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Hyaluronic acid based hydroxamate and conjugates with biologically active amines: *In vitro* effect on matrix metalloproteinase-2

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ABSTRACT

In this study, water soluble hyaluronic acid (HA) based hydroxamate and conjugates with biologically active amines and hydrazides such as *p*- and *o*-aminophenols, anthranilic, 4- and 5-aminosalicylic acids, nicotinic, *N*-benzylnicotinic and isonicotinic hydrazides, *p*-aminobenzenesulfonamide (Streptocide), *p*-aminobenzoic acid diethylaminoethyl ester (Procaine), and 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (4-aminoantipyrene) were examined as matrix metalloproteinase-2 inhibitors (MMPIs). In a dose of 0.27–270 μM, the most efficient MMPIs were HA conjugates with *o*-aminophenol = 4-aminoantipyrine > 4-aminosalicylic acid > 5-aminosalicylic acid. Conjugates with Streptocide, Procaine and HA hydroxamate showed 40–50% inhibitory effect at all used concentrations. Conjugates with anthranilic acid and isonicotinic hydrazide (Isoniazid) in a dose of 0.27 μM inhibited enzyme activity by ~70%, but with the concentration increase their inhibitory effect was decreased.

1. Introduction

Matrix metalloproteinases (MMPs) are a family (more than 20) of extracellular zinc and calcium dependent endopeptidases (collagenases, gelatinases, stromelysins, and the membrane-type MMPs) involved in connective tissue remodeling in both normal physiological and pathological conditions. In pathological processes, such as chronic inflammation, tumor progression, cardiovascular disease, skin ulceration and many others, the level of MMPs is increased causing the weakening and destruction of connective tissue. Therefore, the MMPs inhibition is a strategic objective in the treatment of wide range diseases, and the search of efficient MMP inhibitors (MMPIs) is one of the priorities in the development of modern medicines (Gupta, 2012; Sagi & Gaffney, 2015).

The MMPIs library includes a wide variety of substances with different structures, both naturally occurring and synthetic compounds (Agrawal et al., 2010; Gupta, 2012; Whittaker, Floyd, Brown, & Gearing, 1999). Generally, these compounds contain zinc-binding groups (ZBG) and molecule fragments, which electrostatically interact with MMPs *via* hydrogen bonds or van der Waals forces. Classical MMPIs include carboxylate, hydroxamate, thiol, phosphinate or phosphonate groups; among them hydroxamate type inhibitors are considered as the most promising candidates for use in medical practice (Gupta, 2012). However, despite the large diversity, none of the known MMPIs has been licensed until date, with the exception of doxycycline, approved for the treatment of periodontal disease. This is mainly due to low selectivity, high toxicity, low bioavailability and/or solubility of the majority of MMPIs.

From the viewpoint of improving the solubility (and bioavailability) or decreasing the toxicity, the most suitable carriers for MMPIs are monosaccharides such as glucose (Attolino et al., 2010; Calderone et al., 2006) or water-soluble polysaccharides, for example, hyaluronic acid (HA) (Garg & Hales, 2004; Kogan, Šoltés, Stern, & Gemeiner, 2007; Ponedel'kina, Lukina, & Odinokov, 2008; Prestwich, 2011). This natural non-immunogenic and biodegradable polymer, consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine units, is widely applied in medicine as a synovial fluid substitute (to treat osteoarthritis of the knee *via* injecting into the joint) and viscoelastic for ophthalmic surgery. In order to increase the effectiveness of HA formulations for the treatment of osteoarthritis and like, some MMPIs of complex structure, preferably hydroxamate-type, were conjugated with HA *via* amide bond [EP 1082963][1]; the conjugates contained no more than 4% of

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[1] Also see JP 2004292465, in which carbamate link between HA and hydroxamate-type MMPIs was used.

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covalently attached MMPIs but revealed high inhibitory effect against collagenase-1, stromelysin-1, gelatinase A (MMP-2) and gelatinase B (MMP-9).

On the other hand, among the numerous HA derivatives synthesized worldwide it is possible to find ones, which correspond to requirements for MMPIs. For example, obtained in our previous works HA based hydroxamate (Ponedel'kina, Gaskarova, Lukina, & Odinokov, 2012a) and conjugates with several physiologically active amines and hydrazides (Ponedel'kina, Lukina, Sufiyarova, & Odinokov, 2012b; Ponedel'kina et al., 2005; Ponedel′kina, Sal′nikova, Lukina, Tyumkina, & Odinokov, 2012c), such as *p*- and *o*-aminophenols, anthranilic, 4- and 5-aminosalicylic acids, nicotinic, *N*-benzylnicotinic and isonicotinic (Isoniazid) hydrazides, *p*-aminobenzenesulfonamide (Streptocide), *p*-aminobenzoic acid diethylaminoethyl ester (Procaine) and 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (4-aminoantipyrene), contain groups or moieties capable of complexion with the catalytic site of MMPs (Zn^{2+}) or specific interaction with the enzymes. These HA derivatives are easy synthesized by carbodiimide technique in aqua medium, contain from 12 to 60% modified units (per 100 HA disaccharide units) depending of amine attached to HA backbone and are water soluble compounds. Moreover, they exhibit increased resistance to testicular hyaluronidase (or a reduced biodegradability) compared to intact HA, that is important for the development of drugs with prolonged action (Ponedel'kina, Lukina, Khaibrakhmanova, Sal'nikova, & Odinokov, 2011). To evaluate the potential of these HA based hydroxamate and conjugates as MMPIs, their effect on *in vitro* activity of one of MMPs (MMP-2) was studied in the present work.

2. Experimental

2.1. Materials

Hyaluronic acid (sodium salt) from bacterial source (M_n ~100 kDa) was purchased from "Leko Style" (St.-Petersburg). MMP-2 (62 kDa, human recombinant, expressed in *Escherichia coli*) and MMP-2 fluorogenic substrate MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (MCA, $C_{49}H_{68}N_{14}O_{15}$) were obtained from Sigma-Aldrich (USA). All other chemical reagents were purchased from Acros Organics and used without further purification.

2.2. Synthesis of HA conjugates **2a–j**

Conjugates of HA with *p*-aminophenol (**1a**), *o*-aminophenol (**1b**), anthranilic (*o*-aminobenzoic) acid (**1c**), 4-aminosalicylic acid (**1d**), 5-aminosalicylic acid (**1e**), isonicotinic acid hydrazide (Isoniazid) (**1f**), *p*-aminobenzenesulfonamide (Streptocide) (**1g**), *p*-aminobenzoic acid diethylaminoethyl ester (Procaine) (**1h**), 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (4-aminoantipyrine) (**1i**), and nicotinic hydrazide (**1j**) were obtained as previously reported (Ponedel'kina et al., 2005). In the case of amines **1**c,e,**f**,**g**,**h**,**j** *N*-hydroxybenzotriazole (HOBt) was added to avoid the *O*-hyaluronylisoureas formation.

Briefly, HA (120 mg, 0.3 mmol), corresponding amine **1a–j** (0.3–0.6 mmol) and HOBt (0.3–0.6 mmol, for amines **1**c,e,**f**,**g**,**h**,**j**) were dissolved in distilled water (30 mL) and the pH was adjusted to 4.7–4.8 with 0.1 M NaOH or HCl. Then 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (0.45–0.9 mmol) was added to the vigorously stirred reaction mixture at ambient temperature. To maintain the reaction pH at 4.7–4.8 0.1 M HCl was added if required. After 2 h, the reaction mixture was neutralized with 0.1 M NaOH, and HA conjugates **2a–j** were precipitated with three volumes of methanol. The precipitates were centrifuged, washed with methanol and diethyl ether and dried in vacuum at 60 °C for 2 h. The samples were obtained as white water soluble powders. Content of modified units in conjugates **2a–j** was found from ^{1}H NMR using the integrations of signals at 7.0–8.9 ppm for corresponding amine residue and signals at 1.4–2.0 ppm for acetamido group as reference (Fig. 1 and Figs. S1–S5). Upshifted signals in the area of 1.42–1.89 ppm for CH_3CON group were observed for all conjugates expect **2j** and due to shielding effect of adjacent magnetically anisotropic aromatic ring of arylamide group on the acetamido group (Fig. 1 and Figs. S1–S5). Content of modified units in conjugates **2a–j** is given in Table 2. Yield: 85–94%.

2.3. Synthesis of HA conjugate **2k**

Conjugate **2j** (100 mg) was benzylated with benzyl chloride in dimethylformamide at 80 °C for 20 h (Scheme 1), according to method described in a previous study (Ponedel'kina et al., 2012a,b,c).

^{1}H NMR (500 MHz, D_2O) δ 9.4–8.1 (4*H* Py), 7.45–7.41 (5*H*, Ph), 5.85 (br. s, CH_2, Bn), 4.48–3.27 (HA backbone), 1.96 (3*H*, CH_3CON, HA). The ratio of integral intensities of H-Py, H-Ph and H-CH_2 was 4:5:2 (*i.e.* the benzylation degree was 100%). Yield: 90%.

2.4. Synthesis of hydroxamate **6**

2.4.1. Preparation of O-hyaluronylisoureas **3**

Isoureas were prepared by reaction of HA (*A* units) with EDC (Scheme 2). In short, HA (200 mg, 0.5 mmol in 20 mL H_2O) and EDC (144 mg, 0.75 mmol in 20 mL H_2O) solutions with pH 4.7–4.8 were mixed at vigorously stirring, and the pH was maintained by adding 0.1 M HCl. Reaction was carried out for 2 h at ambient temperature, after that isoureas **3** were isolated by precipitation with methanol and was dried. Content of isoureido residues (*B* units) was found from ^{1}H NMR using the ratio of integral intensities of signals at ~3 ppm for $(CH_3)_2N$ group in *B* units and the signal at ~2 ppm for CH_3CON group. B = 100%; yield: 98%.

2.4.2. Synthesis of HA methyl ester **4**

Ester **4** was prepared according to a patented method (Hirano et al., 2005) (Scheme 2). Initially HA sodium salt was converted to H^+ form using cation-exchange resin Dowex 50X8 and was freeze-dried. Then, a suspension of the prepared HA in H^+ form (100 mg, 0.26 mmol) in 10 mL methanol was reacted with 0.125 mL 2 M trimethylsilyl diazomethane (TMSD) in hexane under stirring for 4 h at room temperature. The solid was separated and purified by precipitation from water solution to methanol. The esterification degree (~100%) was calculated by comparing ^{1}H NMR signals at 2.02 ppm (3H, CH_3CON) and 3.84 ppm (3*H*, OCH_3). Yield: 94%.

2.4.3. Preparation of O-hyaluronylurea **5**

Solution of 100 mg (0.26 mmol) HA (H^+ form) in 2 mL H_2O was mixed with the solution of 80 mg (0.39 mmol) dicyclohexylcarbodiimide (DCC) in 2 mL DMSO and was left overnight. The resulting precipitate was filtered and the transparent solution was dialyzed against 0.15 M NaCl for 24 h. The dialysate was concentrated and three volumes of methanol were added, after then the obtained precipitate was treated as described above for conjugates **2a–j**. Content of ureido residues (24%) was found from the ratio of intensities of signals in area of 1.39–1.96 ppm for cyclohexyl protons and the signal at ~2 ppm for CH_3CON group. Yield: 85%.

2.4.4. Preparation of hydroxamate **6**

Isoureas **3** (100 mg, 0.187 mmol) were suspended in 6 mL methanol and stirred at room temperature for 48 h with a mixed solution (insoluble NaCl was removed by filtration) containing KOH (162 mg, 2.9 mmol) in 0.6 mL methanol and hydroxylamine hydrochloride (150 mg, 2.2 mmol) in 1.8 mL methanol (Scheme 2,

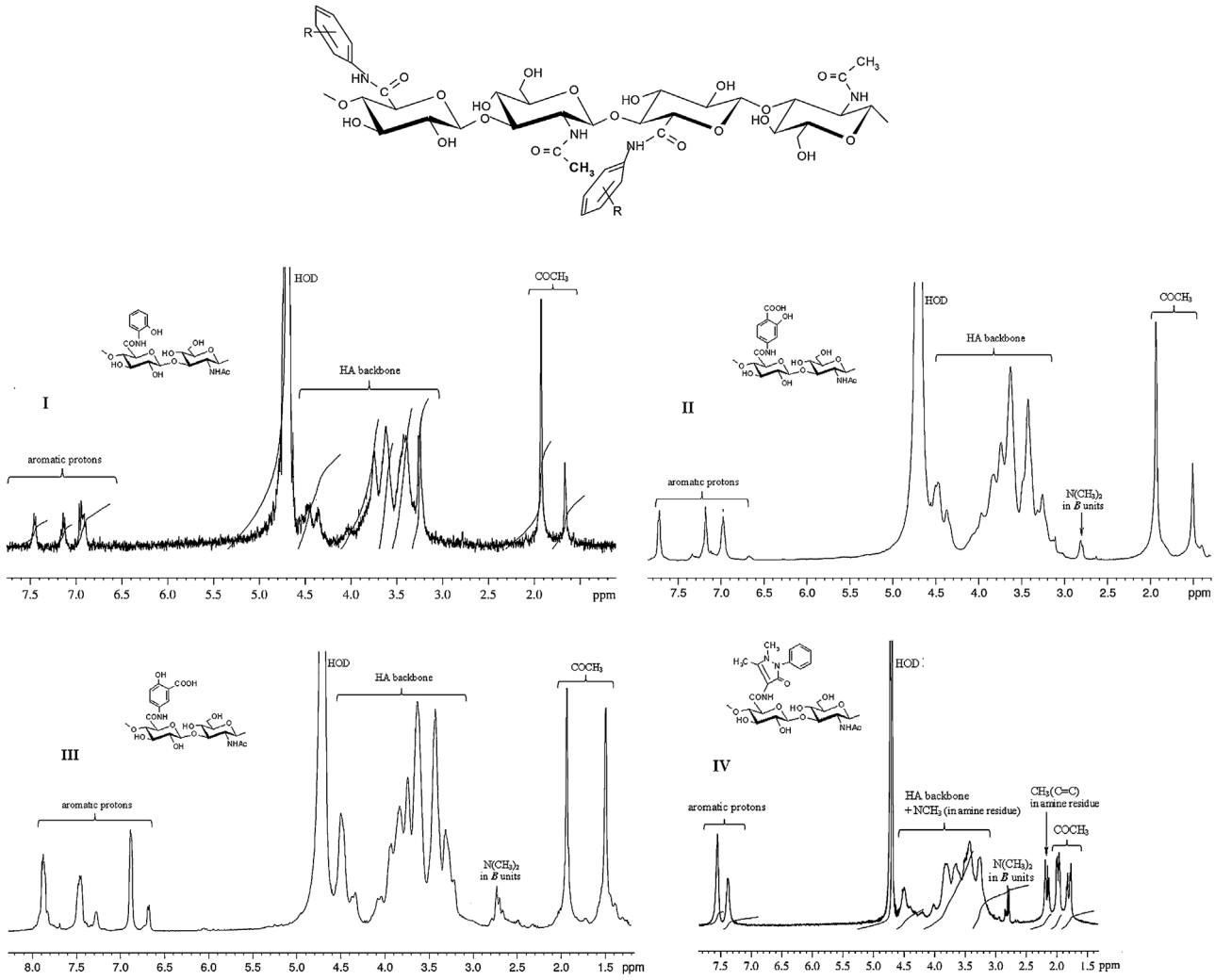
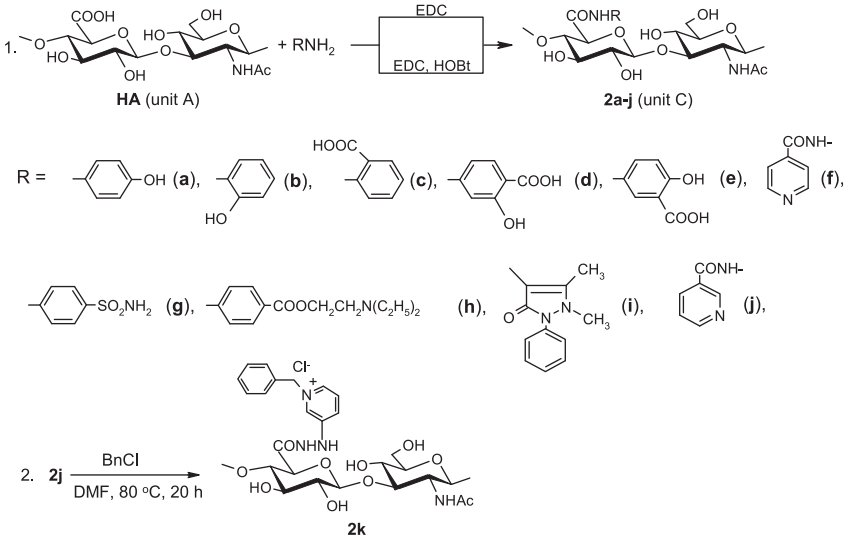
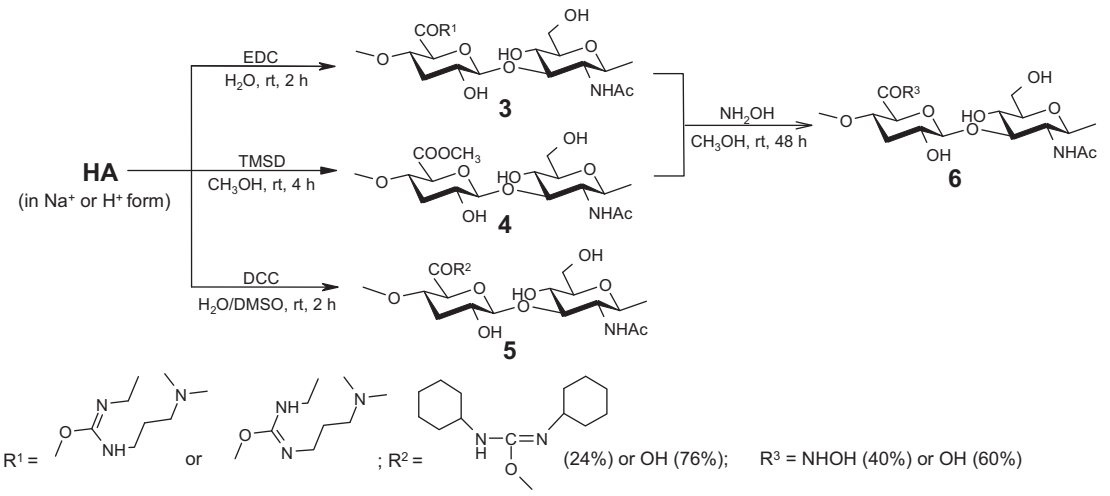


Fig. 1. Shielding effect of adjacent magnetically anisotropic aromatic ring on HA acetamido group and ^{1}H NMR spectra of HA conjugates with (I) *o*-aminophenol **2b**, (II) 4-aminosalicylic acid **2d**, (III) 5-aminosalicylic acid **2e**, and (IV) 4-aminoantipyrene **2i**.



Scheme 1. Synthesis of HA conjugates **2a**–**k**.



Scheme 2. Synthesis of HA hydroxamate.

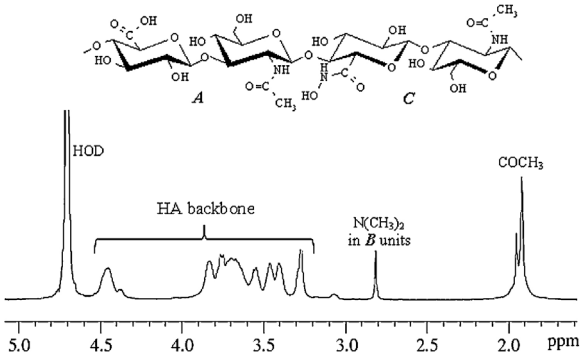


Fig. 2. ^{1}H NMR spectrum of hydroxamate **6**.

see also the work Ponedel'kina et al., 2012a). The solid was purified from impurities by re-precipitation with methanol and was further treated as described above for conjugates **2a–j**. Content of unreacted *B* units was found from ^{1}H NMR as for acylisoureas **3** (see Section 2.4.1.). *B* = 6%. Content of hydroxamic acid residues (*C* units) in product **6** was found using both a color reaction with $Fe(ClO_4)_3$ (λ 520 nm, standard-benzohydroxamic acid) and the ^{1}H NMR integrations of signals at 1.92 and 1.96 ppm for methyl protons in CH_3CON group (Table 2, Fig. 2). Content of *C* units in mol.% (*i.e.* per 100 HA disaccharide units) was calculated according to formula (1) deduced for product containing three types of units—*A*, *B* and *C*.

$$C = \frac{A(100M_A + 134B)}{ac - 15A}, \quad (1)$$

where *A* is the absorbance of the sample solution with concentration *c* (mg/mL), a is the slope of the calibration curve (a = 885 mL/mol), M_A is the molecular weight of unmodified disaccharide unit *A* in HA (M_A = 401), *B* is the content of unreacted *B* units in mol.%, 134 is the difference between the molecular weights of *B* and *A*, 15 is the difference between C and *A*. Deducing formula (1) is shown in SI. Simplified formula (2) can be used for the reaction product with negligible quantity of *B* units.

$$C = \frac{100AM_A}{ac - 15A}. \quad (2)$$

$C = \sim 40\%$; yield : 78%.

Ester **4** (100 mg) was treated with hydroxylamine in methanol and purified as described for isoureas **3**. C = 13%; yield: 76%.

Accordingly another technique, ester **4** reacted with 50-fold excess of hydroxylamine in water at pH 11.7 at ambient temperature for 18–20 h. Product of hydroxamation was recovered as depicted for conjugates **2a–j**. C = 20%; yield: 81%.

2.5. MMP-2 activity assay

MMP-2 (10 μg, active form) was reconstituted in 0.7 mL H_2O. Fluorogenic substrate MCA (1 mg) was dissolved in 1 mL DMSO after that 50 μL of the MCA solution was diluted 20-fold with 0.5 M Tris–HCl buffer containing 0.15 M NaCl, 10 mM $CaCl_2$, 0.05% Brig-35, and 0.02% sodium azide (pH 7.5).

Solutions of conjugates **2a–k** and hydroxamate **6** with varying concentration from 1.485 to 1485 μM were prepared in 0.5 M Tris–HCl buffer. Molarity was calculated by the following formula:

$$\text{Molarity} = \frac{1000m}{M_{av}V},$$

where *m* is the weight of sample (μg), *V* is the volume of solution (mL), and M_{av} is the average molecular weight of disaccharide unit in a corresponding conjugate. M_{av} was calculated by the following formula:

$$M_{av} = \frac{M_C C + M_A(100 - C)}{100},$$

where M_C and C are molecular mass and content (Table 2) of hydroxamic units C, respectively, and M_A—molecular mass of unmodified unit *A*.

Solution of MMP-2 in water (20 μL, final concentration ~41 nM) was incubated with MCA in Tris–HCl buffer (50 μL, final concentration ~1 μM) for 60 min at room temperature in the presence of varying concentrations of sample (20 μL, final concentration from 0.27 to 270 μM). The reaction was then quenched with 0.5 M EDTA (20 μL) and the fluorescence intensity I_s was measured. Excitation and emission wavelengths were 325 and 393 nm, respectively. The experiment was repeated twice, and the error was ±10%.

Inhibitory effect (*I*, %) of hydroxamate **6** on MMP-2 was calculated by the formula:

$$I = \frac{I_{c1} - I_s}{I_{c1}} 100,$$

where I_{c1} is the intensity of positive control solution (without sample).

Inhibitory action of HA conjugates **2a–k** on MMP-2 was calculated with the formula:

$$I = \frac{I_{c1} - (I_s - I_{c2})}{I_{c1}} 100,$$

where I_{c2} is the second control solution containing sample (without MCA and MMP-2). Second control was used to compensate intrinsic emission due to an aromatic moiety in the conjugates.

2.6. Analyses

^{1}H NMR spectra were recorded on a Bruker Avance III 500 MHz (500.13 MHz) spectrometer. Samples were analyzed as solutions in D_2O (15 mg/mL) at room temperature using acetone as internal standard (δ 2.22 ppm for ^{1}H). Fluorescence intensity was measured using FLUOROLOG spectrofluorometer.

3. Results and discussion

3.1. Synthesis of HA derivatives

HA conjugates **2a–j** with amines and hydrazides **1a–j** (some of the properties of amines are given in Table 1) have previously been obtained using a condensing reagent EDC alone (Ponedel'kina et al., 2011, 2005). In this case, conjugates **2a** (with *p*-aminophenol), **2b** (with *o*-aminophenol), **2d** (with 4-aminosalicylic acid), and **2i** (with 4-aminoantipyrine) contained exclusively carboxamide units (Scheme 1), and conjugates **2**c (with anthranilic acid), **2**e (with 5-aminosalicylic acid), **2g** (with Streptocide), **2h** (with Procaine), **2f** and **2j** (with isonicotinic and nicotinic hydrazides) in addition to carboxamide units contained from 10 to 33% isoureido units formed upon EDC coupling with HA carboxyl groups (Ponedel'kina et al., 2005). The presence of the hydrophobic units in the structure of HA conjugates markedly decreased solubility of these in water.

It is known that reagent for peptide synthesis HOBt has a positive influence on the selectivity and yield of the conjugation of polyuronic acids with amines under the EDC action (Kurisawa, Chung, Yang, Gao, & Uyama, 2005; Ponedel'kina et al., 2012a,b,c). Therefore, conjugation of HA with amines **1c**,**e**,**f**,**g**,**h**,**j** was performed in the presence of both EDC and HOBt (Scheme 1). The ^{1}H NMR spectra showed that conjugates **2c**,**e**,**f**,**g**,**h**,**j** practically did not contain isoureido units but included less carboxamide units than conjugates prepared without HOBt (Ponedel'kina et al., 2011). Conjugate **2k** was synthesized by benzylation of **2j** in DMF (Scheme 1) (Ponedel'kina et al., 2012a,b,c). The characteristic of all conjugates is given in Table 2.

For the synthesis of hydroxamate **6**, *O*-hyaluronylisoureas **3** and methyl ester **4** were used. Isoureas **3** were obtained by the interaction of HA with EDC in water (Ponedel'kina et al., 2012a), and the ester **4** was synthesized by the treatment of HA (in H^+ form) with TMSD in methanol (Hirano et al., 2005) (Scheme 2). In both cases the degree of esterification of HA carboxyl groups was 100%. Yet another substrate for hydroxamation, *O*-hyaluronylurea **5**, was obtained by the interaction of HA (H^+ form) with DCC in a mixture of H_2O-DMSO (1:1). It contained only 24% ureido units and therefore was unusable for preparation of HA hydroxamate. The low yield of the HA urea **5** was probably due to more rapid DCC hydrolysis under the action of HA in H^+ form as compared with the reaction of DCC with HA carboxyl groups.

Isoureas **3** were treated with alkaline hydroxylamine in methanol since the reaction yield was expected to be higher in this solvent than in water (see the work, Ponedel'kina et al., 2012a), and methyl ester **4** was hydroxamated both in alcohol and in an aqueous medium at pH 11.7 (Scheme 2).

Completeness of the hydroxamation of esters **3** and **4** was evaluated by the decrease or disappearance of ^{1}H NMR signals at 2.80 or 3.84 ppm corresponding to methyl protons in Me_2N- (in isoureas **3**) or MeO- (in ester **4**) groups, respectively. The content of hydroxamic C units in products was found with a well-known ferric hydroxamate test. After hydroxamation of isoureas **3**, the residual content of *B* units in the reaction product was ~6 mol %, and the content of *C* units calculated with the formula (1) (formula deduction is given in Supplementary Data) was 40% per 100 HA disaccharide units; the most part of B units (54%) was hydrolyzed to initial *A* units under the action of alkali. Treatment of ester **4** with the alcohol hydroxylamine under the same conditions as for the isourea **3** led to a product containing only 13% of *C* units; *O*-methyl groups were found to be absent. Hydroxamation of ester **4** in an aqueous-alkaline medium at pH 11.7 gave a product containing no more than 20% C units. Thus, the preferred substrate for the preparation of HA hydroxamate is *O*-hyaluronylisoureas.

Separately it is necessary to touch the question of determining C units in HA hydroxamate. Although ferric hydroxamate test is methodologically simple reaction, the error of determination of the hydroxamic units in polysaccharides can be high due to residual moisture in samples. They must be carefully dried, that is rather difficult to carry out in the case of HA known by its own moisture-retaining properties. Moreover, ferric hydroxamate reaction gives the result in moles of *C* units per mg sample, and to express it in molar percentage a formula taking into account the sample weight, the slope of the calibration curve, the content of unreacted B units and other parameters is required (see Eqs. (1) and (2), Section 2). Therefore, the ^{1}H NMR is more convenient method because it allows determining the content of modified units in HA in molar percentage. However, there are some limitations on the ^{1}H NMR application. Samples should not contain *B* units in a large amount since the signals of protons in CH_2—CH_2—CH_2 group in *B* units are located in the same region as the signals of protons in MeCON group in HA, and the content of hydroxamic units must be at least 20–25%. Under these conditions, the splitting of methyl singlet for MeCON group into two signals of different intensity at 1.93 and 1.96 ppm becomes noticeable indicating the sensitivity of the acetamido group to the adjacent hydroxamated unit (Fig. 2). The intensity of signal at 1.93 ppm was 37% of total intensity that practically coincided with the result of ferric hydroxamate reaction (40%), and so it was assigned to methyl protons in the glucosamine unit neighboring with hydroxamate unit (Fig. 1).

3.2. The effect of HA based hydroxamate and conjugates on MMP-2 activity

Numerous inflammatory and degenerative diseases, such as arthritis, periodontitis, non-healing leg ulcers as well as burn wound and other dermatological disorders, for treatment of which the natural HA and its derivatives can be used, are characterized by increased level of several MMPs including MMP-2. Moreover, MMP-2 plays an important role in skin aging process. Therefore, MMP-2 was chosen for evaluation of MMPI properties of HA derivatives.

Properties of amines **1a–j** used for conjugation with HA as well as D-glucuronic acid hydroxamate, the structural analog of hydroxamate **6**, are given in Table 1. MMPI properties are known for 4-aminosalicylic acid (towards MMP-1), 5-aminosalicylic acid and *p*-aminophenol (against MMP-2). In contrast to *p*-aminophenol, its analogs *N*-acetyl-*p*-aminophenol and *o*-aminophenol exhibited the weak inhibitory properties. Anthranilic acid moiety and sulfamido group as is known are included in the structure of numerous MMPIs.

The results of the action of HA derivatives in the concentrations from 0.27 to 270 μM on MMP-2 activity are shown in Table 2.

As expected, the conjugate **2a** (with *p*-aminophenol) had virtually no effect on MMP-2 activity (*cf.* with *N*-acetyl-*p*-aminophenol). In contrast, the conjugate **2b** (with *o*-aminophenol) at all concentrations completely inhibited MMP-2, although *o*-aminophenol

Table 1
Properties of amines **1**α**–e**,**g**,**h**, hydrazides **1f**,**j** and glucuronic acid hydroxamate.

Compound	Properties
1α	Is included to the library of fragments for design of potential MMPIs; IC_{50} 10.8 ± 1.7 μM (against MMP-2) and 21.2 ± 10.4 μM (against MMP-9) (Rubino, Maggi, Laghezza, Loiodice, & Tortorella, 2011) **1**α is a precursor for the synthesis of paracetamol (*N*-(4-hydroxyphenyl)acetamide), which was found to be a weak inhibitor of MMPs (Rubino et al., 2011)
1b	Participates in metabolic pathways of tryptophan and in concentration of 100 μM inhibits MMP-2 activity by 34–76% (Rubino et al., 2011)
1c	Participates in metabolic pathways of tryptophan A number of hydroxamic derivatives of **1c** are MMPIs (Gupta, 2012; Verma & Hansch, 2007)
1d	Is included in the library of fragments-chelators for potential MMPIs synthesis, but it was not tested as an *in vitro* inhibitor (Agrawal et al., 2010) Sodium salt of **1d** (PAS) decreases MMP-1 activity by inhibiting a p38 MAPK-PG signaling cascade. This pathway is a therapeutic target to reduce inflammatory tissue destruction in tuberculosis (Elkington, Ugarte-Gil, & Friedland, 2011; Rand, Green, Saraiva, Friedland, & Elkington, 2009)
1e	Is an inhibitor of prostaglandin synthesis and possesses anti-inflammatory activity and is used for treatment of acute ulcerative colitis and Crohn's disease (Warner et al., 1999) It is MMP-2 and MMP-9 inhibitor and suppresses the growth of tumor cells in the large intestine (Kim et al., 2009) **1e** is included to the library of fragments-chelators for synthesis of potential MMPIs, but it was not tested as an *in vitro* inhibitor (Agrawal et al., 2010)
1f	Is used in the treatment of tuberculosis (Isoniazid). Conjugate HA-**1f** is more active against *Mycobacterium tuberculosis* than **1f** alone (Salah, Ahmed, Hala, & Hala, 2013) Due to hydrazide moiety it has scavenging activity for OH, NO and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. These reactive oxygen species (ROS) are known to induce oxidative stress and cause aging and various diseases in the living body (Ogata et al., 2002) It is inhibitor of cytochrome P450 system and lysyl oxidase *in vivo* (Finkel, Clark, & Cubeddu, 2009)
1g	Is a antimicrobial drug and the dihydropteroate synthetase inhibitor (Finkel et al., 2009)
1h	Is a local anesthetic and monoamine oxidase (MAO) inhibitor (Finkel et al., 2009)
1i	Has analgesic activity and antioxidant properties (Ragab, Abdel-Gawad, Georgey, & Said, 2013; Santos, Antunes, Noronha, Fernandes, & Vieira, 2010) **1i** is used for the synthesis of dipyrone and phenylbutazone Conjugates of **1i** with HA and chondroitin sulfates have analgesic effect (Ponedel'kina et al., 2012b). Pyrazolone derivative, 3-methyl-1-phenyl-2-pyrazolin-5-one (Edaravone), is a free-radical scavenger and promising neuroprotective drug for the treatment of ischemic stroke and preventive therapy of abdominal aorta aneurysm (Morimoto et al., 2012; Yagi et al., 2009)
1j	Is a scavenger of OH, NO and DPPH radicals like isoniazid (Ogata et al., 2002)
Glucuronic acid hydroxamate	Has antioxidant and bovine semicarbazide-sensitive amine oxidase inhibitory activity (Liu, Liang, Lee, Tsai, & Hou, 2011)

Table 2
HA derivatives and their effect on MMP-2 activity.

Conjugate	Content of modified units (mol.%)	% of MMP-2 inhibition at different final concentrations of HA derivatives (μM of HA disaccharide units)			
		0.27	2.7	27	270
2a	50	20	7	2	18
2b	46	100	100	100	100
2c	12	66	53	46	28
2d	37	29	39	61	92
2e	40	36	18	29	73
2f	43	69	46	25	15
2g	48	46	49	49	39
2h	27	55	48	56	48
2i	60	100	100	100	100
2j	36	23	20	0	4
2k	36	20	10	6	0
Hydroxamate **6**	40	38	50	42	45

1b itself is a weak inhibitor of MMP. Conjugates **2d** and **2e** (with 4- and 5-aminosalicylic acids) dose-dependently inhibited the enzyme activity, and the conjugate **2d** was somewhat more effective. Conjugates **2j** and **2k** (with hydrazides of nicotinic and *N*-benzylnicotinic acids) did not revealed inhibitory effect (Table 2). Conjugate **2i** (with 4-aminoantipyrine) has shown to be effective MMP-2 inhibitor (Table 2), and this result is not unexpected. 4-aminoantipyrine is a derivative of pyrazolone, a key structure of numerous therapeutically important compounds. Pyrazolone derivatives inhibit the production of tumor necrosis factor-α (TNF-α) and reduce the level of other proinflammatory cytokines (*e.g.*, interleukin) thereby reducing the inflammation and preventing degradation of tissues in such wounds as rheumatoid arthritis, osteoarthritis, and Crohn's disease. However, as it is known from the literature, pyrazolone derivatives have not been tested as potential MMPIs, except Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) (Yagi et al., 2009). Edaravone, as was shown on laboratory animals (rats) with aortic aneurysm, reduces the MMP-2 and MMP-9 levels in vascular walls in 2.1–3.8 and 2–12 times, respectively, as compared with the control group (Morimoto et al., 2012; Yagi et al., 2009). Evidently, pyrazolone derivatives should be included in the library of perspective inhibitors of MMPs along with other MMPIs.

Conjugates **2c** (with anthranilic acid), **2f** (with isoniazid), **2g** (with Streptocide), **2h** (with Procaine) and hydroxamate **6** showed some unusual results. HA derivatives **2g**, **2h** and **6** inhibited the MMP-2 activity to approximately 50%, but not in a dose dependent manner. Conjugates **2c** and **2f** in a dose of 0.27 μM repressed the activity of MMP-2 by 70%, but with further increasing concentration in 10, 100 and 1000 times the inhibitory effect of **2c** was decreased in 1.2, 1.4 and 2.4 times, and the inhibitory effect of **2f** was reduced in 1.5, 2.8 and 4.6 times (Table 2). Decrease of

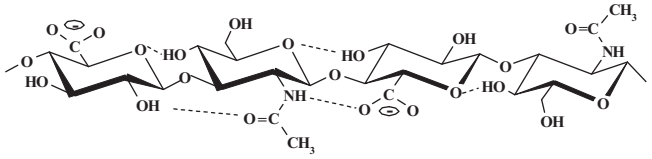


Fig. 3. Secondary structure of HA stabilized by hydrogen bonds.

inhibitory of these HA derivatives, in other words, the increasing their activating effect on MMP-2 could be explained by the influence of fragments of HA remaining unmodified (Isnard, Legeais, Renard, & Robert, 2001; Robert, Robert, & Renard, 2010). Indeed, it is known from the literature that natural HA in a dose of ~2.5 mM is capable of inducing the expression and activity of MMP-2 (and MMP-9). But in dose of 0.27–270 μM, in which HA derivatives were tested and which was by 1–4 orders of magnitude lower than active HA concentration, unmodified HA does not effect on MMP activity (data in Table 2 are not shown). Although, to compare it with modified HA is not quite correct for the following reasons. It is known that both the primary structure of HA (*i.e.* sequence units of D-glucuronic acid and *N*-acetyl-D-glucosamine) and the secondary structure of HA, presenting in aqueous solution a left-handed single helix with two disaccharide residues per turn and stabilized by hydrogen bonds (Fig. 3), have decisive importance in a specific interaction with small and large biomolecules (Scott, 2007). Due to chemical modification and hence disordering the system of hydrogen bonds and occurrence of electrostatic interactions different from that in natural HA, both primary and secondary structure of HA are changed. As a result, the change of conformation of HA macromolecule may lead to alteration of its interaction with MMPs.

4. Conclusion

Thus, the first results on the study of effect of some HA derivatives on MMP-2 activity have been obtained. Conjugates with *o*-aminophenol, 4-aminoantipyrine, and 4- and 5-aminosalicylic acids have proved to be inhibitors of MMP-2 at a concentration of 0.27–270 μM. Conjugates with Streptocide, Procaine and HA hydroxamate showed only 40–50% inhibitory effect independently of the used concentration. Conjugates with anthranilic acid and isoniazid in a dose of 0.27 μM inhibited enzyme activity by almost to 70%, but upon increasing concentrations their inhibitory effect was decreased. The advantages of application of some of these HA derivatives as MMPIs are good solubility in water, and the use of amines, physiological and pharmacological properties of which have been long and well known.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2016.02.022.

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