

A New Range of Chitosan Based Nano-antiviral Agents

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ABSTRACT The current study involves the synthesis of fourteen analogs of oligochitosan and their screening for antiviral potential against human immunodeficiency virus (HIV), respiratory syncytial virus (RSV) and Coxsackie virus. The synthesized oligochitosan analogs were characterized by nuclear magnetic resonance (NMR) and FTIR techniques. HIV-1 p24 ELISA was performed using HIV-1 p24 antigen capture assay in order to estimate the viral infectivity loss. It was observed that sulfated oligochitosan was devoid of antiviral activity as compared to oligochitosan UN102 analog. The rest of UN102 analogs which include N-thiol (UN105), N-glutaryl (UN106), N-Azido (UN111) and N-phthaloyl (UN114) and N-citric analog (UN117) exhibited antiviral activity against HIV. The UN102 also decreased viral infection caused by RSV. In addition, UN102 was found to bind Coxsackie virus, which causes autoimmune myocarditis. The findings were of great interest to proceed for the development of novel antiviral agents.

INTRODUCTION

Diverse factors affect the development of anti-viral drugs including high incidence of a particular viral disease and availability of the vaccine, severity, ease of diagnosis, duration of the acute and chronic viral disease and the drug target (Pardi and Weissman 2020). The adverse effects are accompanied by currently used broad antivirals (Villamagna et al. 2020), the appearance of new emerging viruses (Choi 2021) and resistance against existing antiviral in new virus strains (Irwin et al. 2016) which also suggest an urgent need for the development of safe and broad spectrum of antiviral agents. It is essential

to discover new strategies to overcome the above challenges and improve public health.

Natural products are highly valuable and many medicinally important compounds have been identified among them like polysaccharides which are reported for their antiviral action (Ehresmann et al. 1977; Minari et al. 2011). In particular, sulfated polysaccharides are a class of molecules with favorable tolerable profiles in animals and humans characterized by a plethora of biological activities, including antiviral activity in some studies (Nakashima et al. 1987; Schols et al. 1992; Sugawara et al. 1989). Also, it was reported that selective use of sulfates of chitosan, a chitin derived polysaccharide, resulted in a higher anti-HIV activity (Artan et al. 2010; Satitsri and Muanprasat 2020; Nishimura et al. 1998). However, the preparation of sulfated chitosan remains complex and heterogeneous products make them difficult to use in biological activity. These factors collectively discourage use of sulfated chitosan. Moreover, sulfated polysaccharides only work at lower pH and some of these exhibit altered clotting properties. The high molecular weight polysaccharides are not preferred due to poor tissue penetrating ability (Wang et al. 2012). Therefore, there is need for a pharmaceutical agent that is safe, convenient, effective and preferably a

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cost-effective non-sulfated polysaccharide as an anti-viral agent that can be used to overcome the technical and commercialization challenges facing sulfated polysaccharides. Recently high molecular weighing chitosan has been shown to exhibit anti-viral activity (Zheng et al. 2016). However, studies have shown that high molecular weighing chitosan (100-400 kDa) exhibits aggregation, low solubility under physiological conditions, high viscosity at concentrations used for *in vivo* delivery and slow dissociation or degradation (Zaikov 2005).

Mohapatra's research group has previously reported a 10 kDa on nano - chitosan, referred to here as UN102, which we have characterized and used in diverse genes, drugs and peptide delivery studies. This oligochitosan is well known for its bio - compatibility, biodegradability, favorable physico - chemical properties and chemical modification suitability (Bird et al. 2014; Kong et al. 2005; Zhang et al. 2005). We reasoned that UN102 could lead to the development of potent broad antiviral by making structural modifications. For this purpose, functionality in UN102 was made and the synthesized analogs were characterized by NMR and FTIR techniques. These UN102 analogs were tested for antiviral properties against HIV, RSV and Cocksackie virus.

Objectives

1. To confirm the antiviral activity of oligochitosan against HIV, RSV and Cocksackie virus.
2. To chemically modify the natural occurrence oligochitosan in order to boost its antiviral activity.
3. To compare antiviral activity of the synthesized analogs with the naturally occurrence oligochitosan.

METHODOLOGY

The first batch of analogs were synthesized and tested between fall 2014 and spring 2015, and later on in 2019, UN105, UN106, and UN111 were resynthesized and tested again to confirm activity. The methodology used to prepare these compounds was chosen based on the fact that the amino moiety in the chitosan's ring is very reactive which is the reason why, the researchers decided to protect it with N-Phthaloyl group in dimethyl sulfoxide:water 95/5 for the subsequent

steps, this methodology prevents the formation of O-phthaloyl byproduct. At the end of the procedure, the phthaloyl group is removed using hydrazine monohydrate in order to regenerate the free amino group. This synthetic strategy was used for most of the modifications done to the original chitosan's ring, blocking amino group, working on the hydroxyl groups, and deprotecting the amino group at the end of the procedure.

Synthesis of Oligochitosan Analogs

Oligochitosan 10KD (UN102), a water soluble oligochitosan with approximately 70 units of chitosan monomers linked β -glucan linkages with 15 percent deacetylation and the starting material for all the chitosan's analogs synthesized for this study, was prepared using an acid hydrolysis method from insoluble chitosan (purchased from Sigma) and the product was characterized by ¹³CNMR, ¹H NMR and FTIR. In general, when the modification was done in a different position other than the amino group, the procedure involved protection and deprotection of the amino group using phthalic anhydride in dry dimethylformamide (DMF) for 12 hours and reflux. Alternatively, an additional protection was done over the primary alcohol group on C6 as follows, starting 10K chitosan (100 mg) was suspended in dry 1,4 p-dioxane (0.5M) at room temperature under argon atmosphere with magnetic stirring. Diphenylphosphoryl azide (DPPA) 6.5 μ l, 1.8 equiv) and DBU (3.0 μ l, 2.4 equiv) were then added drop wise. The reaction was completed in 12 hrs.

Synthesis of Thiolated-Chitosan Analog (UN105)

Initially, 500 mg of chitosan UN102 was dissolved in 15 ml of water acidulated with 1% acetic acid. Then 500 μ l of TGA was added followed by the addition of 0.5g, 2.6 μ mol of EDCI and a catalytic amount of DMAP. The reaction was run for 12 hrs. The resulting product was precipitated and washed 3 \times 5ml cold ethanol. The product was then re-suspended in water and dialyzed using Spectra/Por[®] dialysis tubing 6-8K molecular weight cut-offs (MWCO) for 24 hours changing the water after every 4 hours. The final product, a white solid, was obtained in a 67 percent yield and confirmed by NMR and FTIR.

¹H-NMR Analysis

¹H NMR spectra of compounds was taken on a Bruker AVIII500 Spectrometer (Switzerland, provided by Bruker Tech. and Serv. Co., Ltd. Beijing, China) at room temperature. The operation frequency of 500 MHz was set and 99.9 percent Deuterium Oxide (D₂O) was used. MestreNova software. Chemical shifts were reported in parts per million with TMS as internal standard (Tan et al. 2018).

FTIR Analysis

The Jasco-4100 Fourier Transform Infrared Spectrometer (Japan, provided by JASCO Co., Ltd. Shanghai, China) was used to conduct FTIR spectrum. The scanning range of 4000-400 cm⁻¹ was used for spectra generation (Tan et al. 2018).

Synthesis of Glutaryl-Chitosan Analog (UN106)

500 mg of chitosan 10K, UN102, was dissolved in 5 ml of DMSO and 0.39 g, 3.43 mmol of glutaric anhydride was added and a catalytic amount of DMAP followed by 740 µl, 5.28 mmol of trimethylamine. The reaction was completed after 12 hr. The crude product was precipitated in ice-cold water and washed (3 × 10 mL) with cold water and then re-suspended in a mixture of ethanol-water and dialyzed using Spectra/Por[®] dialysis tubing 6-8K molecular weight cut-offs (MWCO) for 24 h and finally freeze-dried for 24 h at -68 °C.

Synthesis of Azido-Chitosan Analog (UN111)

A 100 mg of chitosan UN102 sample was suspended in dry 1,4 *p*-dioxane (0.5M) at room temperature under argon atmosphere with magnetic stirring. DPPA (6.5 µl, 1.8 equivalent) and DBU (3.0 µl, 2.4 equivalent) were then added drop wise. The reaction was completed after 12 Hrs. The reaction mixture was then heated under argon atmosphere in an oil bath after the addition of sodium azide (5 equiv) and 15-crown-5 (0.1 equiv). The reaction mixture was allowed to stir overnight for completion. The solvent was evaporated on rotary evaporator under vacuum at 40°C and the yellow solid was suspended in water (3 × 20 mL) and the solid recovered by centrifugation at 4K g, then washed with ethyl acetate (3 × 20 mL) and the solid recovered by centrifugation at 4K g and

finally washed with ethanol and acetone and the solid recovered as described before.

Virology Studies

Viral cultures and solution preparation: HIV89.6 was obtained from NIH NIAID repository. Stock solutions of 10 mg/ml of UN102 were prepared in water. Stock solutions (10 mg/ml) of the rest of the compounds were prepared using 10% DMSO in PBS due to their solubility. Respective negative controls were used in the study.

Medium/Blood Samples

To ensure uniformity and reproducibility, standards with known viral copy numbers of HIV89.6 (NIH/NIAID repository) were prepared. A known amount of virus was inoculated into cell culture medium in ten-fold serial dilutions. HIV-1 RNA was extracted using the High Pure viral RNA kit (Ambion). Complementary DNA (cDNA) was synthesized with the SuperScript III cDNA Synthesis Kit (Invitrogen), amplified and quantified using primers specific for HIV-1 LTR. Real-time PCR amplification was performed in a CFX96TM Real-Time system (Bio-Rad, Hercules, CA). The primers used were specific to a conserved region of HIV-1 LTR: *5mplification was performed in (LTR sense; position 506 of HxB2) 5LTR sense; position 506 of Hx (LTR antisense; position 626 of HxB2) (Mehta et al. 2009).* HIV-1 specific amplicons were detected using SYBR Green dye (Bio-Rad). The number of HIV-1 RNA copies in each test template was measured by its threshold cycle (Ct) as determined from the curve of serially diluted standards using data analysis software (Bio-Rad CFX Manager). The threshold cycle values were plotted against copy numbers to construct the standard curve. Quantification of HIV-1 RNA in each test sample was back calculated and viral load was expressed as copies/mL. At the end of the assay, the specificity of each amplified product was ascertained by means of melting curve analysis. This eliminated false-positive detections due to primer-dimers or nonspecific amplicons.

HIV p24 Assay

HIV-1 p24 ELISA was performed using HIV-1 p24 antigen capture assay according to

manufacturer's protocol in each test (Advanced Bioscience Laboratories). Real-time PCR assay was performed for the detection and quantitation of HIV-1 from cell culture. HIV-1 p24 ELISA antigen assay was adopted due to its frequent use in antiviral studies (Behbahani et al. 2014).

p24 ELISA and Quantitative PCR from Human Blood

The compounds UN102, UN103, UN104, UN105, UN106, UN107, UN108, UN109, UN110, UN111, UN112, UN113, UN114, UN115 and UN117 were tested for their efficacy in binding to the HIV-89.6 virus in the presence of blood. Human whole blood was acquired from Florida Blood Services and each time 100 μ l (microliters) of blood was inoculated with 1 mg of the compound and 35 pg (picograms) of HIV-89.6. The mix was incubated for 30 minutes at room temperature on a rotating platform. Later supernatant was separated upon centrifugation (12000 rpm for 5 minutes). Part of the supernatant was used to determine p24 levels using HIV-1 p24 antigen capture assay kits (Advanced Bioscience Laboratories). Remaining supernatant was used for isolation of RNA using High Pure viral RNA isolation kit (Roche). The 5 μ g of the isolated RNA was used to synthesize complimentary DNA HIV-1 LTR using Superscript III (Invitrogen). Quantitative real-time PCR was performed using BioRad CFX 96 with the known amounts of HIV-1 RNA as standards. The starting quantity of HIV-1 RNA in the samples treated with different compounds was determined using the standard curve and plotted on the graph.

Neutralization of RSV and Coxsackievirus

A culture supernatant was inoculated with either RSV or coxsackie virus. UN102 was incubated with either 10 μ l RSV (8×10^8 pfu/ml) or Coxsackie virus at room temperature for 30 min and subjected to centrifugation at 12000 rpm for 5 min to pellet the UN102 complexes and RNA was isolated from supernatant after incubation. The RNA from supernatant containing virus, treated with phosphate buffered saline (PBS) served as control. The infectivity of the RSV was examined by releasing virus from the pellet. Thus, compounds were incubated with 1 μ l rg

RSV (8×10^8 pfu/ml) at room temperature for 30 min and subjected to centrifugation at 12000 rpm for 5 min and the pellet was resuspended in 100 μ l of fresh medium and added onto Vero cells plated in 48 well plate (60,000 cells/well). The equal amount of virus incubated in PBS was used as control and treated similarly and the pellet added onto Vero cells.

Statistical Analyses

Statistical analysis was performed using the general linear model (GLM) procedure of the SAS System 9.2 (SAS Institute Inc., Cary, NC, USA). The $P < 0.05$ was considered a significant different.

RESULTS

The synthesized compounds were purified using column chromatography and the purity of the compounds corroborated using NMR protonic and ^{13}C . The more active compounds were chosen for this publication and the characteristic peaks for these compounds are presented as follow:

Synthesis and Characterization of Oligochitosan (UN102)

The oligomeric chitosan polysaccharide obtained as yellowish solid was characterized by ^1H NMR, ^{13}C NMR and FT-IR. ^1H NMR (400 MHz, D₂O): δ 3.72 (5H, m), 3.59-3.53 (3H, m), 2.97 (2H, t), 1.88 (1H, s). ^{13}C NMR (100 MHz, D₂O): δ 177.24, 101.0, 97.77, 76.49, 74.92, 70.27, 60.06, 55.93, 22.24, 20.50-19.72. FTIR: 3340 (O-H stretch); 1620.8 (Allyl C=C); 1514, 1507 and 1378.3 (aromatic C=O, N-H bending and C-N stretching).

Synthesis of Thiolated-Chitosan Analog (UN105)

The final product, a white solid was obtained in a 67 percent yield and confirmed by NMR and FTIR. ^1H NMR and FTIR.

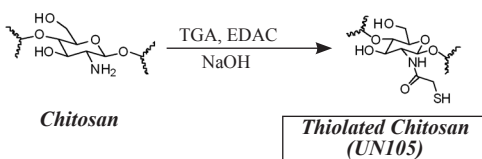
The ^1H NMR (400 MHz, D₂O) δ 3.72 (5H, m), 3.59-3.54 (3H, m), 2.99 (2H, t), 1.87(1H, s), 1.10-0.9 (2H, m), 0.52(1H, s). ^{13}C NMR (100 MHz, D₂O): δ 177.24, 101.0, 97.77, 76.49, 74.92, 70.27, 60.06, 55.93, 47.90, 33.33, 22.24, 20.50-19.72. The chemical shift values between 3.2-3.5 were probably due to H3-H6 connected

to the non-anomeric C3–C6 carbons in the glucopyranose ring. FTIR: 3225 (O-H stretch); 2496 (-S-H stretching); 1681 (N-H bending); 1546 (amide II), 1246 (C-SH stretching), 1149 and 1032 (asymmetric stretching vibration C-O-C), 808 (S-S bisulfide bond).

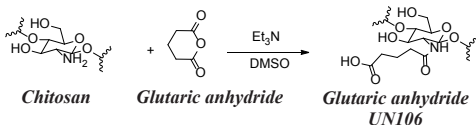
Synthesis of Glutaryl-Chitosan Analog (UN106)

The final product, a whitish solid was obtained in a 54 percent yield and confirmed by NMR and

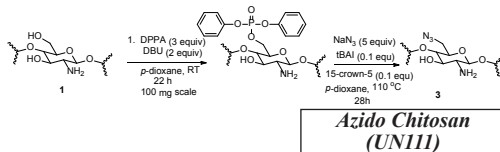
A.



B.



C.



D.

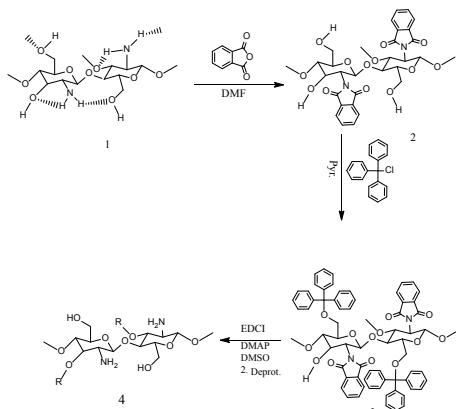


Fig. 1. Schematics of chemical modifications of UN102. A, 2B, 2C.

Synthetic pathways followed to prepare UN105 (A), UN107 (B) and UN111 (C) analogs.

FTIR (Fig. 1.2A, 1.2B and 1.2C). ^1H NMR (400 MHz, DMSO-d₆): δ 7.95-7.92 (1H, s), 2.89 (1H, s), 2.73 (1H, s), 2.54-2.50 (4H, m), 2.29-2.22 (1H, m), 1.72-1.67 (2H, t). The δ values 2.54-2.50 (4H m) represented glutaryl group. ^{13}C NMR (100 MHz, DMSO-d₆): δ 182.50, 141.35, 138.41, 134.20, 133.08, 129.26, 128.13, 126.61, 97.66, 89.03, 76.50, 74.96, 70.23, 60.22, 56.03, 22.3611. FTIR: 3340 (O-H stretch); 1705 (C=O stretch); 1642 (N-H deformation); 1546 (amide II), 1378, 1150 and 1000 (asymmetric stretching vibration C-O-C).

Synthesis of Azido-Chitosan Analog (UN111)

A white solid was obtained in a 78 percent yield and confirmed by ^{13}C , ^1H NMR and FTIR studies. ^1H NMR (400 MHz, D₂O): δ 3.72 (5H, m), 3.59-3.54 (3H, m), 2.99 (2H, t), 1.87(1H, s). ^{13}C NMR (100 MHz, D₂O): δ 177.24, 101.0, 97.77, 76.49, 74.92, 70.27, 60.06, 55.93, 22.24, 20.50-19.72. FTIR: 3340 (O-H stretch); 2361-2341 (-N=N+=N strong vibration); 1681 (N-H deformation); 1546 (amide II), 1379, 1149 and 1032 (asymmetric stretching vibration C-O-C).

Additional UN102 analogs are illustrated in Figure 1. The compounds synthesized involved carboxylation (UN103), ester mannosylation (UN104), N-thiolation (UN105), N-glutamyl addition (UN106), Sulfation (UN107), Oxidation with diamino Starch (UN108), Oxidation with Starch azure (UN109), N-Phthaloyl (UN110), Azido-methyl (UN111), Bromination (UN112), Propargylation (UN113), N-phthaloylation (UN114), conjugation with curcumin glutaryl (UN115), and amidation using citric acid (UN117) whose structures are shown in Figure 2.

UN102 Analogs Tested for Anti-HIV Activity in HIV Culture Supernatants *In Vitro*

To identify the compounds that bind HIV, the HIV containing supernatant from HIV-infected cells was incubated with the desired compound and neutralization of HIV (1 mg of the compound and 35 μg of HIV-89.6), was determined by analysis of the total HIV RNA in culture supernatant. The results of RNA concentration in the supernatant showed that in addition to UN102, several of its analogs such as UN105, UN106, UN111, UN114 and UN117 showed more than 90 percent reduction in levels of HIV RNA in the

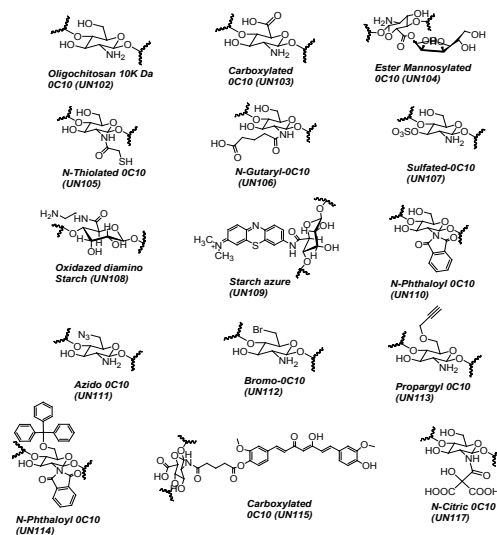


Fig. 2. Chemical structures of the most representative analogs synthesized during this work

Details of the procedure to obtain these analogs is presented in the material and methods section.

supernatant. The rest of **UN102** analogs including UN103, UN104, UN107, UN108, UN109, UN110, UN112, UN113 and UN115 showed less than 20 percent binding or in some cases (Table 1).

Then, HIV p24 was used as a marker to verify the binding potential of synthesized chitosan derivatives. Total RNA level was isolated from supernatant (mixture of PBS, compound and virus, 1 mg of the compound and 35 pg of HIV-89.6). The results showed that compounds UN102, UN105, 106, UN111, UN114, UN111-2 (UN117) decreased p24 levels in the supernatant more than 95 percent and UN103, UN104, and UN113 failed to show such reduction Table 2. These results confirm the ability of some of the **UN102** analogs to neutralize HIV from culture supernatant.

UN102 Analogs Show Anti-HIV Activity in PBMC Culture Ex Vivo

To confirm the activity of the newly synthesized compounds in HIV infected human cells, peripheral blood mononuclear cells (PBMCs) from healthy humans were cultured and parallel assays with PBMCs treated with UN102 and samples with no treatment were also run, followed

Table 1: Screening of compounds for HIV binding in culture supernatant

Compound	Percentage of control
UN102	5
UN103	94
UN104	90
UN105	10
UN106	6
UN107	75
UN108	110
UN109	72
UN110	70
UN111	10
UN112	98
UN113	105
UN114	30
UN115	78
UN117	7
AA control	80

UN102 analogs tested for anti-HIV activity in HIV culture supernatants in vitro UN105, UN106, UN111, UN114 and UN117 show more than 90 percent reduction in levels of HIV RNA in the supernatant while the rest i.e UN103, UN104, UN107, UN108, UN109, UN110, UN112, UN113 and UN115 show less than 20 percent binding. The results of a representative experiment of N=3 are shown. *, P=<0.05 and **, P=<0.01

by infection with HIV-1 89.6. Samples were pelleted on days 0, 2, and 3. Culture supernatants were assayed for p24 concentration. The results show that p24 levels are significantly reduced in UN102-treated vs. non-treated cells on day 3 of the culture Table 3. We also examined the viral RNA copy number from blood of HIV infected patients which were treated with analogs UN102 to UN117. The results show that the compounds UN105, UN106 and UN111 showed the strongest reduction in copy number, followed by UN102, UN114 and UN117.

Table 2: Screening of compounds for HIV binding in culture supernatant

Compound	Viral RNA (Number/ml)
UN102	0.5×10^{12}
UN103	5.8×10^{12}
UN104	7.3×10^{12}
UN105	
UN106	
UN111	0.2×10^{12}
UN113	8.1×10^{12}
UN114	0.3×10^{12}
UN111-2	
10% DMSO control	7.9×10^{12}

Binding potential of synthesized chitosan derivatives. Results show compounds UN102, UN105, 106, UN111, UN114, UN111-2 (UN117) decreased p24 levels in the supernatant by more than 95 percent and UN103, UN104, and UN113 failed to show such reduction. The results of a representative experiment of N=3 are shown. *, P=<0.05 and **, P=<0.01

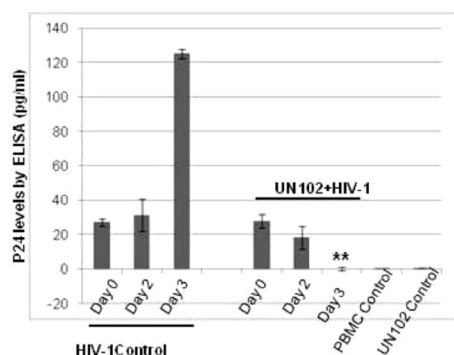


Fig. 3. Effect of UN102-treatment in HIV-infected PBMCs
The results of a representative experiment of N=3 are shown. *, P<0.05 and **, P<0.01

In marked contrast, UN103, UN104 and UN113 did not show any reduction in copy number (Fig. 3).

UN102 Enabled Detection and Neutralization of RSV and Cocksackie Virus

A culture supernatant was inoculated with either RSV or Cocksackie virus and then was incubated with UN102 at room temperature for 30 min and subjected to centrifugation to pellet the UN102 complexes and RNA was isolated from supernatant after incubation. The RNA from supernatant containing virus and treated with PBS served as control. Results in Figure 4A shows that UN102 was able to remove both RSV and Cocksackie virus

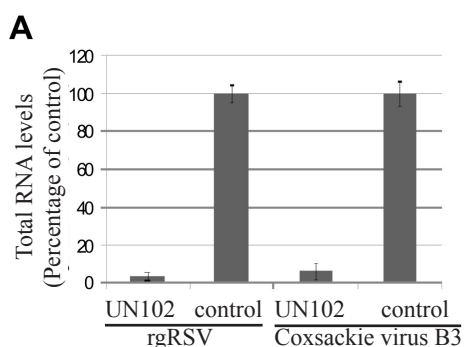


Fig. 4. UN102 Neutralization of RSV and Cocksackievirus
A culture supernatant was spiked with either RSV or coxsackievirus

Fig. 4A. RNA was isolated from supernatant after incubation
The RNA from supernatant containing virus and treated with PBS served as control

Fig. 4B. The results show that the viral RNA was recovered from the pellet. The assay was done in triplicate. The results of a representative experiment of N=3 are shown *, P<0.05 and **, P<0.01

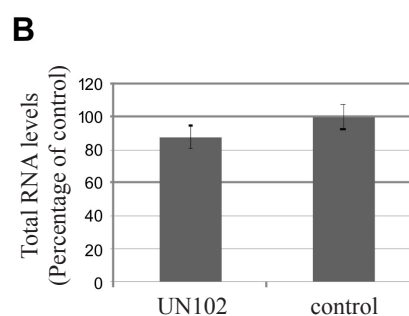
Table 3: Effect of compounds that remove HIV from blood as determined by RNA copy number

Compound	Viral RNA (Copy numbers/ml)
UN102	0.5×10^{12}
UN103	5.9×10^{12}
UN104	7.3×10^{12}
UN105	0.1×10^{12}
UN106	0.1×10^{12}
UN111	0.2×10^{12}
UN113	8.1×10^{12}
UN114	0.3×10^{12}
UN117	0.2×10^{12}
Veh. control	7.9×10^{12}

The assay was done in triplicate. Viral RNA copy number from blood of HIV infected patients treated with analogs. Results show compounds UN105, UN106 and UN111 show the strongest reduction in copy number, followed by UN102, UN114 and UN117. In contrast UN103, UN104 and UN113 did not show any reduction in copy number. The results of a representative experiment of N=3 are shown. *, P<0.05 and **, P<0.01

from the culture supernatant to the extent of 95-99 percent. The total RNA was isolated from the pellets representing complexes of UN102 and RSV, as shown in Figure 4B. The results show that the viral RNA was recovered from the pellet.

UN102 was used to absorb the virus (recombinant RSV expressing green fluorescence reporter protein) from the culture supernatant to test the binding ability of UN102 in terms of infectivity loss (Fig. 5). The infectivity of the virus of Vero cells was examined by releasing virus from the pellet.



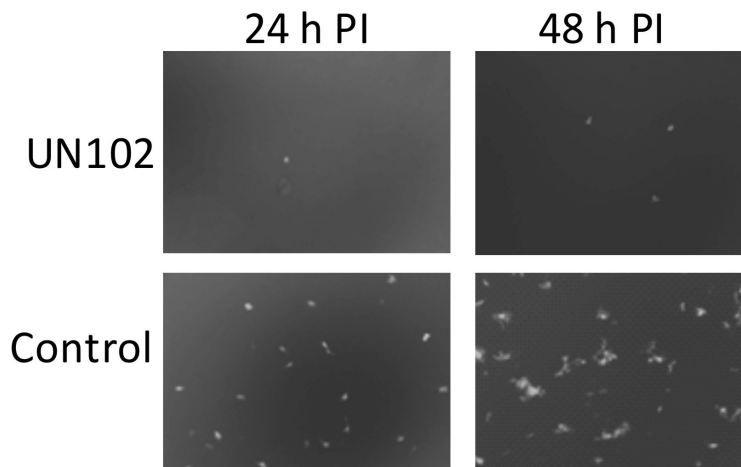


Fig. 5. Inactivation of virus following UN102 binding to the virus

UN102 was used to absorb the virus (recombinant RSV expressing green fluorescence reporter protein) from the culture supernatant. The infectivity of the virus was examined by releasing virus from the pellet. Thus, UN102 was incubated with 1 μ l rg RSV (8×10^8 pfu/ml) at room temperature for 30 min and subjected to centrifugation and the pellet was resuspended in 100 μ l of fresh medium and added onto Vero cells plated in 48 well plate (60,000 cells/well). The equal amount of virus incubated in PBS was used as control and treated similarly and the pellet added onto Vero cells. Pictures were taken at 24 h/48 h post treatment of the cells. The assay was done in triplicate. The results of a representative experiment of N=3 are shown.

The pellet was re-suspended in fresh medium and added onto Vero cells plated in 48 well microplate. The equal amount of virus incubated in PBS was used as control and treated similarly and the pellet added onto Vero cells. Photomicrographs were taken at 24 h/48 h post treatment of the cells. No RSV infected cells were found in pellet obtained after treatment of pellet with UN102, showing the virus found on the pellet was unable to infect cells.

DISCUSSION

The studies reported herein comprise synthesis of a number of UN102 oligochitosan derivatives, which have been utilized for neutralization of viruses including HIV, RSV and Coxsackie virus. Based on the literature, we reasoned that this oligochitosan may be better suited to develop a sulfated derivative and other chemical derivatives to identify the ones with best antiviral activity. Surprisingly, sulfated 10 kDa oligochitosan did not exhibit any antiviral activity, whereas other analogs were identified with comparatively better antiviral activity. The synthesized UN102 analogs were characterized

by ^1H NMR and ^{13}C NMR and FTIR techniques and signals or peaks obtained in respective spectra confirmed the structural modifications as a result of chemical reactions.

The selection of the viruses to be used for this study was based on prevalence, pathogenicity, virulence, impact in population, availability and experience. Three different viruses including HIV, RSV and the Coxsackie virus were chosen to test antiviral activity of UN102 and its synthesized derivatives. First, HIV types 1 and 2 (HIV-1 and HIV-2) have been identified as the primary etiologic agents of AIDS and its associated disorders (AIDS Portal 2017), which causes a chronic viral disease lasting the lifetime (Harrison et al. 2010). Treatment with antiretroviral therapy (ART) increases the life expectancy of people infected with HIV from 1 year without ART to >10 years (Humphreys et al. 2010). ART decreases viral load and increases immuno - competence but once HIV becomes resistant to ART, a combination of antiretroviral drugs are used which often increases the drugs' side-effects, opportunistic infections, autoimmune diseases (French 2007; Hirsch 2004) and treatment costs. Secondly, RSV is an acute infection that

occurs in immuno - compromised patients causing ~64 million infections and killing ~166,000 persons annually including infants worldwide (Thompson et al. 2003; World Health Organization 2010) and despite its discovery in 1955, no vaccines or effective anti-viral treatments are available (Karron 2008). Third, the Coxsackie viruses comprising of ~29 different viruses cause several symptoms from hand, mouth and foot diseases to severe conditions like meningitis, encephalitis, paralysis, myocarditis and pericarditis (Koh et al. 2016).

A major and promising finding of these studies is the remarkable improvement on antiviral activity displayed by some of the UN102 analogs: glutaryl, thiolated and azido chitosan. However, it was observed that unmodified UN102, exhibited comparatively better antiviral activity than sulfated UN102. The reason behind this remained unveiled. Although, a previous study revealed that sulfated chitosan showed significant antiviral activity against Newcastle disease virus (Karthik et al. 2016). The evidence that HIV concentration can be reduced in blood by more than 90 percent, opens a possibility to explore the use of these chitosan analogs after fully satisfying all FDA requirements, as a potential treatment to be prescribed after detection of a viral agent in a bodily fluid, to reduce viral load in blood and may be useful in treating or preventing conditions including AIDS and other viral infections. The chitosan, its derivatives and some other polysaccharides were reported to have many medicinal properties including anti-HIV (Vo and Kim 2010). The results of HIV RNA and p24 analysis in HIV infected blood show that the mentioned analogs were effective in neutralizing >50 percent of HIV from blood, suggesting that these new chemical compounds could be in pharmaceutical preparations to control viral infections. Further study on these compounds may provide a novel range of chitosan derivatives to treat viral infections.

Another important finding is that the antiviral effect of these new compounds UN102, UN105, UN106 UN111, etc. are not HIV specific. Studies with RSV and Coxsackie viruses have shown the compounds may also decrease these viral titers. A key advantage of this treatment is that it can be personalized by using required amount of the compounds, as determined from *ex vivo* neutralization.

CONCLUSION

The Covid 19 pandemic has been a major challenge for the contemporary world; it has critically affected many areas of life and has revealed our vulnerability to new agents of infection. The analogs synthesized in this study could eventually be tested against Covid 19 and its variants to evaluate the antiviral activity. The synthesis of these analogs do not require special conditions and therefore can be performed at room temperature using standard laboratory equipment. Additionally, the methodology is susceptible to being scaled up, which gives a comparative advantage as the cost of most of the antiretroviral drugs that are vastly purchased by NGOs and humanitarian bodies to supply for free to third world countries that have a high number of HIV infected people. The discovery of how to reduce the viral counts of some diseases will be able to help governments and individuals save when it comes to medical expenses and in the case of Covid, life can go back to normal without the risk of infection that might lead to death in some cases.

RECOMMENDATIONS

The recommendations are as follows:

First, there is an urgent need to have safe and broad spectrum of antiretroviral agents that are affordable. The antiretroviral agents being affordable will enable various institutions to carry out more research with different agents at their disposal without worry of being over budget. The agents also need to be safe so as to not put the lives of the scientists conducting the laboratory tests in danger during and after the duration that they work with them.

Second, proper funding and adequate government support especially the FDA is vital in these studies. Funding will enable the scientists to conduct widespread research using various chitosan derivatives that might even be in different locations and countries. Proper funding will lead to the discovery of drugs that can be used to fight or reduce the viral counts in viral diseases in human beings.

Third, more research that is in depth needs to be conducted. This is so that there can be effective and efficient results that are produced and in a timely manner.

Finally, the research needs to involve other countries especially the third world countries that

are mostly affected by the mentioned viruses in huge numbers especially HIV. Involving scientists in third world countries will give the countries not only more knowledge but also the independence they need to improve the health of their citizens.

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ABBREVIATIONS

EDCI: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

HIV: Human Immunodeficiency Virus

DMAP: 4-Dimethylaminopyridine

DMF: Dimethylformamide

DMSO: Dimethyl Sulfoxide

DPPA: Diphenylphosphoryl azide

NMR: Nuclear Magnetic Resonance

PBS: Phosphate-buffered saline

RNA: Ribonucleic acid

RSV: Respiratory Syncytial Virus

REFERENCES

- AIDS Portal 2017. The War on AIDS: Current global Statistics For The Information Age. From <<https://aidsportal.org/current-global-statistics/>> (Retrieved on 21 December 2020)
- Artan M, Kaadeniz F, Karagozlu M, Kim M 2010. Anti-HIV-1 activity of low molecular weight sulfated chitooligosaccharides. *Carbohydrates Res*, 345(5): 656-662.
- Behbahani M, Sayedipour S, Shanehsazzadeh M et al. 2014. In vitro anti-HIV-1 activities of kaempferol and kaempferol-7-O-glucoside isolated from *Securigera securidaca*. *Research in Pharmaceutical Sciences*, 9(6): 463.
- Bird G, Boyapalle S, Wong T, Opuko-Nsiah K et al. 2014. Mucosal delivery of a double-stapled RSV peptide prevents nasopulmonary infection. *J Clin Invest*, 124(5): 2113-2124.
- Choi Y 2021. Emerging and re-emerging fatal viral diseases. *Exp Mol Med*, 53: 711-712.
- Ehresmann E, Deig F, Hatch M, DiSalvo et al. 1977. Antiviral substances from California Marine Algae. *Journal of Phycology*, 13(1): 37-40.
- French MA 2007. Disorders of immune reconstitution in patients with HIV infection responding to antiretroviral therapy. *Curr HIV/AIDS Rep*, 4(1): 16-21.
- Harrison KM, Song R, Zhang X 2010. Life expectancy after HIV diagnosis based on national HIV surveillance data from 25 states, United States. *J Acquired Immune Deficiency Syndrome*, 53(1): 124-130.
- Hirsch VM 2004. What can natural infection of African monkeys with simian immunodeficiency virus tell us about the pathogenesis of AIDS? *AIDS Rev*, 6(1): 40-53.
- Humphreys EH, Chang LW and Harris J 2010. Antiretroviral regimens for patients with HIV who fail first-line antiretroviral therapy. *Cochrane Database Syst Rev*, 6: CD006517.
- Irwin KK, Renzette N, Kowalik TF, Jensen JD 2016. Antiviral drug resistance as an adaptive process. *Virus Evolution*, 2(1): 14.
- Karron RA 2008. Respiratory syncytial virus and parainfluenza virus vaccines. In: SA Plotkin, W Orenstein, PA Offit (Eds.): *Vaccines*. Elsevier-Saunders: New York, pp. 1283-1293.
- Karthik R, Manigandan V, Saravanan R, Rajesh R et al. 2016. Structural characterization and in vitro biomedical activities of sulfated chitosan from *Sepia pharaonis*. *International Journal of Biological Macromolecules*, 84: 319-328.
- Koh WM, Bogich T, Siegel K, Jin J et al. 2016. The epidemiology of hand, foot and mouth disease in Asia: A systematic review and analysis. *Pediatric Infectious Disease J*, 35(10): e285-300.
- Kong X, Hellerman G, Mohapatra S et al. 2005. An immunocompromised BALB/c mouse model for respiratory syncytial virus infection. *Viral J*, 2: 3.
- Minari M, Rincao V, Sores S, Ricardo N 2011. Antiviral properties of polysaccharides from *Agaricus brasiliensis* in the replication of bovine herpes virus 1. *Acta Virologica*, 55(3): 255-259.
- Mehta N, Trzmielina S, Nonyane B, Eliot M et al. 2009. Low-cost HIV-1 diagnosis and quantification in dried blood spots by real time PCR. *PLoS One*, 4(6): e5819.
- Nakashima H, Kido Y, Kobayashi N, Motoki Y et al. 1987. Purification and characterization of an avian myeloblastosis and human immunodeficiency virus reverse transcriptase inhibitor, sulfated polysaccharides extracted from sea algae. *Antimicrob Agents Chemother*, 31(10): 1524-1528.
- Nishimura I, Kai H, Shinada K, Yoshida T et al. 1998. Regioselective syntheses of sulfated polysaccharides: specific anti-HIV-1 activity of novel chitin sulfates. *Carbohydr Res*, 306(3): 427-433.
- Pardi N, Weissman D 2020. Development of Vaccines and Antivirals for Combating Viral Pandemics. *Nature Biomedical Engineering*. From <<https://doi.org/10.1038/s41551-020-00658-w>> (Retrieved on 10 June 2021).
- Satitsri S, Muanprasat C 2020. Chitin and Chitosan derivatives as biomaterial resources for biological and biomedical applications. *Molecules Journal*, 25(24): 5161.
- Schols D, Pauwels R, Witvrouw M, Desmyter J et al. 1992. Differential activity of polyanionic compounds and castanospermine against HIV replication and HIV-Induced syncytium formation depending on virus strain and cell type. *Antiviral Chemistry and Chemotherapy*, 3(1): 23-29.
- Sugawara I, Itoh W, Kimura S, Mori S et al. 1989. Further characterization of sulfated homopolysaccharides as anti-HIV agents. *Experientia*, 45(10): 996-998.
- Tan W, Li Q, Dong F, Zhang J et al. 2018. Novel cationic chitosan derivative bearing 1, 2, 3-triazolium and pyridinium: Synthesis, characterization, and antifungal property. *Carbohydrate Polymers*, 182: 180-187.
- Thompson WW, Shay DK, Weintraub E, Brammer L et al. 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA*, 289(2): 179-186.
- Villamagna A, Gore S, Lewis J, Doggett J 2020. The Need for Antiviral Drugs for Pandemic Coronaviruses from a Global Health Perspective. *Frontiers in Medicine*. From <<https://doi.org/10.3389/fmed.2020.596587>> (Retrieved on 10 June 2021).
- Vo TS, Kim SK 2010. Potential anti-HIV agents from marine resources: An overview. *Marine Drugs*, 8(12): 2871-2892.

- Wang W, Wang S, Guan H 2012. The antiviral activities and mechanisms of marine polysaccharides: An overview. *Marine Drugs*, 10(12): 2795-2816.
- World Health Organization 2010. *The Initiative For Vaccine Research: Report 2008-2009*. Geneva: World Health Organization.
- Zaikov G 2005. *Chemistry of Polysaccharides*. UK: Taylor and Francis.
- Zhang W, Yang H, Kong X, Mohapatra S et al. 2005. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nat Med*, 11(1): 56-62.
- Zheng M, Qu D, Wang H, Sun Z et al. 2016. Intranasal administration of Chitosan against Influenza A (H7N9) virus Infection In A Mouse Model. *Sci Rep*, 6: 28729.

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