Streptococcus hyaluronlyticus and even phages of group A streptococci produce HAase (Baker et al., 2002). HAase production by streptococci is related to their virulence. Streptocymes HAase is often used as a standard in HAase assays (Stern and Stern, 1992; Lokeshwar et al., 2000, 2001). We have recently shown that HYAL1-type HAase promotes bladder tumor growth, muscle invasion, and angiogenesis (Lokeshwar et al., 2005b). Therefore, potent HAase inhibitors would be useful as contraceptives, anti-tumor agents and possibly have anti-bacterial and anti-venom/toxin properties.

Many synthetic and naturally occurring compounds act as HAase inhibitors. These include high molecular mass poly (styrene-4-sulfonate) (PSS), gossypol, sodium aurothiomalate, fenoprofen, glycerrhizic acid, fatty acids, plant-derived compounds, heparin, and O-sulfated HA (sHA) (Balazs et al., 1951; Perreault et al., 1980; Wolf et al., 1984; Joyce et al., 1986; Yuan et al., 1995; Furuya et al., 1997; Toida et al., 1999; Anderson et al., 2000, 2002; Mio and Stern, 2002; Zaneveld et al., 2002; Yingprasertchai et al., 2003). For example, more than half a century ago, Balazs (1951) reported that sHA and heparin inhibit testicular HAase. HAase inhibitors such as high molecular mass PSS and sodium cellulose sulfate (Ushercell) are being tested as contraceptive agents, because they inhibit the activity of testicular HAase (Anderson et al., 2002). These compounds also inhibit sexually transmitted disease-causing bacteria but not the normal vaginal flora (Anderson et al., 2000; 2002; Zaneveld et al., 2002). Similarly, sodium aurothiomalate has been shown to reduce local tissue damage and prolong survival time in mice infected with different venoms (Yingprasertchai et al., 2003). The anti-inflammatory agent glycerrhizic acid from licorice has been shown to inhibit testicular HAase (Furuya et al., 1997). O-Sulfated derivatives of HA inhibit urinary HAase, which is identical to HYAL1 (Csoka et al., 1997; Frost et al., 1997; Lokeshwar et al., 1999; Toida et al., 1999).

Although several inhibitors of HAase are known, it is unknown whether these inhibitors show variability in inhibiting the activity of different types of HAases (i.e., testicular, HYAL-1, bacterial, and venom-derived [e.g., bee venom]). If different HAases show differential sensitivity toward various HAase inhibitors, HAase inhibitors can be selected to inhibit the activity of one type of HAase without inhibiting others. Such selectivity may help in designing HAase inhibitors as therapeutics for diseases, contraceptives, and venom antidotes, without significantly affecting HAase functions that are required in normal physiology.

In this study, we compared the sensitivity of HYAL-1, testicular, bee, and Streptomyces HAases with 21 HAase inhibitors, using the HAase enzyme-linked immunosorbent assay (ELISA)-like assay and enzyme kinetic analysis.

### Results

#### Effect of PSS compounds on HAase activity

To study the effect of PSS compounds on HYAL-1, testicular, Streptomyces, and bee HAases, we used an activity-based HAase ELISA-like assay. All four HAases were assayed at their optimum pH, and the pH optimum value for each HAase was determined by generating pH activity profile curves (14, 26, and data not shown). HAase activity is usually expressed as the number of glycosidic linkages split per time unit. It is noteworthy that although the HAase ELISA-like assay is specific and allows assaying of several specimens simultaneously, it does not give information on the number of glycosidic linkages split per time unit. Therefore, all of the constants, that is, IC$_{50}$, $K_m$, $V_{max}$, and $K_i$ values reported in this study should be considered as apparent. In addition, the molecular weights of each compound (e.g., 990,000 dalton for PSS 990,000, 1400 dalton for PSS 1400) were used in the calculations of IC$_{50}$ values and other constants.

As shown in Figure 1A, PSS 990,000 inhibits the activity of all four HAases in a dose-dependent manner. However,
HYAL-1 and bee HAases are more sensitive to inhibition by PSS 990,000 than testicular and Streptomyces HAases. From the inhibition curves, we determined the IC$_{50}$ values for each inhibitor. The IC$_{50}$ values of PSS 990,000 for testicular HAase (0.042 μM) and Streptomyces HAase (0.39 μM) are 4.2-fold and 40-fold higher than the IC$_{50}$ values for HYAL-1 (0.0096 μM) and bee HAase (0.0091 μM), respectively (Table I). VERSA-TL 502 is the sodium salt of PSS of molecular weight ∼10$^6$ dalton, which was used by Anderson et al. (2000) to demonstrate that PSS inhibits testicular HAase activity and blocks fertilization. As summarized in Table I, IC$_{50}$ values of VERSA-TL 502 for HYAL-1 (0.0088 μM), bee (0.0081 μM), testicular HAase (0.042 μM), and Streptomyces HAase (0.32 μM) mirror those for PSS 990,000.

Next, we determined whether the length of the PSS polymer affects its ability to inhibit the activity of different HAases. All four HAases showed a progressive decrease in susceptibility to inhibition by PSS polymers of decreasing lengths, when IC$_{50}$ values were calculated on the real molecular weight basis. In addition, the HAases also showed differential sensitivity to various PSS inhibitors. For example, PSS 210 (a monomer) does not inhibit the activity of any of the four HAases (Table I). As shown in Figure 1B and Table I, although HYAL-1 and bee HAase are inhibited completely with PSS 1400 (IC$_{50}$ = 8.2 and 6.6 μM, respectively), the activity of testicular HAase is blocked only partially (IC$_{50}$ = 68 μM). The activity of Streptomyces HAase is not inhibited by PSS 1400. PSS 4300 and PSS 6800 also show differences in their ability to inhibit HYAL-1 (IC$_{50}$ = 3.3 and 2.0 μM), bee (IC$_{50}$ = 2.6 and 1.9 μM), testicular (IC$_{50}$ = 4.9 and 6.7 μM), and Streptomyces (no inhibition) HAases. The IC$_{50}$ values for HYAL-1 and bee HAases closely resemble those for testicular HAase for PSS compounds between PSS 17,000 and PSS 150,000 (Table I). It is noteworthy that although significant differences were observed in the sensitivity of testicular HAase to higher molecular mass PSS compounds, when compared with PSS1400, there appears to be very little difference in the inhibitory power of PSS compounds when the IC$_{50}$ values for HYAL1 and bee HAases are calculated on a weight concentration basis.

**Effect of sHA on HAase activity**

O-Sulfation of glycosaminoglycans, including O-sHA and heparin, inhibit both urinary and testicular HAases (Balazs et al., 1951; Wolf et al., 1984; Toida et al., 1999). In fact, the anti-HAase activities of sHA and heparin against testicular HAase were compared as early as 1951 by Balazs et al.

### Table I. IC$_{50}$ values of 21 HAase inhibitors for HYAL-1, bee, testicular, and Streptomyces HAases

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>HYAL1 IC$_{50}$ (μM)</th>
<th>Bee venom HAase IC$_{50}$ (μM)</th>
<th>Testicular HAase IC$_{50}$ (μM)</th>
<th>Streptomyces HAase IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>990,000</td>
<td>0.0096</td>
<td>0.0091</td>
<td>0.042$^a$</td>
<td>0.39$^a$</td>
</tr>
<tr>
<td>VERSA-TL 502</td>
<td>0.00876</td>
<td>0.0081</td>
<td>0.0416$^a$</td>
<td>0.316$^a$</td>
</tr>
<tr>
<td>350,000</td>
<td>0.037</td>
<td>0.013</td>
<td>0.074</td>
<td>NI</td>
</tr>
<tr>
<td>150,000</td>
<td>0.07</td>
<td>0.047</td>
<td>0.074</td>
<td>NI</td>
</tr>
<tr>
<td>77,000</td>
<td>0.19</td>
<td>0.13</td>
<td>0.39</td>
<td>NI</td>
</tr>
<tr>
<td>49,000</td>
<td>0.31</td>
<td>0.34</td>
<td>0.23</td>
<td>NI</td>
</tr>
<tr>
<td>32,000</td>
<td>0.42</td>
<td>0.53</td>
<td>0.54</td>
<td>NI</td>
</tr>
<tr>
<td>17,000</td>
<td>0.89</td>
<td>1.0</td>
<td>0.89</td>
<td>NI</td>
</tr>
<tr>
<td>6800</td>
<td>3.3</td>
<td>2.6</td>
<td>4.9</td>
<td>NI</td>
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<td>4300</td>
<td>1.97</td>
<td>1.9</td>
<td>6.7</td>
<td>NI</td>
</tr>
<tr>
<td>1400</td>
<td>8.2</td>
<td>6.6</td>
<td>67.6$^a$</td>
<td>NI</td>
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<td>PSS 210</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>sHA2.0</td>
<td>0.019</td>
<td>0.019</td>
<td>0.078$^a$</td>
<td>NI</td>
</tr>
<tr>
<td>sHA2.5</td>
<td>0.017</td>
<td>0.018</td>
<td>0.049$^a$</td>
<td>NI</td>
</tr>
<tr>
<td>sHA2.75</td>
<td>0.0083</td>
<td>0.012</td>
<td>0.038$^a$</td>
<td>NI</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.39</td>
<td>0.41</td>
<td>17$^a$</td>
<td>NI</td>
</tr>
<tr>
<td>Gossypol</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Fenoprofen</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>1-Tetradecane sulfonic acid</td>
<td>63$^a$</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Glycerrhizic acid</td>
<td>39.4$^a$</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Sodium aurothiomalate</td>
<td>NI</td>
<td>NI</td>
<td>94$^a$</td>
<td>NI</td>
</tr>
</tbody>
</table>

NI, no inhibition.

The IC$_{50}$ values for each of the 21 inhibitors were calculated by generating an inhibition curve for each inhibitor, as shown in Figures 1 and 3 for PSS and sHA compounds, respectively.

$^a$The differences in the IC$_{50}$ values of a particular HAase inhibitor among different HAases are statistically significant ($P < 0.05$; unpaired t test).
Therefore, we tested the effect of sHA and heparin on the activity of HYAL-1, testicular, bee, and Streptomyces HAases. We also determined whether the number of sulfate groups on sHA affected its ability to inhibit HAase activity.

sHA compounds containing varying degrees of sulfation were prepared using the tributylamine salt of HA and various amounts of SO$_3^–$ pyridine, as reported by one of us (i.e., Barbucci et al., 1995). Depending on the sulfate content, we designated these sHA species as sHA2.0, sHA2.5, and sHA2.75. To determine the degrees of sulfation in each of the sHA compounds, we performed %S and %N analysis. As summarized in Table II, based on our previously described procedure, we are able to obtain sHA species with increasing degrees of sulfation by increasing the SO$_3^–$-pyridine to the tributylamine HA ratio. The %S in sHA2.0 (9.74%) is close to the theoretical %S value (i.e., 10.3%) calculated from a sHA disaccharide (molecular weight = 620 dalton) that contains two sulfate groups; the experimental and theoretical values for %N are identical (i.e., 2.2%). The %S in sHA2.5 (i.e., 11.8%) is slightly lower than the theoretical %S value (i.e., 12.2%), and the %N value in sHA2.5 is slightly higher (i.e., 2.2%) than the theoretical %N value (i.e., 2.1%). These differences may be because of experimental variation, which was about 10%. sHA2.5 (molecular weight of the disaccharide = 656 dalton) means that 50% of disaccharides in the HA molecule most likely contain two sulfate groups and the remaining 50% contain three sulfate groups. Nonetheless, the degree of sulfation in sHA2.5 is lower than that in sHA2.0. In case of sHA2.75 (disaccharide molecular weight = 680 dalton), the %S value (12.5%) is closer to the theoretical value 12.9%, and the %N value is almost the same (2.01%) as that of the theoretical value (2.05%). This suggest that in the sHA2.75 polymer, 75% of the HA oligosaccharides contain three sulfate groups and the remaining 25% contain two sulfate groups. As summarized in Table II, increasing the SO$_3^–$-pyridine to the tributylamine HA to 15:1 does not result in a further increase in HA sulfation.

We also confirmed the presence of sulfate groups in each sHA compound using the dimethylmethylene blue (DMMB) assay and the HA ELISA-like assay. Sulfated glycosaminoglycans, but not HA, are detected by the DMMB assay. As shown in Figure 2A, HA is not detected by the DMMB assay. However, sHA derivatives, sHA2.0, sHA2.5, and sHA2.75, are detected by the DMMB assay, and the optical density increases in a dose-dependent manner. Furthermore, sHA derivatives are detected by the DMMB assay in a manner very similar to that of other sulfated glycosaminoglycans assayed by the DMMB method (data not shown). As shown in Figure 2B, HA is detected by the HA ELISA-like assay; and the maximum limit of HA detection by the ELISA-like assay is 10 ng/mL of HA. However, none of the sHA compounds are detected by the HA ELISA-like assay at that concentration. Furthermore, even at >100,000-fold excess concentration (i.e., 100–300 μg/mL), the detection of sHA2.0, sHA2.5, or sHA2.75 in the HA ELISA-like assay is <50% of the maximum optical density reading obtained at 10 ng/mL concentration of HA. It is noteworthy that sulfated glycosaminoglycans are also not detected by the HA ELISA-like assay. These three analytical analyses establish that we have synthesized sulfated derivatives of HA.

![Figure 2. Characterization of sHA compounds. (A) DMMB assay. Various concentrations of HA and sHA compounds were mixed with DMMB reagent, and the O.D. was measured at 515 nm. (B) HA ELISA-like assay. Various concentrations of HA and sHA compounds were measured by the HA ELISA-like assay, as described in Materials and methods.](https://academic.oup.com/glycob/article-abstract/16/1/11/651381)
As summarized in Table I, both HYAL-1 and bee HAases can be inhibited by heparin with IC$_{50}$ values of 0.39 μM for HYAL-1 and 0.41 μM for bee HAase, respectively. However, the IC$_{50}$ of heparin for testicular (IC$_{50}$ = 17 μM) is ~40 times higher, and Streptomyces HAase is resistant to inhibition by heparin.

**Effect of small molecular weight inhibitors on HAase activity**

As summarized in Table I, gossypol and fenoprofen do not inhibit the activity of any of the four HAases when tested up to a concentration of 100 μg/mL. Both 1-tetradecane sulfonic acid (IC$_{50}$ = 63 μM) and glycerrhizic acid (IC$_{50}$ = 39 μM) weakly inhibit HYAL-1 activity (Table I). Sodium aurothiomalate inhibited only testicular HAase with an IC$_{50}$ of 94 μM (Table I).

The results described above demonstrate that among the 21 HAase inhibitors that were tested, PSS and sHA are the two categories of inhibitors that are the most effective in inhibiting HYAL-1, bee HAases and to a lesser degree testicular HAase. The activity of Streptomyces HAase is not inhibited by all but two (i.e., PSS 990,000 and VERSA-TL 502) HAase inhibitors.

**Enzyme kinetic studies**

To determine the mechanism by which PSS and sHA compounds inhibit the activity of HYAL-1, bee, and testicular HAases, we performed enzyme kinetic studies. For kinetic studies, we slightly modified the ELISA-like assay by coating microtiter wells with HA at concentrations from 0.5 to 10 μg/mL. This range of substrate concentration was chosen to be in the linear range of product formation.

Figures 4 and 5 show Lineweaver-Burke plots for HA degradation by HYAL-1 (Figure 4) and testicular (Figure 5) HAases in the presence of four different concentrations of PSS 990,000, PSS 1400, and sHA2.5. As shown in Figure 4A–C, the double reciprocal plots for HA degradation by HYAL-1 in the presence or absence of PSS 990,000, PSS 1400, and sHA2.5 are linear, indicating that none of the HAase inhibitors induce any cooperativity. However, in the presence of each of these inhibitors, the slopes and extrapolated Y- and X-intercepts increase, suggesting that PSS 990,000, PSS 1400, and sHA2.5 inhibit HYAL-1 through a mixed inhibition mechanism (i.e., competitive and uncompetitive inhibition). Both sHA2.0 and sHA2.75 also inhibit HYAL-1 activity through a mixed inhibition mechanism.

As summarized in Table III, both HYAL-1 and bee HAases are hygroscopic and the molecular weight of sHA2.0 (774,000 dalton), sHA2.5 (818,000 dalton), and sHA2.75 (848,000 dalton) are estimates, the IC$_{50}$ values presented in Table I for each sHA compound may be off by a factor of 2 (or 50% error).

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**Fig. 3.** Effect of sHA on HAase activity. Approximately 40 mU/mL of HYAL-1, bee, testicular, or Streptomyces HAase were incubated on an HA-coated microtiter well plate in the presence or absence of various concentrations of sHA2.0 (A), sHA2.5 (B), or sHA2.75 (C). The HAase ELISA-like assay was performed as described in Materials and methods. HAase activity, in the absence of any inhibitor was considered as 100% (control), and the data are expressed as % of control.
As summarized in Table III, both testicular and bee HAases also appear to be inhibited by PSS and sHA compounds through a mixed inhibition mechanism. Except for PSS 1400, the other inhibitors are equally good as competitive and uncompetitive inhibitors of testicular HAase (Table III). For bee HAase, except for PSS 990,000, the $K_i(s)$ of PSS 1400, sHA2.0, sHA2.5, and sHA2.75 as competitive inhibitors are $\sim$25-fold higher than the $K_i(s)$ of the same inhibitors as uncompetitive inhibitors, suggesting that these compounds are better uncompetitive inhibitors.

**Discussion**

HAases are expressed in both prokaryotes and eukaryotes and are involved in bacterial pathogenesis, spread of toxins and venoms, acrosomal reaction/ovum fertilization, and cancer progression (Tu and Hendon, 1983; Henrissat, 1991; Gmachl and Kreil, 1993; Kreil, 1995; Henrissat and Bairoch, 1996; Anderson et al., 2000, 2002; Lokeshwar et al., 2000, 2001, 2002b, 2005b; Zaneveld et al., 2002; Stern, 2004). Thus, inhibition of HAase activity by HAase inhibitors could be used as an antidote for toxins/venoms, contraceptives, and anti-tumor therapy. For example, using bladder and prostate cancer model systems, we have recently shown that HYAL1 is a molecular determinant of tumor growth, invasion, and angiogenesis (Lokeshwar et al., 2005a,b). Lack of HYAL1 arrests cells in the G2-M phase of the cell cycle, and the tumors generated are 10-fold smaller, less angiogenic, and resemble benign neoplasia. Thus, if an HAase inhibitor is more specific for HYAL1, it could be used to design anti-cancer therapies. Several reports have shown that the sodium salt of PSS, sodium cellulose sulfate, and other HAase inhibitors have a broad spectrum of anti-microbial activity against organisms which cause sexually transmitted diseases and are effective as both contraceptive agents and as antitoxins for cobra and pit viper venoms (Anderson et al., 2000, 2002; Zaneveld et al., 2002; Yingprasertchai et al., 2003). The availability of selective inhibitors, which target one type of HAase, without affecting other HAases, is desirable because HAases have normal physiologic functions. These examples suggest a practical value for surveying HAase inhibitors for specific clinical uses.

In this study, we show that there is some selectivity among the different inhibitors with respect to blocking the activity of different HAases. For example, we found that HYAL-1 and bee HAases show similar sensitivity to all but two HAase inhibitors (i.e., 1-tetradecane sulfonic acid and glycerrhizic acid). HYAL-1 and bee HAases have an acidic pH optimum and share a 27.2% amino acid identity. However, the amino acid identity cannot explain the similarity and differences of various HAases to inhibition by different HAase inhibitors. For example, testicular HAase is more resistant than HYAL-1 and bee HAase to inhibition by 20 of the 21 HAase inhibitors that were tested in this study. Similarly, Streptomyces HAase, which has a pH optimum at 5.0, is resistant to all but two (PSS 990,000 and VERSA-TL...
Table III. Effect of PSS and sHA on HAase activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>HYAL1</th>
<th>Bee venom HAase</th>
<th>Testicular HAase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Competitive ($K_i$ μM)</td>
<td>Uncompetitive ($K_i$ μM)</td>
<td>$V_{\text{max}}$ pmol/min</td>
</tr>
<tr>
<td>None</td>
<td>—</td>
<td>—</td>
<td>0.45 ± 0.22</td>
</tr>
<tr>
<td>PSS 990,000</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>PSS 1400</td>
<td>91 ± 7</td>
<td>3.5 ± 2.1</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>sHA2.0</td>
<td>0.08 ± 0.03</td>
<td>0.004 ± 0.002</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>sHA2.5</td>
<td>0.04 ± 0.01</td>
<td>0.002 ± 0.001</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>sHA2.75</td>
<td>0.03 ± 0.02</td>
<td>0.002 ± 0.001</td>
<td>0.02 ± 0.01</td>
</tr>
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</table>

Kinetic data were generated for PSS 990,000, PSS 1400, sHA2.0, sHA2.5, and sHA2.75 from the double reciprocal plots for each inhibitor as have been presented for PSS 990,000, PSS 1400, and sHA2.5 for HYAL-1 (Figure 4) and testicular (Figure 5) HAases. The $K_m$ and $V_{\text{max}}$ values (mean ± SD) for each enzyme are calculated assuming the molecular mass of HA as 500,000 dalton. $K_i$ values for each inhibitor are calculated using the molecular mass of each inhibitor.
502) of the HAase inhibitors tested in this study. Thus, although the reason is unknown at this time, it appears that HAases active at pH ≥ 5.0 are resistant to several different classes of HAase inhibitors. However, consistent with our conclusion that different HAase inhibitors have different selectivity toward HAases, Botzki et al. (2004) showed that l-ascorbic acid 6-hexadecanoate is 14- to 25-fold more potent in inhibiting HAase from *Streptococcus agalactiae* than it is for inhibiting *Streptococcus pneumoniae* and testicular HAase. Because sodium-PSS, cellulose sulfate, heparin, and gossypol have been shown to inhibit the acrosomal reaction, it is important to bear in mind that when used clinically, these inhibitors will inhibit HYAL-1 which is present in serum (Wolf et al., 1984; Yuan et al., 1995; Lokeshwar et al., 1999; Anderson et al., 2000, 2002; Zaneveld et al., 2002).

At this time, it is unknown why the bacterial HAase (i.e., Streptomyces HAase) is resistant to all but two HAase inhibitors that were tested. Because the sequence of *Streptomyces hyalurolyticus* is unknown, we performed an amino acid sequence alignment between HYAL-1 (GenBank Accession number BC035695.1) and *Streptomyces griseus* HAase (GenBank Accession number AB028210.1). Although, there is ~26% amino acid identity between these two proteins, the identical amino acids occur randomly in both sequences. In addition, large stretches of amino acid sequences present in one protein are absent in the other and vice versa. Similar observations were made when we aligned the Streptomyces HAase sequence with either PH20 (GenBank accession number NM003117.3) or bee HAase (GenBank accession number A47477; Markovic-Housley et al., 2000). It is noteworthy that we have identified a 30 amino acid sequence that is well conserved in all six human HAases and in bee HAase. This sequence is required for HAase activity (Lokeshwar et al., 2002a). We noted that within this 30 amino acid sequence, identical or conserved amino acid substitutions are present at positions 2, 3, 6, 7, 14, 15, and 17–22 in all mammalian and bee HAases. However, this 30 amino acid sequence is not well conserved in Streptomyces HAase. These observations suggest that higher eukaryotic HAases have significantly diverged from bacterial HAases, and this may explain why the bacterial HAase is resistant to most of the HAase inhibitors that were tested in this study. The crystal structures of *S. agalactiae*, testicular HAase, bee venom HAase and chondroitin ABC hyase have been deciphered recently (Markovic-Housley et al., 2000; Huang et al., 2003; Botzki et al., 2004). These structures show notable similarities and differences in the catalytic sites of these glycosaminoglycan-degrading enzymes. Because many of the HAase inhibitors are uncompetitive inhibitors, it appears that they most likely do not bind directly to the catalytic domain. The sequences of these enzymes in other parts of the molecule are less conserved, suggesting that it may be possible to identify natural compounds or synthesize new ones that specifically inhibit a particular glycosaminoglycan-degrading enzyme.

It has been previously shown that fully O-sHA can inhibit urinary HAase activity. In this study, we find that modification of HA with two sulfate groups is as effective in inhibiting HAase activity as is the sHA with a mixture of HA disaccharides with two and three sulfate groups. It is interesting that although theoretically HA disaccharides can contain up to four sulfate groups, experimentally we could not achieve this degree of sulfation even after increasing the SO$_3^-$-pyridine to the tributylamine HA ratio from 1:10 to 1:15. We could not increase this ratio further, because of the solubility of SO$_3^-$pyridine, at higher concentrations. It is noteworthy that Balazs et al. (1951) reported 13.2% S content in their sHA preparation. This experimental %S value is also less than the theoretical %S value (13.8%) for a HA disaccharide that contains three sulfate groups, suggesting that maximally sHA polymer is most likely a mixture of disaccharides containing two or three sulfate groups. In the case of PSS compounds, the length of the polymer is the factor that decides the efficacy of HAase inhibition. Therefore, if these or cellulose-sulfate compounds are to be used as contraceptives, the optimum concentration will depend upon the length of the polymer and how it inhibits testicular HAase.

It has been previously reported that fully O-sHA inhibits urinary HAase activity through both competitive and non-competitive mechanisms (Toida et al., 1999). Since in that study, the $K_m$ values in the absence or presence of inhibitor were not reported, it is unclear whether the authors observed a non-competitive or uncompetitive inhibition. In our study, most inhibitors caused a slight increase in the $K_m$, suggesting a mixed inhibition consisting of competitive and uncompetitive mechanisms. Since both sHA and PSS compounds are more effective as uncompetitive inhibitors than as competitive inhibitors, these would be effective in vivo, as their efficacy will be independent of the HA concentration present in target tissues and tissue fluids. This finding is particularly important for designing anti-HAase treatments for cancer, because in many tumor tissues HA concentration is elevated (Setala et al., 1999; Auvinen et al., 2000; Lokeshwar et al., 2000, 2001, 2002b; Pirinen et al., 2001; Posey et al., 2003).

Taken together, our study demonstrates that although all HAases degrade HA, they show differences in their sensitivity to different classes of HAase inhibitors. HAase inhibitors displaying mixed inhibition may prove to be effective in inhibiting HAase activity in various pathophysiologic conditions.

**Materials and methods**

**Materials**

Sodium salts of PSS of molecular weight 210 (PSS 210), 1400 (PSS 1400), 4300 (PSS 4300), 6800 (PSS 6800), 17000 (PSS 17000), 32000 (PSS 32000), 49000 (PSS 49000), 77000 (PSS 77000), 150000 (PSS 150000), 350000 (PSS 350000), and 990000 (PSS 990000) were purchased from Fluka Biochemicals (Buchs, Switzerland). VERSA-TL 502, a PSS of molecular weight $1 \times 10^6$ dalton was kindly provided by Alco Chemicals, a Division of National Starch Company (Chattanooga, TN). Gossypol, fenoprofen, 1-tetradecane sulfonic acid, glycercrizic acid, and heparin were purchased from Sigma Chemicals (St. Louis, MO).

sHA derivatives sHA2.0, sHA2.5, and sHA2.75 were prepared as discussed previously (Barbucci et al., 1995) with the following modifications: 100 mg of human umbilical
cord HA–sodium salt was dissolved in distilled water and desalted on a Cellex-P matrix (BioRad, Hercules, CA). HA was then mixed with three molar equivalents of tributylamine at room temperature for 2 h. Following incubation, the HA solution was alkalinized (pH 8.0), dialyzed extensively against distilled water, and lyophilized. Hundred milligrams of the HA–tributylamine salt was suspended in anhydrous dimethylformamide and mixed with various amounts of anhydrous SO₃·pyridine under a stream of nitrogen. The amount of SO₃·pyridine determines the number of O-sulfated groups in the sHA polymer (Barbucci et al., 1995).

The sHA was then precipitated, dialyzed against water, and lyophilized. The molecular mass of the various sHA compounds was determined using a Sepharose S-300 (GE Healthcare, Piscataway, NJ) gel filtration column (1.5 × 120 cm) eluted with phosphate-buffered saline + 0.05% Tween 20; 1.6 mL/fraction. The column was calibrated using HA species of various molecular mass (2.0 × 10⁶ D to 8 kDa; Genzyme, Cambridge, MA). The elution volume for sHA2.0 derivatives was similar to 0.5 mU/mL activity was expanded in T-75 cm² flasks. At et al.

HYAL-1 cDNA encoding the full-length protein (Lokeshwar et al., 2005b). Briefly, a stable HYAL1 clone expressing HYAL-1, testicular, bee, or Streptomyces HAase in the presence or absence of various HAase inhibitors in respective assay buffers. For the HYAL-1 activity assay, we used 0.1 M sodium formate, 0.15 M NaCl, 0.02% bovine serum albumin buffer, pH 4.2. Testicular HAase assay buffer contains 0.1 sodium acetate, 0.15 M NaCl, 0.02% bovine serum albumin, pH 5.5. Bee HAase was assayed in the same buffer as that used for HYAL-1, whereas Streptomyces HAase was assayed in 0.1 M sodium acetate, 0.15 M NaCl, 0.02% bovine serum albumin, pH 5.0.

The effect of HAase inhibitors on the activity of HYAL-1, testicular, bee, and Streptomyces HAases was tested at 0, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80, and 100 μg/mL concentrations of each inhibitor. The plate was incubated at 37°C for 16 h. Following incubation, the degraded HA was washed off, and the HA remaining on the microtiter plate was determined using a biotinylated HA-binding protein and an avidin-biotin detection system (Vector Laboratories, Burlingame, CA) (Lokeshwar et al., 2001). Each plate was developed for 3 min, and the reaction was terminated by adding 3 N HCl. HAase activity (mU/mL) was calculated from a standard graph, prepared by plotting known amounts of Streptomyces HAase (10⁻⁴ U/mL), versus (control [no enzyme]) O.D.₄₀₅ nm – sample O.D.₄₀₅ nm). IC₅₀ for each inhibitor was calculated as the concentration (μM) of an inhibitor required to inhibit 50% of the activity of the HAase tested. Molecular weights of each compound (e.g., 990,000 dalton for PSS 990,000, 1400 dalton for PSS 1400, 774,000 for sHA2.0) were used in the calculations of IC₅₀ values in μM. About 100% activity was defined as the enzyme activity obtained in the absence of any inhibitor. The data presented are mean ± SEM from duplicate measurements in two independent experiments.

Enzyme kinetic assays

Microtiter plates were coated with 100 μL of 0, 0.5, 1, 2.5, 5, 7.5, and 10 μg/mL of human umbilical cord HA at 37°C for 4 h. Following incubation, each well received ~20 μU/mL of HYAL-1, Streptomyces, testicular, or bee HAase and 0, 6, 10 or 20 μg/mL of an inhibitor, in a total volume of 100 μL of an appropriate HAase assay buffer (as described above). Control wells received inhibitor at a particular concentration but no enzyme. The plate was incubated at 37°C for 4 h and then developed as described above. The amount of product (i.e., HA degraded) was calculated by subtracting the amount of HA remaining on the wells from the amount of HA that was used to coat the wells. The amount of HA remaining on the wells was calculated from O.D.₄₀₅ nm in control wells (i.e., wells containing no enzyme) and O.D.₄₀₅ nm in sample wells (i.e., wells containing enzyme + inhibitor). Kₘ, Vₘ₉₉, and Ki (for both competitive and uncompetitive inhibition) were calculated by plotting Lineweaver-Burke double reciprocal plots. The Kₘ value was expressed in μM by assuming the molecular mass of HA to be 500,000 dalton. The molecular mass of HA was determined by gel filtration chromatography performed on a Sepharose S-300 column. The Vₘ₉₉ was expressed as pmole/min. The graphs were plotted using the GraphPad Prism Software Program (version 3.1, GraphPad Software, Inc., San Diego, CA), and Ki values for each inhibitor were calculated from the extrapolated X- and Y-intercepts and the slope. Ki for the competitive inhibition component of mixed inhibition was calculated from the equation: α = 1 + I/Ki, where α = 1/Vₘ₉₉. The Ki for the uncompetitive inhibition
component of mixed inhibition was calculated as \( \alpha' = 1 + \frac{I_i}{Ki} \), where \( \alpha' = Y\text{-intercept} \times \frac{1}{I_{\text{max}}} \). The \( Ki \) values were expressed as \( \mu \text{M} \) concentration of the inhibitor and were calculated from the molecular mass of the inhibitor.

### DMMB assay

Various concentrations of HA and sHA in a final volume of 0.1 mL were mixed with 0.5 mL of DMMB reagent. The optical density of the color developed was determined at 515 nm (Farnsdale et al., 1982).

### HA ELISA-like assay

HA ELISA-like assay was performed, as described previously (Lokeshwar et al., 2000, 2001). Briefly, 25 \( \mu \text{g/mL} \) human umbilical cord HA was coated on microtiter wells of a 96-well plate. Following the blocking of nonspecific sites using 1% bovine serum albumin, various concentrations of HA or sHA compounds were incubated in HA-coated wells in phosphate-buffered saline, containing 0.05% Tween 20 for 16 h at room temperature. Following incubation, the unbound HA-binding protein was washed off, and the HA-binding protein bound to microtiter wells was measured using an avidin–biotin detection system (Vector Laboratories). HA present in each sample (ng/mL) was determined from a standard graph plotted as (control O.D.405 nm – sample O.D.405 nm) versus concentration.

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