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Synthesis, antiviral and contraceptive activities of nucleoside–sodium cellulose sulfate acetate and succinate conjugates

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ABSTRACT

Chemical conjugates between sodium cellulose sulfate (CS), displaying contraceptive and HIV-entry inhibiting properties, and nucleoside reverse transcriptase inhibitors (NRTIs) (3′-azido-2′,3′-dideoxythymidine (AZT), 3′-fluoro-2′,3′-dideoxythymidine (FLT), or 2′,3′-dideoxy-3′-thiacytidine (3TC)) were designed to simultaneously provide contraceptive and anti-HIV activity. Two linkers, acetate and succinate, were used to conjugate the nucleoside analogs with CS. The conjugates containing cellulose sulfate-acetate (CSA) (e.g., AZT–CSA and FLT–CSA) were found to be more potent than CS and other conjugates (e.g., AZT–succinate–CS, and FLT–succinate–CS). The presence of both sulfate and the acetate groups on cellulose were critical for generating maximum anti-HIV activity. In addition to showing equal potency against wild-type and multidrug resistant HIV-1, the AZT–CSA conjugate displayed significant contraceptive activity in an animal model, providing the initial proof-of-concept for the design and synthesis of dual-activity compounds based on these combinations.

Unplanned pregnancies and sexually transmitted infections, including HIV/AIDS, affect millions of women each year providing the rationale for the development of novel multi-purpose prevention technologies.[1] Sulfonate and sulfate polyanionic compounds inhibit HIV entry into host cells[2–5] and also display sperm-function inhibiting properties.[6,7] Polyanionic sulfates are polymers, such as dextran, cellulose, styrene, with sulfate groups in their structure.[3,5,8,9] In our attempt to develop novel dual-activity compounds, we conjugated polyanionic cellulose derivatives with known HIV nucleoside reverse transcriptase inhibitors (NRTIs).

The viral envelope protein gp120 is known to have positively charged residues in its V3 loop. HIV entry in host cell depends on the virus interactions with the negatively charged surfaces of the co-receptors CXCR4 and CCR5.[10] Polyanionic sulfates exhibit their inhibitory activity by blocking these interactions.[11–14]

Different strains of HIV are known to have different gp120 V3 loops. X4-strains of HIV, for instance, possess a higher positive charge density on their V3 loop than R5 strains.[8,14,15] This property of X4 makes them more susceptible to polyanionic inhibitors. Certain polyanionic derivatives like dextran sulfate and heparin show weak inhibitory activity against R5 HIV strains.[11,16]

Sodium cellulose sulfate (Ushercell, CS, **1**) is a polyanionic non-cytotoxic microbicide and its 6% gel has been studied as a vaginal contraceptive and a broad-spectrum antimicrobial agent, both preclinical and clinically.[7,17,18] Similar to other polyanionic derivatives, CS (**1**) interacts with the positively charged viral envelope proteins, and prevents virus cell entry. Various preclinical studies showed that CS possesses significant anti-HIV activity against both strains of virus, but it is more active against X4 tropic than R5 tropic HIV.[16,19]

In addition to antimicrobial activity, CS also showed contraceptive activity because of its ability to inhibit sperm enzymes (e.g., hyaluronidase; IC_{50} = 1.7 mg/ml), which are important for sperm–egg interaction.[17] CS also inhibits sperm penetration of cervical mucus.[17] In a non-comparative contraceptive effectiveness trial of 6% CS gel involving 200 couples who used the product for 6 month as their only method of contraception, CS showed similar effectiveness rates to those of commercial spermicides.[7]

CS showed promising results in the preclinical studies and phases I and II clinical trials sponsored by CONRAD. In preclinical studies, CS prevented conception in rabbits when applied vaginally before insemination, and was also found to inhibit HIV, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis*.[6,17,20] Results from phase I clinical trials showed that CS was very safe and non-irritating to penile and vaginal application.[21,22] CS vaginal gel (6%) was well tolerated as vaginal microbicide in both healthy and HIV-infected women, producing similar results to the commercial lubricant K-Y jelly.[7,23–25]

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Despite the promising preclinical results showing anti-HIV activity, CS failed to protect women in two large phase 3 clinical trials.[18,26] Several hypotheses are still being investigated, but low antiviral potency and poor tissue penetration have been suggested as possible causes for the lack of correlation between clinical and preclinical results.

The microbicides field has moved on to the development of more potent and specific compounds, selecting a few nucleoside and non-nucleoside reverse transcriptase inhibitors as its lead candidates (e.g., tenofovir, dapivirine, and UC781). As a result of such change, the current candidates display only antiviral activity and they are more susceptible to develop resistant viruses.[27] The selective activity of these drugs against HIV will leave women who have unprotected intercourse exposed to other sexually transmitted infections and pregnancy. Although CS is no longer being developed as an anti-HIV microbicide, it represents a good candidate to use as proof-of-concept for a substituted compound with dual, contraceptive and antimicrobial activity. Evaluation of novel CS derivatives or conjugates provides insights for designing the second generation of compounds with more optimal biological profile.

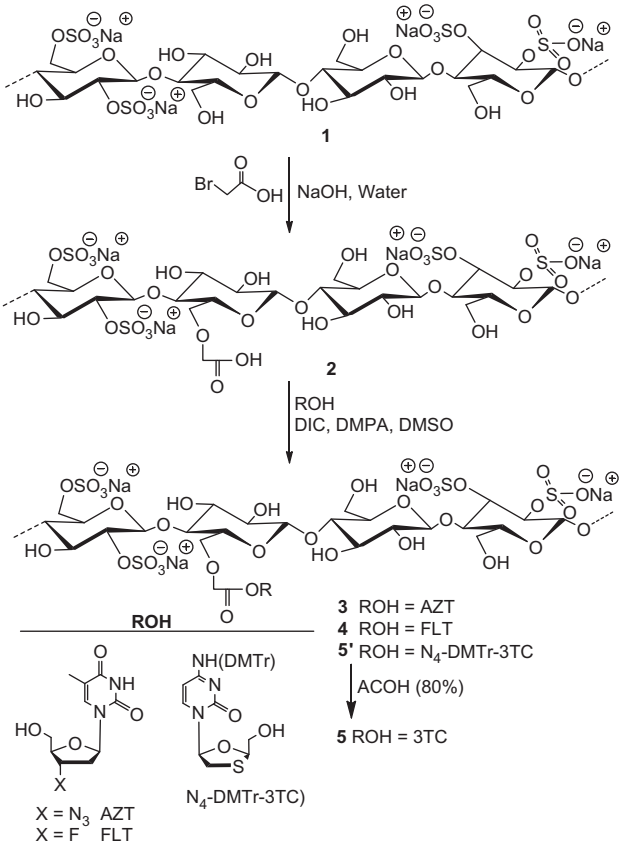
Therefore, as proof-of-concept for a novel dual-activity compound with potent anti-HIV activity, higher barrier to resistant virus and contraceptive activity, we herein report the synthesis and biological evaluation of bifunctional sodium cellulose sulfate conjugates with NRTIs, 3′-azido-2′,3′-dideoxythymidine (Zidovudine, AZT), 3′-fluoro-2′,3′-dideoxythymidine (Alovudine, FLT), and 2′,3′-dideoxy-3′-thiacytidine (Lamivudine, 3TC). Two different linkers (acetate and succinate) were used to conjugate the nucleoside analogs with CS in different loading values. Both nucleosides and CS contain alcohol functional groups and functionalization with linkers containing carboxylic acids allows conjugation through the formation of an ester moiety. Furthermore, it was expected that the ester conjugates would be cleaved by hydrolytic activity of cellular esterase enzymes, resulting in two separate components: CS (HIV entry blocker) and nucleoside analogs (NRTIs). Conjugates were also expected to exert contraceptive activity mediated by the released CS.

The synthesis of cellulose sulfate acetate conjugates of AZT, FLT and 3TC was accomplished using building block synthetic strategy. For the synthesis of nucleoside–CSA conjugates, CS (**1**) was reacted first with 2-bromoacetic acid in presence of sodium hydroxide to render cellulose sulfate acetate (CSA, **2**). CSA was then reacted with AZT, FLT, or N_4-DMTr-3TC to yield cellulose sulfate acetate conjugates of AZT (**3**, AZT–CSA, 1.78% loading), FLT (**4**, FLT–CSA, 1.43% loading), or N_4-DMTr-3TC-CSA (**5′**), respectively. Deprotection of DMTr group in **5′** afforded 3TC–CSA (**5**, 1.07% loading) (Scheme 1).

For the synthesis of sodium cellulose sulfate conjugates linked to AZT or FLT through a succinate linker, AZT and FLT were first reacted with succinic anhydride to synthesize AZT succinate (**6′**) and FLT succinate (**7′**), which were then reacted with cellulose sulfate to afford cellulose sulfate succinate conjugates of AZT (**6**, 18.48% loading) and FLT (**7**, 7.87% loading) (Scheme 2).

The percentage of the loaded nucleosides on the polymer through the acetate or succinate linkers was determined using the size exclusion chromatography (SEC) method based on the calibration curves of different concentrations of nucleosides versus area under the curve. The polymer–nucleoside conjugates were eluted at 9.15–9.50 min. It was assumed that the nucleosides were mainly conjugated through acetate or succinate linkers to the more reactive primary alcohols, but conjugation on secondary alcohols through linkers may also contribute to the percentage of loading. Both conjugations contribute to the overall anti-HIV activity after hydrolysis of the conjugate to the parent nucleosides.

CS exhibited approximately 10-fold higher activity against X4 virus (IIIB strain, IC_{50} = 5.9 μg/ml) than R5 virus (BaL strain, IC_{50} = 62.5 μg/ml) (Table 1). These data were consistent with previously reported data on the weaker antiviral potency of anionic substances against macrophage-tropic R5 HIV-1.[8,14,15] X4 strains possess higher number of positive charges on the V3 loop of gp120 compared to those of R5 strains, making them more susceptible to inhibition by anionic polymers.

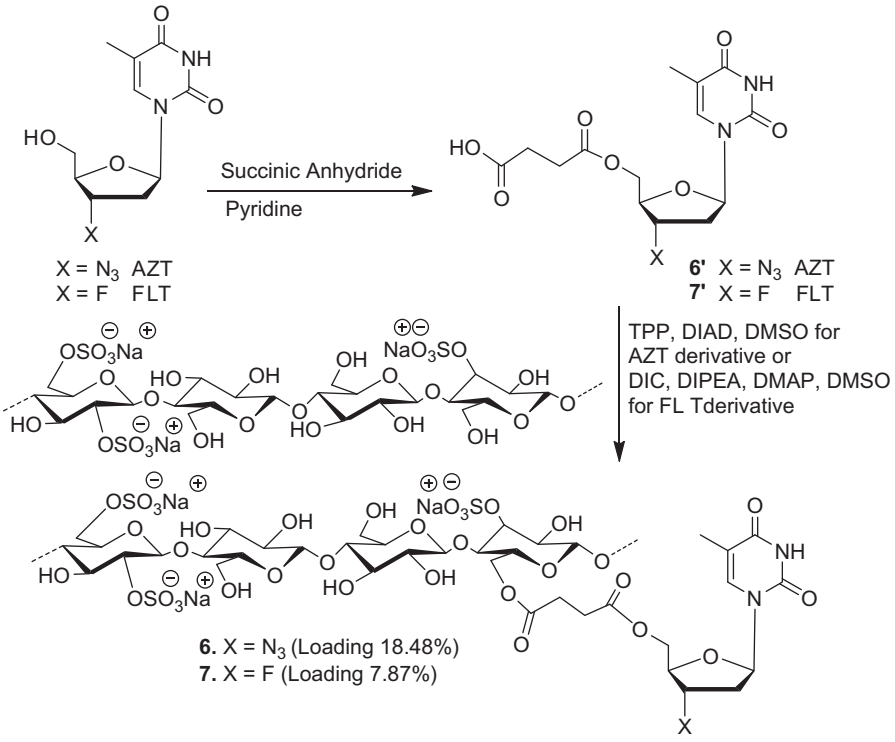


Scheme 1. Synthesis of cellulose sulfate acetate conjugates of AZT (**3**), FLT (**4**), and 3TC (**5**).

In the present study, cellulose sulfate was conjugated with NRTIs, AZT, FLT, and 3TC, through different linkers to synthesize CS–nucleoside conjugates as bifunctional anti-HIV agents targeting different events in HIV life cycle. The conjugates were evaluated for their anti-HIV activity against lab-adapted, X4 and R5, cell-free HIV and wild-type and MDR clinical isolates and for contraceptive properties in an animal model. Cytotoxicity assays exhibited that none of the conjugates and the corresponding physical mixtures were cytotoxic at the highest concentration tested of 100 μg/mL.

Table 1 shows the antiviral activities of the cellulose sulfate–nucleoside conjugates with different loading percentages compared to those of CS, AZT, and FLT. Conjugation of CS with nucleosides improved CS antiviral activity against R5 strains.

Nucleoside–CSA conjugates (AZT–CSA, **3**, 1.78%; FLT–CSA, **4**, 1.43%) demonstrated higher anti-HIV activity than that of CS (**1**). The improved anti-HIV activity of **3** (IC_{50} = 8.1 μg/ml) and **4** (IC_{50} = 1.5 μg/ml) compared to CS (IC_{50} = 62.5 μg/ml) could be due to the release of two anti-HIV agents with different mechanisms of action and/or the presence of additional negatively charged acetate in the structure. Cellulose sulfate-acetate (**2**) was tested for anti-HIV activity as a control. Similar to **3** and **4**, CSA (**2**) exhibited higher potency than CS (**1**), especially against the R5 strain, and surprisingly it was almost equally active to **3** and **4**. The higher activity of **2** and its conjugates, **3** and **4**, may be attributed to the presence of free negatively-charged carboxylate groups from the acetate groups substituted on CS, which enhance the overall negative charge density of the molecule. Although the substitution of acetate group on



Scheme 2. Synthesis of AZT–succinate–CS (**6**) and FLT–succinate–CS (**7**) conjugates.

Table 1
Anti-HIV activity of nucleoside–cellulose sulfate conjugates

Compd	Chemical name (% loading)	Cytotoxicity	Antiviral activity	
			IIIB	BaL
1	Sodium cellulose sulfate (Mol. wt. 2,000,900 Da)	>100	5.9	62.5
2	Cellulose sulfate acetate (CSA)	>100	1.3	1.8
3	AZT–cellulose sulfate acetate (AZT–CSA, 1.78%)	>100	2.5	8.1
4	FLT–cellulose sulfate acetate (FLT–CSA, 1.43%)	>100	2.3	1.5
5	3TC–cellulose sulfate acetate (3TC–CSA, 1.07%)	>100	92.4	75.1
6	AZT–succinate–cellulose sulfate (AZT–Suc–CS, 18.48%)	>100	2.2	9.9
7	FLT–succinate–cellulose sulfate (FLT–Suc–CS, 7.87%)	>100	6.2	6.1
AZT	Zidovudine	>100	2.4	4.2
FLT	Alovudine	>100	<0.1	<0.1
3TC	Lamivudine	>100	7.5	2.6

Anti-HIV activity of compounds evaluated in single-round infection assays using HIV-1 IIIb (X4) and BaL (R5) and galactosidase-transfected P4R5 cell line. Data are expressed as IC_{50} (antiviral) or CC_{50} (cytotoxicity) in μg/ml.

cellulose sulfate increased the anti-HIV activity of CS, cellulose acetate (**14**) was completely inactive (Table 2), suggesting that the presence of sulfate is critical in maintaining the anti-HIV activity of the polymer.

Compared with AZT–CSA and FLT–CSA conjugates, 3TC–CSA (**5**, 1.07% loading) showed a significantly different anti-HIV profile. Conjugate **5** revealed almost 37-fold less anti-HIV activity against X4 strain when compared with **3**. The poor anti-HIV activity of 3TC conjugate could be due to the possible intermolecular interaction of free positively charged 4-amino group of 3TC with the negatively-charged groups on CSA, which reduced the available free negative charge of the conjugate for binding to V3 loops of the virus.

To determine the contribution of sulfate group in generating anti-HIV activity of CS, cellulose phosphate and dextran acetate were studied as controls. These compounds were found to be totally inactive in viral inhibition assays (Table 2), suggesting that negatively-charged acetate and phosphate alone are not sufficient for efficient interaction with HIV gp120 V3 loop.

Table 2
Anti-HIV activities of cellulose acetate, dextran acetate, cellulose phosphate, and physical mixtures of nucleosides with CS, CSA, and cellulose

Compd	Chemical name (% loading)	Cytotoxicity	Antiviral activity	
			IIIB	BaL
8	AZT (1.78%) + CSA	>100	1.7	2.5
9	FLT (1.43%) + CSA	>100	0.7	0.3
10	3TC (1.07%) + CSA	>100	0.7	1.7
11	AZT (18.48%) + CS	>100	16.2	15.3
12	FLT (1.43%) + CS	>100	6.2	7.1
13	FLT (7.85%) + CS	>100	0.7	0.3
14	Cellulose acetate	>100	>100	>100
15	Dextran acetate	>100	>100	>100
16	Cellulose phosphate	>100	72.1	>100
17	AZT (1.78%) + cellulose acetate	>100	75.5	8.5
18	FLT (1.43%) + cellulose acetate	>100	6.8	6.5
19	3TC (1.07%) + cellulose acetate	>100	73.9	22.4

Anti-HIV activity of compounds evaluated in single-round infection assays using HIV-1 IIIb (X4) and BaL (R5) and galactosidase-transfected P4R5 cell line. Data are expressed as IC_{50} (antiviral) or CC_{50} (cytotoxicity) in μg/ml.

The anti-HIV activity of nucleoside–succinate–CS conjugates, AZT–succinate–CS (**6**, 18.48%) and FLT–succinate–CS (**7**, 7.87%) was also evaluated. Both conjugates demonstrated at least six-fold higher anti-HIV activity against R5 strains than CS, suggesting the contribution of the nucleoside analog in anti-HIV activity (Table 1).

Although **6** and **7** had 10 and 6 times higher loading values than the corresponding conjugates substituted with acetate (**3** and **4**), respectively, the cellulose sulfate succinate conjugates were generally less active than cellulose sulfate acetate conjugates (Table 1). The lower anti-HIV activity of conjugates containing succinate linker, despite of their higher nucleoside loading, compared to those containing acetate linker could be due to incomplete hydrolysis of **6** and **7** to parent nucleosides or the hydrolysis of the conjugate to generate inactive nucleoside–succinate derivatives instead of free nucleosides. Furthermore, upon hydrolysis of conjugates containing acetate linker, the acetate group will remain intact on the CS, contributing to the overall negative charge of the anionic polymer. The acetate is attached CS through methylene group and cannot be hydrolyzed like succinate ester conjugate. Further studies on the stability of the conjugates in vitro and in vivo will provide insights about the amount of the parent nucleosides and CS or CSA released and correlation with the anti-HIV activity.

Table 2 shows the anti-HIV activity of the nucleoside–CS conjugates compared with the corresponding physical mixtures. The physical mixture of AZT (1.78%) + CSA (**8**) showed slightly better anti-HIV activity against HIV than the corresponding AZT–CSA conjugate (**3**). When CSA in **8** was replaced by CS in the physical mixture with AZT in **11** (18.48%), the anti-HIV activity was significantly reduced even with a higher loading of AZT in **11** because of the overall reduction of negative charge density in CS and the major contribution of CSA in overall activity.

FLT–CSA conjugate (**4**) and 3TC–CSA (**5**) were compared with the corresponding physical mixtures, **9** and **10**, respectively. Similarly FLT–CSA conjugate (**4**) and 3TC–CSA (**5**) were less potent than their corresponding physical mixtures, **9** and **10**. Physical mixture of CSA and FLT (**9**, 1.43%) exhibited approximately 3–5 fold higher anti-HIV activity against VBI (cell-free virus) when compared with the corresponding FLT–CSA conjugate (**4**, 1.43%). In the case of **10**, the free amino group was not able to reduce the anionic interactions of sulfate group as described above in the conjugate **5**. In general, the physical mixtures of **8**, **9**, and **10** exhibited better anti-HIV activity against HIV than the corresponding conjugates, **3**, **4**, and **5**, respectively, possibly because of the contribution of free negatively-charged acetate group of CSA in the physical mixtures. This is further confirmed by the fact physical mixtures containing CS (**11**, **12**) were significantly less potent than those containing CSA (**8**, **9**).

For example, the overall anti-HIV activity of **9** was reduced by 8–22 times against when CSA in **9** (1.43%) was replaced with CS in **12** (1.43%), suggesting the importance of CSA in overall anti-HIV activity of these conjugates. Confirming the importance of the sulfate groups on the cellulose backbone, the physical mixture of AZT + cellulose acetate (**17**, 1.78%) showed significantly less antiviral activity than AZT–CSA conjugate (**3**, 1.78%) and AZT + CSA (**8**, 1.78%). Similarly, the physical mixture of FLT + cellulose acetate (**18**, 1.43%) showed 9–21 fold less activity against cell-free virus when compared with FLT + CSA (**9**, 1.43%). These results were not surprising since cellulose acetate is an inactive polymer and the percentage of AZT or FLT were low in the physical mixture.

In general, the physical and chemical combination of nucleosides with CS provided better antiviral activity against both X4 and R5 HIV-1 strains. The incorporation of acetate group on CS also improved anti-HIV activity, possibly by creating new negative charges after hydrolysis to free acetate groups on the polymer.

Table 3
Anti-HIV activities of AZT–CSA and FLT–CSA conjugates against R5 and multidrug resistant HIV-1 clinical isolates

Compd	Chemical name	Type of virus	IC_{50} (μg/ml)
3	AZT–CSA (1.78%)	R5	3.52
		MDR	4.22
4	FLT–CSA (1.43%)	R5	2.67
		MDR	0.50
1	CS	R5	>20.0
		MDR	1.61
AZT	Zidovudine	R5	0.03
		MDR	0.3
FLT	Alovudine	R5	0.002
		MDR	0.004

Assay endpoint = p24 level (ELISA); IC_{50} = the minimum drug concentration that inhibits HIV-induced cytopathic effect by 50%, calculated by using a regression analysis program for semilog curve fitting. HIV-1 clinical isolates: R5 = 92TH014; MDR = Multidrug resistant virus 7324-1.

The succinate spacer was less optimal than the acetate group for linking nucleosides and cellulose derivatives.

AZT–CSA (**3**) and FLT–CSA (**4**) conjugates were further evaluated for their anti-HIV activity against wild-type (R5) and MDR clinical isolates and the data were compared with controls, CS and nucleosides (Table 3). CS (**1**) was active against MDR virus (IC_{50} = 1.61 μg/ml), but showed less activity against R5 wild-type (WT) HIV (IC_{50} >20 μg/ml). Although FLT was equally potent against these WT and MDR viruses, as expected, AZT was less active against the MDR strain.

On the other hand, AZT–CSA (**3**) showed almost similar anti-HIV activity against R5 and MDR viruses. Furthermore, AZT–CSA (**3**) was more effective than CS against the R5 HIV-1 lab-adapted strain BaL. Conjugation of CSA and nucleosides improved both the R5 inhibitory activity of CS and the activity of AZT against MDR viruses.

AZT–CSA conjugate (**3**) was selected for in vivo contraceptive efficacy testing in rabbits. Ex vivo mixing of CS and **3** prior to insemination of fertile female rabbits prevented pregnancy in 100% of the animals (Table 4). CS has been shown to display contraceptive properties and this result indicates that the contraceptive properties of CS are retained after AZT conjugation to the polymer. Thus, nucleoside–CS conjugates may have application as potential anti-HIV and contraceptive agents.

CS conjugates of AZT and FLT with acetate linker (AZT–CSA (**3**) and FLT–CSA (**4**)) and the physical mixture of AZT or FLT with CSA (**8** and **9**) exhibited higher anti-HIV activity than their corresponding conjugates with succinate linker (AZT–succinate–CS (**6**) and FLT–succinate–CS (**7**)), CS, and the physical mixtures of CS + AZT or FLT (**11** and **12**), possibly due to the creation of additional negative charges provided by the carboxylic acid of the acetate group.

Unlike AZT and other nucleosides not tested in this study, the above-described conjugates were equally active against MDR virus and R5 WT HIV, suggesting a synergistic activity between entry and replication inhibitory mechanisms afforded by CS and nucleoside derivatives, respectively. A similar type of synergistic activity,

Table 4
Contraceptive efficacy of AZT–CSA conjugate **3**

Group	Concentration (mg/ml)	No. of pregnant females/total	Pregnancy rate (%)
Medium control	0	4/4	100
1	1	0/5	0
3	1	0/5	0

Female rabbits were inseminated with pooled rabbit semen containing 1 mg/ml of test compound or medium control.

albeit mechanistically different, was reported by Gantlett et al.[28], when physically combining two other polyanions, PRO2000 and dextran sulfate, with other entry inhibitors such as the neutralizing antibody IgG1b12, the peptide-based fusion inhibitor T20, the CCR5 antagonist TAK779 and the cyanobacterial protein cyanovirin-N.

These data demonstrate the feasibility of conjugating potent anti-HIV nucleosides with polymeric cellulose derivatives to produce compounds that display antimicrobial and contraceptive properties. In addition to enhanced antiviral activity against R5 and multidrug resistant viruses, the CSA–nucleoside combinations showed in vivo contraceptive activity, laying the foundation for the development of novel, dual-protection products.

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Supplementary data

Supplementary data (including experimental procedures and characterization of compounds, anti-HIV and cytotoxicity assays, and rabbit contraceptive efficacy study) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.133.

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