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Interpreting the behavior of concentration–response curves of hyaluronidase inhibitors under DMSO-perturbed assay conditions

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ABSTRACT

Hyaluronan-degrading enzyme (hyaluronidase) is involved in tumor growth and inflammation, and as such, hyaluronidase inhibitors have received recent attention as potential therapeutics. The previous studies have successfully discovered a wide range of inhibitors, but unfortunately most of them are dissimilar to original ligand hyaluronan and the mode of action is poorly understood. The present study mechanistically characterized these structurally unrelated inhibitors by interpreting the behavior of concentration–response curves under several in vitro assay conditions. Detergent-addition conditions definitely identified aggregation-based inhibitors. Subsequently, DMSO-perturbed conditions, though preliminary, highlighted the inhibitors that might bind to enzyme non-specifically. Here, an intriguing implication of the latter description is that DMSO-perturbed conditions would generate non-productive but not-denatured enzyme that is an assembly of effective species to capture non-specific binding molecules, and thereby would attenuate their inhibitory activities.

Hyaluronidase (HAase) is a class of glycosidase that preferentially degrades hyaluronan (HA), a negatively charged liner polysaccharide composed of repeating disaccharide units of (β-1,4)-D-glucuronic acid (β-1,3)-*N*-acetyl-D-glucosamine (Fig. 1).[1,2] Mammalian HAase (E.C. 3.2.1.35) hydrolyzes β-1,4 glycosidic linkages, producing HA fragments with *N*-acetyl-D-glucosamine at the reducing end, and also shows a limited activity to degrade chondroitins, chondroitin sulfates, and dermatan sulfates. Biologically, HAase has been known to play critical roles in embryonic development, cell motility, tumor growth, invasion, angiogenesis, wound healing, inflammation, and other critical functions in our body.[3] Therefore, HAase inhibitors have received considerable attentions in both biological and pharmaceutical studies.

Fascinated with these potentials, the previous studies have discovered a wide range of HAase inhibitors,[3,4] often dissimilar to original ligand HA as is seen in early stage of drug discovery. Even though these initial hits show reproducible inhibitory activities in a concentration-dependent manner, they are subsequently found to have a peculiar inhibition property owe to the lack of specificity. They also show the inhibitory activity against a wide range of structurally unrelated enzymes often at micromolar concentration, and thus are referred to as 'promiscuous' inhibitors.[5,6] Therefore, a reliable identification of genuine hits, that is, a removal of false positive hits, has been a tremendously important task especially in early stage of drug discovery.

Over the past two decades or so, several studies have been devoted to elucidating the mechanistic insights into promiscuous enzyme inhibitors. The prevalent explanations for promiscuity are the inherent chemical reactivity toward proteins,[7,8] and the interferences with assay readouts.[9] The molecules with such promiscuous properties can be discriminated to some extent by the structural features such as functional groups or skeletons, and thus the rules of thumb have also been developed.[10] Another significant explanation for promiscuity is an aggregation-based inhibition.[6] It has been proposed that low molecular weight compound forms colloidal aggregate by self-association, then the resulting aggregate holds enzyme on its surface and partially unfolds native enzyme, ultimately causes promiscuous inhibition of enzyme at least in biochemical buffers at micromolar concentrations.[11,12] Based on this mechanism, an aggregating inhibitor can be highlighted by analyzing the behavior of concentration–response curves under several in vitro assay conditions. First, it shows steep or bell-shaped concentration–response curves.[13–15] Second, it exhibits the dependence of the inhibitory activity on ionic strength, concentration of enzyme, or incubation time.[6] Third, the inhibitory activity is attenuated in the presence of detergents such as Triton X-100, bovine serum albumin (BSA), or saponin.[16–19] However, whereas these behaviors seem to be more or less dependence on assay conditions,[14,20,21] the correlation between

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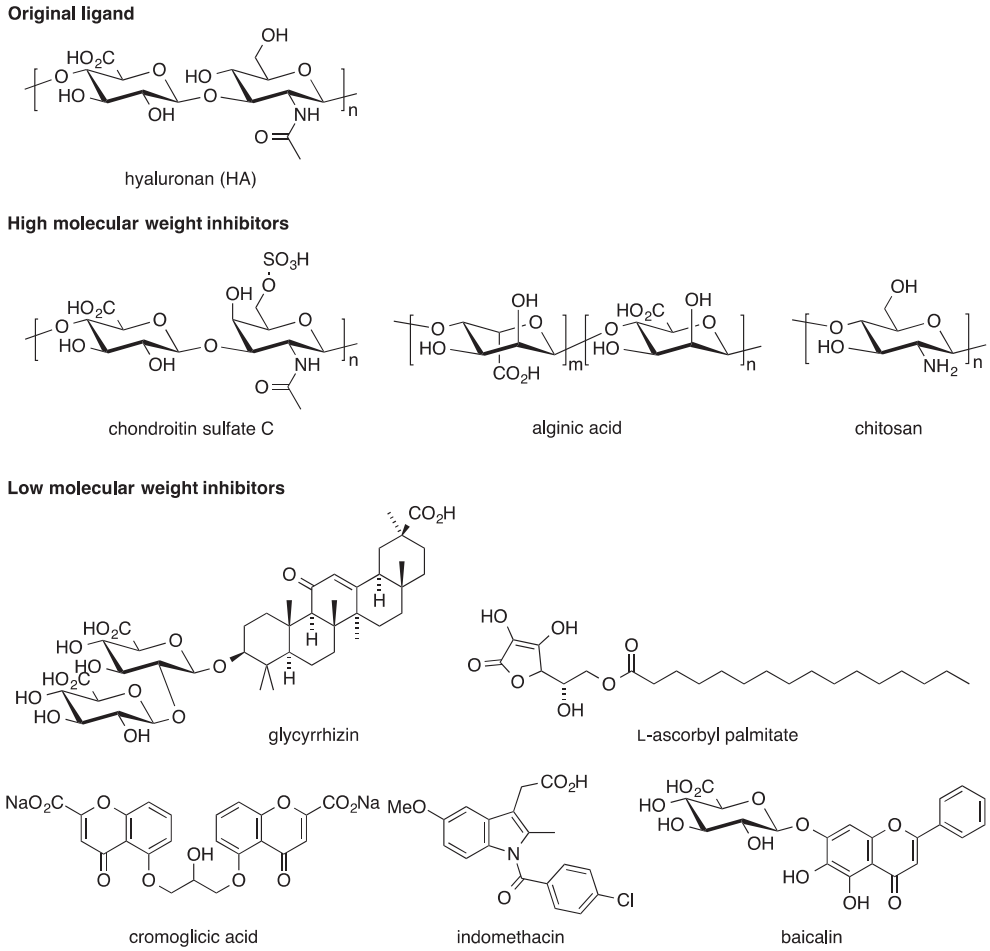

Figure 1. Structures of original ligand and inhibitors of hyaluronidase.

inhibition through aggregation and promiscuity should be carefully evaluated, and there still remains an urgent need for further study to develop a robust methodology to evaluate promiscuity.

Back to our interest in HAase inhibitor, we assumed that the structural dissimilarity between well-known HAase inhibitors and original ligand HA would imply promiscuity, that is, lack of specificity. In the present study, we fortunately noticed a specific change in the behavior of concentration–response curves of HAase inhibitors under dimethyl sulfoxide (DMSO)-addition conditions, where it is assumed that a native enzyme is perturbed to produce non-productive but not-denatured species in a DMSO-concentration dependent manner. This observation was then successfully bound to develop into a novel methodology to highlight non-specific binding inhibitors from initial screening hits by the attenuation of inhibitory activity under DMSO-addition conditions. The proof of concept was extensively established using structurally unrelated well-known HAase inhibitors, in combination with other complementary models.

First of all, we selected eight structurally unrelated inhibitors for consideration (Fig. 1). The similarity with original ligand HA marked three high molecular weight polysaccharides including chondroitin sulfate C,[22,23] polyanionic alginic acid,[24] and polycationic chitosan.[25] On the other hand, low molecular weight inhibitors included naturally occurring glycyrrhizin[26] and baicalin, anti-inflammatory indomethacin[25] and cromoglicic acid,[27] and synthetic L-ascorbyl palmitate.[28,29] Apparently, these low molecular weight inhibitors seem to be less relevant to the original ligand HA. Nevertheless, all inhibitors did show the reproducible inhibitory activity in a concentration-dependent manner under classical assay conditions (Table 1, conditions A, and Figs. S1 and S2, Supplementary material). The less relationships between structure and inhibitory activity prompted us to characterize these structurally unrelated inhibitors mechanistically under several in vitro assay conditions as described below.

Our study began with reconsideration of classical assay conditions. Since HAase exists in an inactive form, it is usually activated by an activator such as NaCl, $CaCl_2$, or compound 48/80 in biological study.[27] The classical assay conditions involve the preincubation of HAase with inhibitor prior to the activation of HAase (Table 1, conditions A). Therefore, it was assumed that the inhibitory activity determined under classical assay conditions would be at least composed of two distinct mode of action, that is, the inhibition of the activation and the inhibition of activated HAase, as sometimes described in the literature.[24,27] This ambiguity in site of action called for consideration under the modified assay conditions; HAase was activated before the addition of inhibitor (Table 1, conditions B). As a result, all high molecular weight inhibitors showed similar IC_{50} values regardless of assay conditions (entries 1–3), while all low molecular weight inhibitors exhibited the significant attenuation of inhibitory activities under conditions B as compared with conditions A (entries 4–8). Indeed, this attenuation was observed even for the potent low molecular weight inhibitors glycyrrhizin and L-ascorbyl palmitate. Glycyrrhizin showed the IC_{50} values of 29 μM for activated HAase, more than three times higher (worse) than the values determined under conditions A (entry 4). L-Ascorbyl palmitate also exhibited the statistically