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Hyaluronic acid alkyl derivative: A novel inhibitor of metalloproteases and hyaluronidases

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ABSTRACT

Extracellular matrix (ECM) degradation, one of the main features of osteoarthritis, is driven by at least two major classes of enzymes: matrix metalloproteases (MMPs) and hyaluronidases. Among certain glycosaminoglycans, including natural and chemically cross-linked HAs, which are currently used as viscosupplements, the hyaluronic acid (HA) alkyl-amides (Hyadd) were here selected as the strongest MMP and hyaluronidase inhibitors. We used *C. histolyticum* collagenase (*Ch*C) and bovine testicular hyaluronidase (BTH) as representative models of human MMPs and hyaluronidases, respectively. The role of the alkyl moiety was investigated using HA derivatives with varying alkyl lengths and degrees of derivatization. The selected compound was then screened against 10 different human MMPs *in vitro*, and the results were validated *ex vivo* in human synovial fluid. Hyadd-C16, identified as a lead compound, showed the highest inhibition potency against MMP13 and MMP8. The *in vitro* results were confirmed by the inhibition of human MMP13 (K_i = 106.1 μM) and hyaluronidase-2 in the synovial fluid of patients with osteoarthritis. This study demonstrates the unique properties of Hyadd-C16, including its remarkable enzymatic inhibitory activity, which is conferred by the hydrophobic chain, and its high biocompatibility and water solubility of the HA backbone.

1. Introduction

Hyaluronic acid is a glycosaminoglycan (GAG) that occurs naturally in the articular capsule of the knee joint and is administered intra-articularly to treat knee pain in patients with osteoarthritis (OA). Osteoarthritis is the most common form of arthritis and affects nearly 27 million Americans [1]. The major characteristic of OA is the breakdown of articular cartilage, although the progression of the disease also affects the synovial membrane and the subchondral bone [2,3]. The process of OA involves inflammation in the early stage of the disease, which is initiated in the articular cartilage and then occurs in the underlying bone tissue [4,5].

Many mediators, including IL-1β, TNF-α, and IL-6, are involved in the pathophysiology of OA. These mediate the expression of genes that encode several inflammatory proteins and induce the activation of cartilage-degrading enzymes, such as MMPs and ADAMTSs, through different signalling pathways. In particular, the activation of the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling pathway [2,6] and of the complement system results in the formation of the membrane attack complex (MAC) [7].

The overexpression of MMPs (*e.g.*, MMP1, MMP3, MMP13) results in an imbalance of MMPs and endogenous inhibitors (α2-macroglobulin and TIMPs) [4,8]; the consequent increased MMP activity is assumed to be among the major causes of the degradation of type II collagen in OA. Thus, two of the clearest signs of disease onset in patients with OA are synovial inflammation and the reduction of the average molecular weight (MW) and concentration of endogenous HA in the synovial fluid (SF), with the consequential reduction in its mechanical function [9]. Physiologically, high MW HA works synergically with lubricin to provide boundary lubrication [10], while low MW HA leads to a decrease in synovial viscosity and may interact with TLR-4 and CD44 receptors in human chondrocytes to induce the production of inflammatory cytokines [11] and the increased expression of MMP13 [12].

Generally, HA turnover is well regulated. For example, in the synovium of the human knee, HA is synthesized by three isoforms of HA synthase (HAS-1, -2 and -3) and metabolized by at least three homologous isozymes (Hyal-1, -2 and -3) [13]. However, this process is not well understood, and recently, a unique hyaladherin (KIA1199) was discovered in the arthritic synovium, and it plays a key role in HA catabolism that functions independently of the CD44 and Hyal enzymes [9].

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While the pathway that results in cartilage breakdown is still unclear, there is strong evidence that hyaluronidases (specifically Hyal-2) [14] and MMPs (*e.g.*, MMP13) [15,16] are important for the degradation of the ECM. MMP inhibition has been extensively investigated, leading to the classification of three groups of inhibitors: peptidomimetics (collagen mimetics), non-peptidomimetics and tetracycline. The first two groups have a functional moiety that is able to coordinate the catalytic zinc atom (hydroxamates, thiols, carboxylates and phosphonic acids), whereas the third group is an antibiotic that has multiple types of action [17]. Hydroxamate inhibitors have been shown to have the highest activity, with IC_{50}-values that are in the picomolar range. Yet, the same group of inhibitors also exhibited unfavourable pharmacokinetics and chronic toxicity (*i.e.* musculoskeletal syndrome) [18], which is attributed to non-selective MMP inhibition. While some MMP inhibitors are currently in development for use in the treatment of arthritis (in addition to cancer and neoplasia), other candidates were withdrawn during clinical trials. For example, the hydroxamate inhibitor Trocade RO32-3555 and the carboxylate inhibitor Tanomastat BAY12-9566 were both withdrawn during Phase II [17]. Most of the inhibitors bear a hydrophobic extension (P'_1 group) that binds the S'_1 pocket in the catalytic site of the MMP. This hydrophobic pocket is variable in depth among different MMPs [19] and is therefore one of the determining factors for substrate specificity [20]. Although there have been substantial efforts to block the destructive activity of MMPs over the past years, no effective MMP inhibitor that can be used in the treatment of OA has yet been approved.

Here, we report a new class of intra-articular alkyl derivatives of HA that act as MMP and hyaluronidase inhibitors for use in OA treatment [21]. The polymer structure of these derivatives suggests that the alkyl side chain could selectively insert into the hydrophobic S_1' pocket of the enzyme binding site and that the HA carboxyl group could act as a zinc-coordinating moiety in the MMP catalytic domain [19]. Moreover, the intra-articular administration of the polymer could overcome the medical complications of unfavourable pharmacokinetics, thereby guaranteeing a sufficient *in vivo* residence time and an absence of toxicity [22]. Furthermore, due to previous reports by Botzki et al. [23], in which L-ascorbic acid 6-hexadecanoate is a potent inhibitor for the hyaluronidase class of enzymes, we expected that the HA alkyl derivatives would also strongly inhibit hyaluronidase. In this study, we first verified that an eventual loss of enzymatic activity in the presence of HA alkyl derivatives or other natural and synthetic cross-linked GAGs (*e.g.*, HA, chondroitin sulphate) that are already used in OA intra-articular therapy did not originate from a simple physical entrapment of the enzymes. As representative models of the human MMPs and hyaluronidases, we used *Chlostridium histolyticum* collagenase (*Ch*C), which is a multi-unit protein with a conserved HExxH zinc-binding motif that is similar to the vertebrate MMPs [24], and bovine testicular hyaluronidase (BTH). These enzymes have already been used for the *in vitro* screening of human enzyme inhibitors [23,24]. Next, we studied six amide derivatives of HA that contain C8, C12, C15, C16, or C18 linear alkyl amines as substituents [25] as well as benzyl amine. In each of these, the inhibitory activity against *Ch*C and BTH was tested. The mechanism of inhibition was elucidated, and the best performing compound was screened against 10 different human MMPs. Finally, the inhibitory activity against Hyal-2 and MMP13 was validated in an *ex vivo* study in the synovial fluid (SF) from patients with OA and inflammatory arthritis.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt (HA) was provided by Fidia Farmaceutici S.p.A. Enzo Life Science supplied a Fluorimetric MMP Inhibitor Profiling Kit. The SensoLyte® Plus 520 MMP-13 Assay Kit was provided by AnaSpec and Proteos Biotech provided the Pure100 Collagenase G/H (*Ch*C). All other reagents were supplied by Sigma and used without further purification.

2.2. Synthesis of HA derivatives

We synthesized hyaluronan 1,4-butanediol-diglycidyl-ether (BDDE) cross-linked polymer (HBC, synthesized with 10% mol/mol BDDE *vs* HA monomeric unit) and the HA alkyl derivatives (Hyadd) as previously reported [25]. The HA benzyl amide (Hyadd-Bz) derivative was synthesized following the same protocol that was used for the synthesis of Hyadd (see SI). Hyaluronan divinylsulphone (DVS) cross-linked polymer (HDC, 10% mol/mol) was synthesized by dissolving DVS (29.4 mg; Sigma–Aldrich) in 0.25 M NaOH (7.4 mL) and then added to the sodium salt of HA (1 g, 700 kDa; Fidia Farmaceutici). The mixture was stirred for 25 min at room temperature and then for one hour at 45 °C. Next, the solution was neutralized with 0.1 M HCl to a pH of approximately seven and then diluted with saline to reach a final HA concentration of 8 mg/mL. The final product was a transparent gel that was characterized by rheological and ICP-OES measurements (see SI).

Every HA derivative was formulated at a concentration of 8 mg/mL in PBS at pH 7 and sterilized by heat. The degree of

Table 1
MW and derivatization degree of the tested polymers.

Sample	Non depolymerized		Depolymerized for 24 h at 105 °C	
	M_w (kDa)	Derivatization degree (% mol/mol)	M_w (kDa)	Derivatization degree (% mol/mol)
Chondroitin sulphate	35	–	<10	–
High M_w HA	2000	–	58	–
HBC 10%	>4000	4.7	91	4.8
HDC 10%	>4000	4.4	87	4.5
Hyadd-Bz	698	7.3	66	7.4
Hyadd-C8	793	7.0	89	6.9
Hyadd-C12	921	8.3	64	8.1
Hyadd-C15	826	8.1	75	8.3
Hyadd-C16	752	7.4	86	7.5
Hyadd-C18	890	7.3	98	7.0
Mean	813	7.6	80	7.5
Standard deviation	83.8	0.5	13.5	0.6
Hyadd-C16	752	7.6	86	7.5
Hyadd-C16 (low degree)	781	3.0	68	2.9
Hyadd-C16 (high degree)	805	17.0	72	17.2