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Modification of Hyaluronic Acid with Hydrazine & Hydrazone; And Using their New Conjugates as Potential Antimicrobial Agents

KEYWORDS: Hyaluronic acid/ Isonicotinyl hydrazine/ benzophenone hydrazone/ biological activity/ ^{1}H NMR.

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ABSTRACT *Modification of the carboxylic acid moieties of hyaluronic acid (HA) with mono-functional hydrazine or hydrazone leads to biochemical probes, biopolymers with altered physical and chemical properties, tethered drugs for controlled release. Isonicotinyl hydrazine and benzophenone hydrazone were condensed with HA to produce conjugates (A) and (B). The reactions were activated by 2-(Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluroborate (TBTU). Both conjugates were characterized through ^{1}H NMR spectroscopy to indicate their formation and degree of substitution (SD). The antimicrobial tests of the conjugates indicated higher activity and long acting effect.*

Introduction:
The increasing number of biomedical uses for HA has encouraged the development of a broad range of HA-based derivatives with enhanced or modulated properties. HA has been the subject of many previous reviews focusing on its biological functions and medical applications and drug delivery (Gaffney et al., 2010). HA is a linear polysaccharide, made of repeating disaccharide units of d-glucuronic acid and N-acytyl glucosamine linked by (1,4) and (1,3) glucosidic bonds (Figure 1). In physiological conditions, HA is in the form of a sodium salt, therefore negatively charged and referred to as sodium hyaluronate. In these conditions, it is highly hydrophilic, surrounded by a sphere of water molecules linked by hydrogen bonds (Day and Sheehan; 2001).

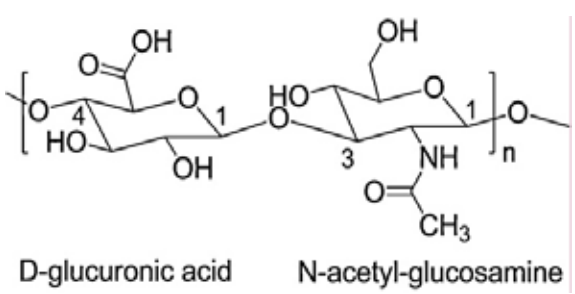

Figure 1: Chemical structure of hyaluronic acid and target sites for chemical modification

HA is naturally present in vertebrate organisms, as well as in bacteria. Its highest occurrence in the human body is in the extracellular matrix (ECM) or connective tissues. HA can be chemically modified in two different ways; cross-linking or conjugation. They based on the same chemical reactions and only differ in that, in the conjugation, a compound is grafted onto one HA chain by a single bond only. The chemical modification of HA can be performed on the two available functional sites of HA, the carboxylic acid and the hydroxyl group (Figure 1). An amino group can also be recovered by deacetylation of the N-acetyl group. It is not known which of the hydroxyl groups reacts, though it is reasonable to assume that the reaction occurs mainly on the hydroxyl of C6 of the N-acetyl-glucosamine moiety of HA because of the better accessibility of reagents to primary alcohols. Numerous methods have been reported for HA cross-linking or conjugation. Some methods are performed in water while others, since they use reagents sensitive to hydrolysis; need to be performed in organic solvents, such as dimethyl-formamid (DMF) or acitonitrite (AN). In this case, the native HA sodium salt first needs to be converted into either its acidic form or a tetrabutylammonium (TBA) salt for solubilization in organic solvents. This requires an additional step, increasing the chances of HA chain fragmentation associated with chemical and physical treatments. Since HA is soluble in water, the easiest method is to perform the reaction in water. However, in aqueous conditions, some reactions are pH-dependent and need to be performed in acidic or alkaline conditions, which have been shown to induce significant HA chain hydrolysis (Maleki et al. 2008). These aspects have encouraged researchers to explore new synthetic routes for the development of HA derivatives with appropriate characteristics according to their specific needs (Yeom et al. 2010).

The biological role of HA has been extensively investigated. It has become attractive in biomaterial applications due to its unique physicochemical properties, non-immunogenicity and high availability. Native HA has limited use due to short turnover rate and poor mechanical stability. Therefore, chemically modified derivatives were developed to obtain biomaterials with tailored properties (Shui et al. 2004). HA would be an excellent candidate as a drug delivery agent due its biocompatibility and specific binding to some cell-surface receptors. Generally, this is achieved by targeting the carboxylic group on the glucuronic moiety and hydroxyl groups found on both moieties (McDonald and Hascall, 2002).

There has been considerable interest in the development of novel compounds with antimicrobial and anti-mycobacterial activities (Sevim and Guniz Kueukguzel, 2007). Hydrazine and hydrazones are of wide interest because of their diverse biological and clinical applications. Hydrazones possessing an azometine–NHN=CH- proton constitute an important class of compounds for new drug development (Balasubramanian et al. 2010). Therefore, many researchers have synthesized compounds as target structures and evaluated their biological activities. These observations have been guiding for the development of new hydrazine and hydrazones that possess varied biological activities. In the present study, we

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have made an attempt to prepare hyaluronic acid conjugates with isonicotinyl hydrazine and benzophenone hydrazone, (primary amines). The biological activities of these two conjugates and their using as potential antimicrobial agents against Mycobacterium tuberculosis M. TB and Candida albicans C. albicans let us hoping they have more potent effect and low toxicity.

MATERIALS AND METHODS

Sodium hyaluronate $(C14H20NO11Na)_n$, isonicotinyl hydrazine, benzophenone hydrazone and TBTU were purchased from Sigma-Aldrich and used without further purification. ^1H NMR measurements were performed using a JEOL ECP 400 MHZ spectrometer, the spectra were carried out in D_2O at the National research center, Dokki, Giza, Egypt. The integrated signals of the substituted groups of the acetyl group of HA were compared to calculate the degree of substitution (SD) for each of conjugates A and B.

Synthesis: General procedure for preparation of conjugates A and B: Sodium hyaluronate (100 mg, 0.25 mmol of –COOH) was dissolved in 9 mL water using a magnetic stirrer until clear solution was obtained. TBTU (100 mg, 0.3 mmol) in 6 mL acetonitrile were added while stirring continued for 15 min at room temperature. The pH was adjusted to 4.75 by 1 N HCl. Isonicotinyl hydrazine (34 mg, 0.24 mmol) or benzophenone hydrazones (49 mg, 0.25 mmol) was then added. The mixture was stirred for further 1 h, while maintaining the pH at 4.75. The reaction was stopped by raising the pH to 7.0 (1 N NaOH). The reaction mixture was transferred into dialyzed tubing with MW cutoff 3.5 kDa and exhaustively dialyzed against water. The clear transparent was filtered through 0.45 mum membrane to yield the corresponding conjugates A (98 mg, 72%) and B (94mg, 68%). (Figure 2, 3, 4 & Table 1).

Antimicrobial activities: Organisms**:** 1- M.TB growth sample recruited from Chest Hospital in Menoufyia governorate. It was diagnosed as active pulmonary TB based on the presence of recent clinical symptoms of TB, positive smear for acid-fast bacilli from sputum and positive culture for TB on Lowenstein-Jensen glycerol (L.J) medium.

Culture and resistance test**:** L.J medium was prepared as follows: 2.4 gm potassium dihydrogen phosphate (anhydrous), 0.24 gm magnesium sulphate, 12 gm magnesium citrate, 3.6 gm L-Asparagine, 12 ml glycerol and 600 ml distilled water, all reagents were added together and dissolved then autoclaved at 121 °C for 10 minutes. Then 1 liter of 2% malachite green solution and whole egg 20 ml were added using a mixer to complete medium preparation. The pH was adjusted to 6.9, and then the medium was dispensed in screw-capped bottles with a volume of 25 ml and in a slanting position for being coagulated at 85 °C for 1 hour (Elbir et al., 2008). The representative loopful of the growth on the primary diagnostic culture is taken with a loop of 3 mm external diameter made from thick nichrome wire, (the amount of this growth is about 2 mg on the loop) and then placed on each of 3 slopes of L.J medium containing respectively, 1 µg/ml of isonicotinyl hydrazine, 1 µg/ml of conjugate A, and 3rd one was free from each of them act as control. After incubation for 28 days at 37 °C, numbers of colonies were determined, 10-15, 2-8 & <85, respectively (Table 2). Distinction should be made between total resistance (growth as in the controls) and partial resistance (growth markedly less than in the controls). In this test, it was shown that isonicotinyl hydrazine is highly resistance than the conjugate A according to the number of the appeared colonies. Thus, it is indicated that the M.TB is more susceptible to conjugate A than the isonicotinyl hydrazine alone. Therefore, M.TB is partially resistant to isonicotinyl hydrazine while it is susceptible to conjugate A. So, conjugate A is considered that it has higher inhibitory effect than isonicotinyl hydrazine. This test was carried out in triplicates.

2- Candida albicans was obtained from The National Liver Institute, Menoufiya University, Egypt.

Culture and inhibition zone**:** Sabouraud agar was prepared by combined peptone 5 gm, glucose 20 gm and agar 7.5 gm in 450 ml of de-ionized water adjusted to pH 5.6 with HCl and adjusted final volume to 500 ml, autoclave 20 minutes at 121 °C, then cooled to 50 °C, and poured into Petri dishes. A loop with a 2 mm^3 (approximately 2 mg moist weight of Fungus (C. albicans) was inoculated in a plate containing Sabouraud agar. The 3 paper discs (5 mm) were let to absorb 1 µg/ml of benzophenone hydrazone, 1 µg/ml of conjugate B, and the 3rd disc without each of them, act as a control disc. The discs were left at room temperature till dryness and then applied in the inoculated plate. It was incubated at 37 °C. The inhibition zones around the discs were measured after 12, 18, and 24 hours (Table 3). The test was carried out in triplicates and the mean values of the measurements were taken.

Results and discussion:

Hyaluronic acid was modified with isonicotinyl hydrazine (isoniazide) and benzophenone hydrazone to produce a new conjugates (A and B) they have a potential higher inhibitory activity against M.TB and Candida albicans, respectively than isoniazide and benzophenone hydrazone alone. Both compounds were selected to react with HA under mild condition to produce the new conjugates. The primary amino-group of each of them could be condensed with the carboxylic group of glucuronic acid moieties. Oh et al., (2010) reported that the amidation in water with carbo-di-imides is one of the most widely used methods for HA modification, this is to convert the carboxyl groups of polysaccharides including HA into amides. They used 1-ethyl-3-[3-(dimethylamino)-propyl]-carbo-di-imide (EDC) at pH 4.75 to activate the carboxyl groups, which then reacted with an amino-acid ester while Bergman (2007) activated the reaction with 2-chloro-4,6-dimethoxy 1,3,5-triazine (CDMT). In this study, 2-(1H-benzotriazole-1-yl)-1,2,3,3-tetramethyluranium tetrafluroborate (TBTU) was used as activator, it is an uranium salt that acts as a highly efficient coupling reagent used in peptide chemistry for a wide variety of peptide sequences, including the synthesis of some pharmaceutical peptides. An effective reagent should have the following characteristics: high efficiency, work in stoichiometric quantities, and solubility in the currently-used solvents. TBTU is one of the most common coupling reagents and additives used in peptide chemistry. The two tautomeric forms of TBTU, one of them reacts with the carboxylate ion (Sewald and Jakubke; 2003). The high efficiency and ease of product isolation, prompted us to investigate its use for the direct coupling reaction of the carboxylic group of HA with each of isonicotinyl hydrazine and benzophenone hydrazones for the synthesis of appropriate products (conjugates A and B) at room temperature (Figure 2). Each reaction was carried out in a mixture of water and acetonitrile (9:6) it was found capable of dissolving both HA and TBTU. Using a combination of polar and non-polar solvents, the introduction of both hydrophilic and hydrophobic substances is made possible. The reaction of TBTU with –COOH of HA and isonicotinyl hydrazine or/and benzophenone hydrazone proceeds according to the proposed synthetic root presented in (Figure 2). The structures of the 2 conjugates products (A and B) were established from their spectral ^1H NMR. The methyl (CH3) signal of the N-acetyl glucosamine moiety appeared at (δ = 2.65) for conjugate A (Figure 3), while it appeared at (δ = 1.97) for conjugate B (Figure 4). This shift may be due to the proximity of pyridine nitrogen to the acetamido group, the appearance of pyridine signals at ((δ = 7.46, 7.57, 7.68, 7.83) indicated the substitution of isonicotinyl hydrazine on HA in conjugate A, and the aromatic multiplates at (δ = 7.51-7.74) indicated the condensation of benzophenone hydrazone with HA in conjugate B. Degree of substitution (SD) were determined from ^1H NMR by comparing integrated signals from the substituted groups with the corresponding methyl (CH3) signals of the N-acetyl glucosamine moiety (Figure 3,4 & Table 1). Low degree of substitution was observed in conjugate B may be due to lower activity of the hydrazone group resulted from tautomerism.

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The biological activity of conjugates A and B were tested against M.TB and fungus C. albicans, the tests were carried out on Lowenstein-Jensen and Sabouraud media, respectively.

The results reported in (Table 2, 3) showed more potent effects of conjugates A and B in comparison with the pure compounds. Highly effective inhibition of conjugate A in medium of L-J inoculated with M. TB, (where the number of colonies growth was 2-8) than isonicotinyl hydrazine alone, (where the number of colonies growth was 10-15) was observed after 28 days incubation (Table 2), and the larger inhibition zones around the discs of conjugate B (14, 22, 24 mm), respectively were showed in Sabouraud agar inoculated with C. albicans than the inhibition zones around the discs of benzophenone hydrazone alone (12, 19, 21 mm), respectively after 12, 18, and 24 hours incubation, respectively (Table 3). The biological tests were carried out under the same amounts of the conjugates and the pure compounds (1 µg/ml). The conjugates with low degree of substitution of the active constitutes, performed increased activities than the pure compounds. The higher activities of the conjugates may be attributed to the high activity of the freshly released active constitutes from the conjugates to bind with the specific receptors. The lower dose of the compounds on hyaluronic acid should be lead to decreased toxicity. Long acting effect may be due to slow release of the drug from the conjugates.

Conclusion:

For its valuable physicochemical properties, HA is currently widely used in a number of therapeutic applications. The design and synthesis of innovative HA derivatives for biomedical applications is still of major interest for the improvement of drug efficacy and targeting. The chemical modification method described above offer for the synthesis of new derivatives with various physicochemical properties. This review may be used as a tool for the design or improvement of HA derivatives for existing or new applications in the future.

Table 1: Specification for synthesized hyaluronic acid conjugates

Conjugate	substituent	Yield%	SD%
A	Isonicotinyl hydrazone	72	19.4
B	Benzophenone hydrazone	68	8.7

SD: Degree of substitution

Table 2: Number of colonies growth under the effect of anti-M.TB and new conjugate A with control containing L-J medium.

Compound in culture 1µg/ml	Number of colonies/ 28 days	status
Isonicotinyl hydrazine	10-15	++
Conjugate A	2-8	+
control	>85	++++

++++: Confluent growth

++ : Discrete colonies according amount of growth (partially resistant)

+ : Isolated colonies

(<10 isolated colonies is considered effective inhibition)

Table 3: Determination of inhibition zone

Compound	Organism	Inhibition zone (mm) 12 hr 18 hr 24 hr
Benzophenone hydrazone	C. albicans	12 19 21
Conjugate B	"	14 22 24

Control disc: No inhibition

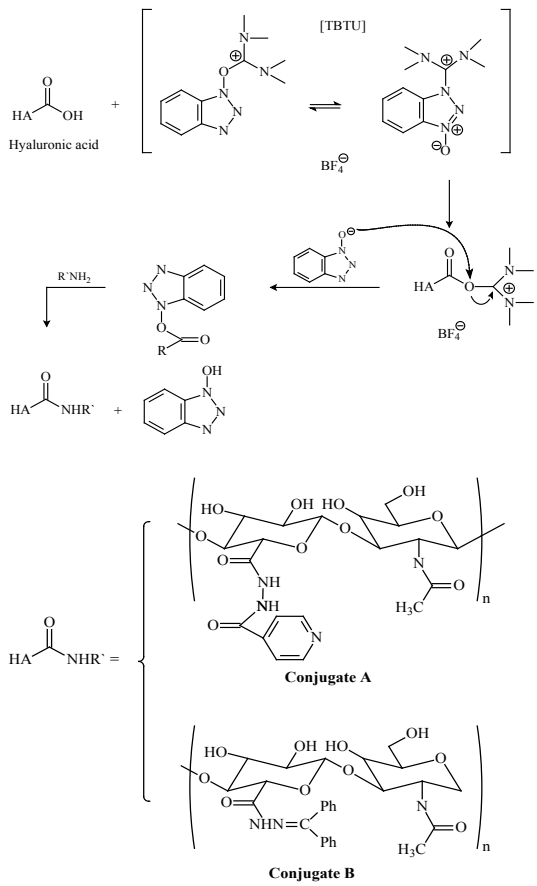

Figure 2: Proposed general synthetic route for TBTU activated reaction of hyaluronic acid with isonicotinyl hydrazine and benzophenone hydrazone

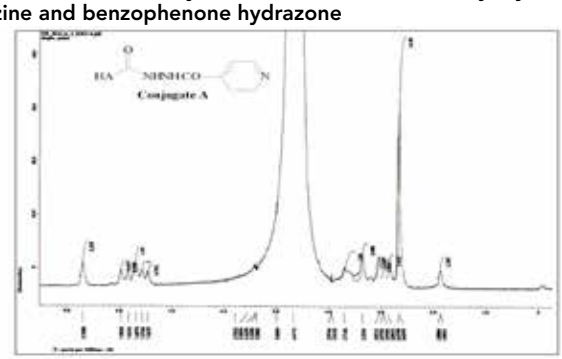

Figure 3: ^{1}H NMR spectra of conjugates A recorded in D2O

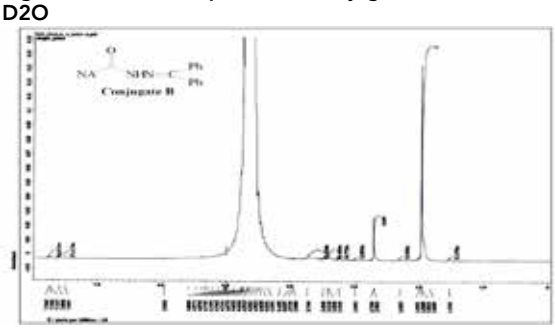

Figure 4: ^{1}H NMR spectra of conjugates B recorded in D2O

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