Improvement of Hepatocyte-Specific Gene Expression by a Targeted Colchicine Prodrug

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Colchicine, an established tubulin inhibitor, interferes with the trafficking of endocytotic vesicles and thereby promotes the escape of lysosome-entrapped compounds. To improve its potency and cell specificity, a targeted prodrug of colchicine was synthesized by conjugation to a high-affinity ligand (di-N_α,N_ε-(5-(2-acetamido-2-deoxy-β-D-galactopyranosyloxy)pentanomido)lysine, K(GalNAc)$_2$) for the asialoglycoprotein receptor on parenchymal liver cells. The resulting colchicine – K(GalNAc)$_2$ conjugate bound to this receptor with an affinity of 4.5 nM. Confocal microscopy studies confirmed rapid uptake and receptor dependency of a prodrug conjugated with fluorescein isothiocyanate. Colchicine – K(GalNAc)$_2$ substantially increased the transfection efficiency of polyplexed DNA in parenchymal liver cells in a concentration- and receptor-dependent fashion. Colchicine – K(GalNAc)$_2$ was found to enhance the transfection efficiency by 50-fold at 1 nM, whereas the parental colchicine was ineffective. In conclusion, this nontoxic colchicine – K(GalNAc)$_2$ conjugate can be a useful tool to improve the transfection efficiency of hepatic nonviral gene transfer vehicles.

KEYWORDS:

asialoglycoprotein receptor · drug delivery · drug targeting · gene therapy · tubulin

Introduction

Gene therapy requires the development of DNA carriers that can deliver therapeutic genes or drugs to the targeted tissue and, once there, allow the therapeutic agents to act efficiently. Although viral vectors are generally very efficient, nonviral gene delivery systems offer clear advantages in that they are less toxic, less immunogenic, and easy to prepare. A major limitation of nonviral vectors, however, involves their suboptimal extra- and intracellular handling, which results in a poor efficacy of gene expression in the target tissue in vivo.[1] As shown by various research groups, the former can be effectively tackled by directed gene delivery to specific receptors on the target cell.[1–4] In an attempt to further improve the transfection capacity of nonviral vectors, strategies have been developed to facilitate the intracellular transport of the DNA from the lysosomal compartment to the nucleus.[5, 6] In particular, fusogenic peptides have been the subject of thorough study in this regard and they were shown to be very effective in improving intracellular DNA trafficking.[7–11]

In this study we have explored the potential of the tubulin inhibitor colchicine to improve gene delivery. Colchicine is an established antiinflammatory drug, which is used in the treatment of gout.[2] Disruption of microtubule formation leads to mitotic arrest and interferes with the intracellular trafficking of endocytotic vesicles.[2] These properties render colchicine valuable in mechanistic studies of endocytosis, as well as in therapeutic settings where it improves the lysosomal escape of drugs or DNA.[2] Chowdhury et al.[12] argued that transient pharmacological disruption of microtubules may enhance the persistence of internalized DNA and, thus, transgene expression in the target cell. Nevertheless, application of colchicine is hampered by its systemic toxicity.[13] This has prompted the design of less-toxic colchicine derivatives, of which colchicinamide, bearing an amino instead of a methoxy group at the C10 position, is considered to be one of the most promising.[14]

Here, we have pursued a different strategy to prevent the systemic side effects of colchicine and to enhance the tissue specificity of tubulin inhibition. We have synthesized a targeted

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colchicine derivative that was equipped at the C10 position with a ligand for the asialoglycoprotein receptor (ASGPr) on parenchymal liver cells. As a ligand we used di-N_α,N_ε-(5-(2-acetamido-2-deoxy-β-D-galactopyranosyloxy)pentanomido)lysine (K(GalNAc)$_2$),[5, 15, 16] which has previously been shown to be effective in redirecting (oligo)nucleotides to this receptor.[3, 4, 17, 18] K(GalNAc)$_2$ was conjugated to colchicine through a disulfide bond, which can be cleaved in the lysosomal compartment to release the parental colchicine. Reduction of disulfides in the lysosomes was reported by Arunachalam et al.[19] to be mediated by a lysosomal thiol reductase. Another advantage of the disulfide connection is its versatility: besides colchicine, other compounds can easily be conjugated to the K(GalNAc)$_2$ part. Here, it was shown that the glycoconjugated colchicine displays a high affinity for the ASGPr and strongly improved polyplex-based gene expression to parenchymal liver cells in an ASGPr-dependent fashion.

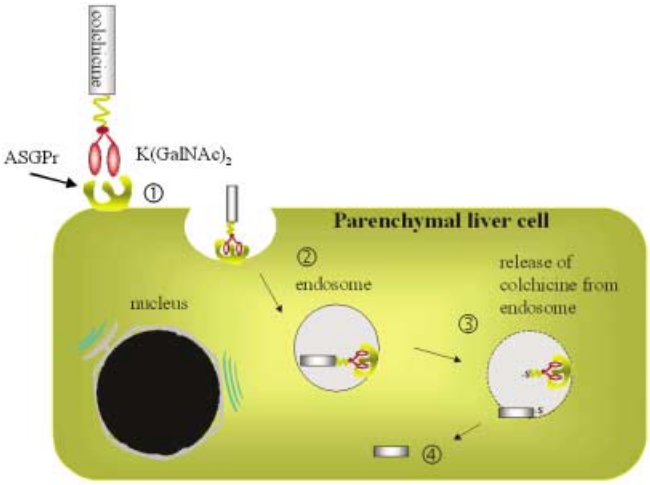


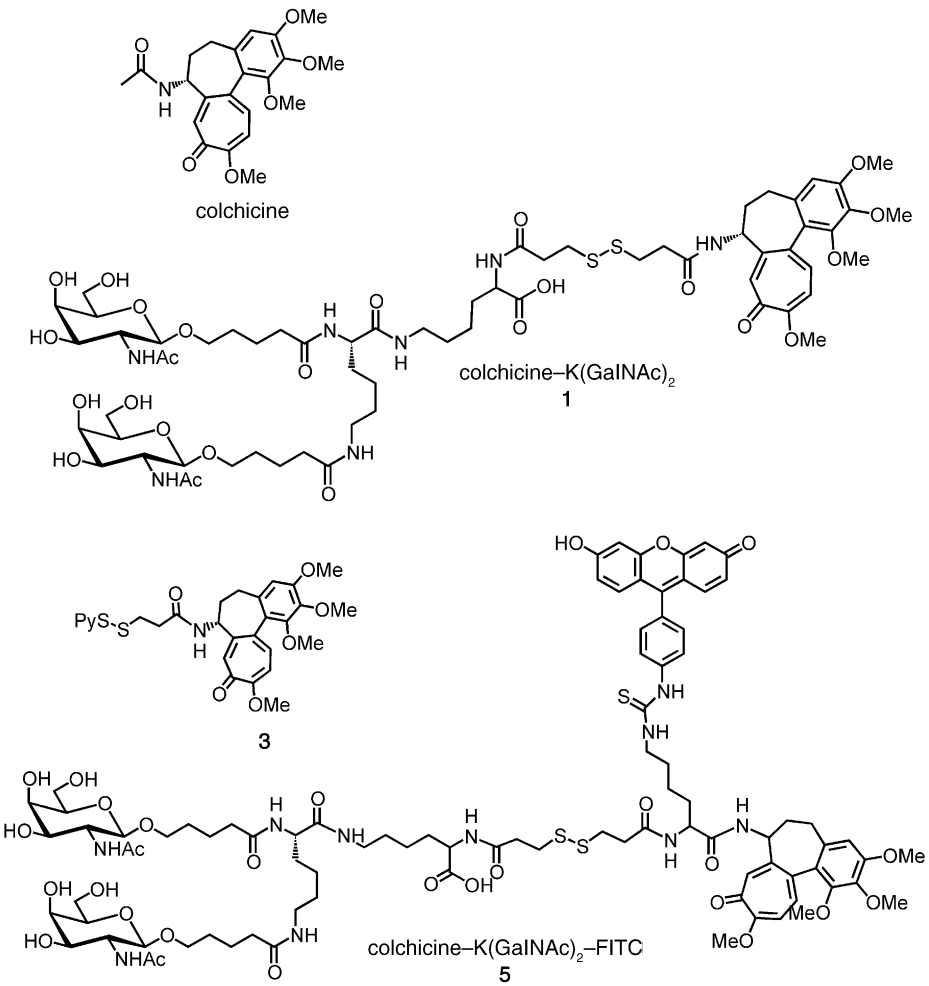
Figure 1. Concept of targeting colchicine – K(GalNAc)$_2$ to the asialoglycoprotein receptor (ASGPr). The (GalNAc)$_2$ unit of the colchicine prodrug is recognized by the ASGPr on parenchymal liver cells (①).After recognition by the ASGPr the colchicine prodrugs are taken up through receptor-mediated endocytosis (②). In the endosome, the disulfide bond of the colchicine – K(GalNAc)$_2$ conjugate will be cleaved by lysosomal thiol reductases, and the parental colchicine will be released from the glycoconjugate (③), to exert its action by inhibiting tubulin activity (④).

Results and Discussion

Increasing the intracellular transgene persistence by disruption of microtubules is an effective strategy to improve nonviral gene therapy protocols.[12]. In this study we have used an established microtubulin inhibitor, colchicine, for this purpose in order to increase the transfection yield of polyplexed DNA. To improve transgene expression in a cell-specific manner and to avoid ectopic toxicity, we have synthesized a glycoconjugated prodrug that can be targeted to the ASGPr on parenchymal liver cells. Hereto, colchicine was coupled to K(GalNAc)$_2$, a high-affinity ligand, which was previously shown to be effective in redirecting drugs and oligonucleotides to this receptor.[17] The presumed action of the colchicine – K(GalNAc)$_2$ conjugate is schematically depicted in Figure 1.

Synthesis

We initially set out to prepare the colchicine-conjugated glycoside—in which the individual components are connected by a disulfide bridge—by a solution-phase approach. This approach proved unsuccessful, however, it guided us in the design of target compound **1** (Scheme 1). Retrosynthetic analysis indicated that conjugate **1** was accessible through formation of a disulfide bond between a suitably



*Scheme 1. Chemical structures of colchicine, the starting compound **3** for synthesis of the prodrug, colchicine – K(GalNAc)$_2$, and colchicine – K(GalNAc)$_2$ – FITC. Py = pyridyl.*

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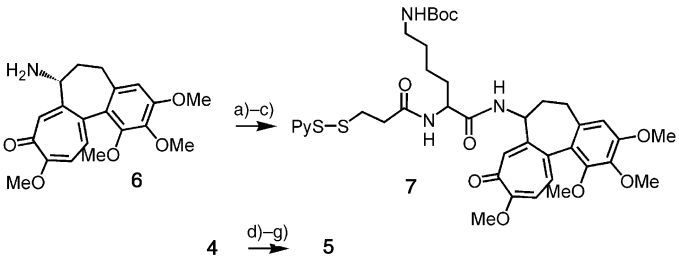
protected, sulfhydryl-activated cluster galactoside and a sulfhydryl-functionalized colchicine derivative. Previous work from our laboratory described the successful solid-phase synthesis of multivalent 2-deoxy-2-acetylaminogalactosides clustered to a lysine core.[16] The incorporation of a lysine core in this case provided a handle for on-resin attachment of the cluster galactoside as well as the colchicine moiety. The requisite resin **2**, functionalized with a divalent lysine-based cluster galactoside (Scheme 2), was available,[9] and colchicine derivative **3** containing an activated sulfhydryl group (Scheme 1) was prepared following known procedures.[2]

For the subsequent assembly of target conjugate **1** by disulfide formation, resin **2** was extended with a sulfide-containing spacer arm to give resin **4** (Scheme 2). The Fmoc group was removed from resin **2**, a sulfide-containing spacer was added, and the immobilized thiopyridyl functional group obtained was reduced to provide the desired resin **4**. Disulfide formation was then immediately performed by addition of a tenfold excess of colchicine derivative **3** to resin **4**. The on-resin conjugate obtained was cleaved from the resin and this resulted in concomitant deprotection of the sugar residues. The nearly pure product **1** was further purified by HPLC and was characterized by NMR spectroscopy, LC/MS, and MALDI-TOF spectrometry.

The preparation of labeled cluster **5** (Scheme 1) is very similar to the synthesis described above. Activated sulfhydryl compound **7** (Scheme 3) was used in the conjugation reaction with resin **4**, as an alternative to the colchicine derivative **3** that had been used in the preparation of unlabeled conjugate **1**. Fmoc/Boc-protected lysine was coupled to the deacetylated colchicine derivative **6**, and the subsequent removal of the Fmoc protecting group allowed the introduction of a sulfhydryl spacer arm to give compound **7**. Subsequent to the disulfide-bond formation between immobilized cluster **4** and colchicine derivative **7**, removal of the Boc group enabled the addition of FITC to the resulting free N^{ε} amine moiety. Application of the cleavage conditions as described in the preparation of **1** gave the desired conjugate **5** (characterized by LC/MS). The product was purified by HPLC.



*Scheme 3. Preparation of **5**. a) FmocLys(Boc)OH, HATU, DIPEA; b) piperidine; c) SPDP, DMAP, CH_2Cl_2; d) **7**, MeOH, CH_3CN; e) TFA, CH_2Cl_2 (1:1); f) FITC, DMF, DIPEA; g) 0.4 M NaOH, 4 °C. Boc = tert-butoxycarbonyl, DIPEA = diisopropylethylamine, DMF = N,N-dimethylformamide, FITC = fluorescein isothiocyanate, HATU = N-[(dimethylamino)-1H-1,2,3-triazole-[4,5-b]-pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate, TFA = trifluoracetic acid.*

In vitro binding studies

The affinity of colchicine – $K(GalNAc)_2$ for the ASGPr was monitored by an in vitro competition assay of ^{125}I-Asialoorosomucoid (^{125}I-ASOR) total binding to mouse parenchymal liver cells. As can be concluded from Figure 2, colchicine – $K(GalNAc)_2$

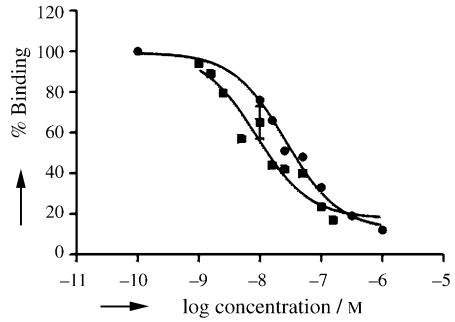
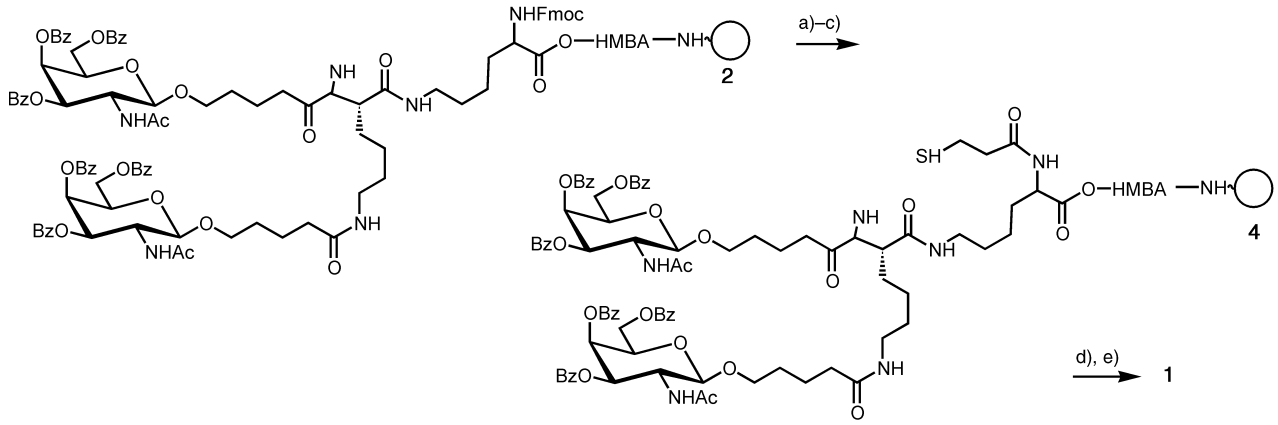


Figure 2. Competition of ^{125}I-ASOR binding to mouse parenchymal liver cells by colchicine – $K(GalNAc)_2$ (■) or $K(GalNAc)_2$ (●). Parenchymal liver cells (1×10^6) were incubated for 2 h at 4 °C with 5.5 nM ^{125}I-ASOR in the presence or absence of colchicine – $K(GalNAc)_2$ or $K(GalNAc)_2$ at nine concentrations ranging from 1 nM to 5 μM. Displacement binding data were analyzed according to a single site model by using a computerized nonlinear-fitting program (graphpad Prism 3.0). The affinity of colchicine – $K(GalNAc)_2$ was calculated to be 4.5 ± 2.2 nM.



*Scheme 2. Assembly of conjugate **2**. a) Piperidine; b) SPDP, DMAP, CH_2Cl_2; c) DTT, Et_3N, MeOH; d)**3**, MeOH, CH_3CN; e) 0.4 M NaOH, 4 °C. Bz = benzyl, DMAP = 4-dimethylaminopyridine, DTT = dithiothreitol, Fmoc = 9-fluorenylmethoxycarbonyl, HMBA = 4-hydroxymethylbenzoic acid, SPDP = O-succinimidyl ester of S-pyridyl-protected mercaptopropionic acid.*

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gave complete and monophasic inhibition of ^{125}I-ASOR binding with an inhibition constant (K_i) of 4.5 ± 2.2 nM, which is considerably lower than that of K(GalNAc)$_2$ (K_i = 30 nM[20]).

Localization of colchicine – K(GalNAc)$_2$ – FITC

Upon internalization of polyplexed DNA by hepatocytes the bulk of the DNA will be degraded within four hours. Therefore any intervention aimed at increasing the persistence of the internalized DNA should be effected within the first few hours after DNA administration. To allow us to monitor the colchicine prodrug after uptake, it was conjugated to FITC and incubated with parenchymal liver cells. Conjugation of FITC to compound **1** results in compound **5** (Scheme 1). Although it may influence the intracellular distribution pattern of the colchicine compound, the FITC group is located on the colchicine unit of the conjugate and will enable us to monitor the intracellular handling of the colchicine unit after uptake. Colchicine – K(GalNAc)$_2$ – FITC conjugate **5** was rapidly taken up and within ten minutes it was found to be evenly distributed over the parenchymal liver cell (Figure 3 a and b). The conjugate mainly accumulated in the cytoplasm, but after longer incubation times (> 30 min) some nuclear localization could also be observed (Figure 3 c). No uptake of the FITC-labeled conjugate was observed at 4 °C (Figure 3 e), which suggests that uptake is mediated through an active transport process. The presence of functional receptors on the parenchymal liver cells was confirmed by incubation with tetramethylrhodamine-5-isothiocynate – asialoorosomucoid (TRITC-ASOR; Figure 3 f).

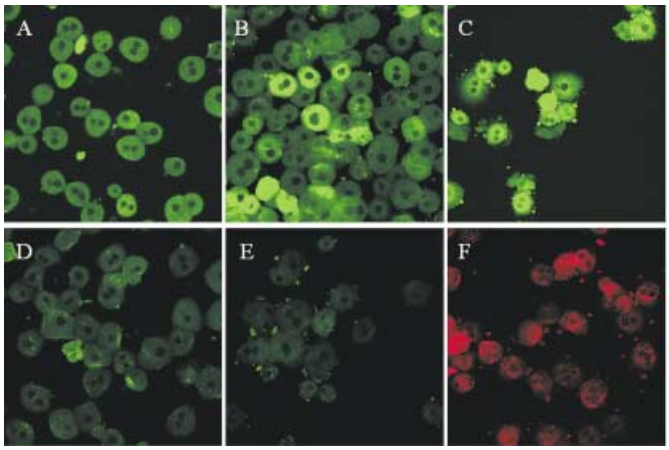

Figure 3. Confocal laser scanning microscopy of cellular uptake of colchicine – K(GalNAc)$_2$ – FITC by mouse parenchymal liver cells. Cells (2 × 10^5) were incubated at 37 °C with 100 nM colchicine – K(GalNAc)$_2$ – FITC for A) 5 min, B) 10 min, and C) 30 min. D) Untreated parenchymal cells or E) parenchymal cells that were incubated for 10 min at 4 °C in the presence of 100 nM colchicine – K(GalNAc)$_2$ – FITC were taken as the controls. F) Parenchymal liver cells incubated with 40 nM TRITC-ASOR for 1 h at 37 °C were taken as a reference for ASGPr-mediated uptake.

Effect of colchicine – K(GalNAc)$_2$ on polyplex-mediated gene transfer to mouse parenchymal liver cells

We have evaluated the effect of colchicine – K(GalNAc)$_2$ on gene transfer to mouse parenchymal liver cells by using an established nonviral vector based on the cationic oligopeptide KWK$_8$AKY (K8).[6] DNA polyplexed with these small-sized synthetic oligocations may be more fit for systemic applications, as the derived condensates are generally smaller and less immunogenic than polymeric condensed DNA.[21, 22] Moreover, the K8-derived polyplexes do not activate the complementary system, are non-aggregating, and are readily unpacked intracellularly.[21, 22] We assessed whether the colchicine glycoconjugate was able to promote the release of lysosomally entrapped DNA to the cytosol and, thus, to enhance the transfection level. Mouse parenchymal liver cells were transfected with plasmid DNA (pCMVluc) polyplexed with K8 (N:P ratio of 4:1) in the absence or presence of colchicine or colchicine – K(GalNAc)$_2$. Preincubation of polyplexed DNA with colchicine did increase the transfection efficiency in a dose-dependent manner up to 100-fold (Figure 4).

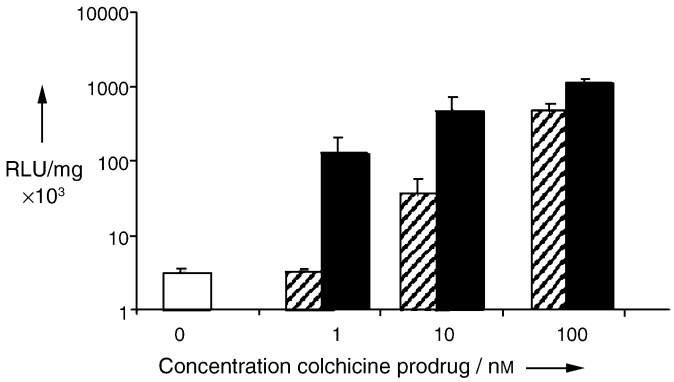

Figure 4. Effect of colchicine or colchicine – K(GalNac)$_2$ on nonviral gene transfer to mouse parenchymal liver cells. Plasmid DNA (pCMVLuc; 1 μg well^{-1}) was condensed in HEPES-buffered saline (HBS) with K8 peptide (N:P ratio of 4:1), and the polyplexes were incubated in the absence (empty bar) or presence of colchicine (hatched bar) or colchicine – K(GalNAc)$_2$ (solid bar) at various concentrations. After 48 h, cells were lysed and the luciferase activity was determined and corrected for protein content. Data given are the mean values ± the standard deviation of nine results.

Colchicine – K(GalNAc)$_2$ displayed an even higher capacity to enhance the transfection efficiency, especially at concentrations below 100 nM. While colchicine did not affect the transfection yield at 1 nM, the glycoconjugated colchicine was found to improve the transfection efficiency by 50-fold. The stimulatory effect of the colchicine glycoconjugate leveled off at 100 nM (the highest concentration tested), while that of colchicine itself steadily increased over the whole concentration range (1 – 100 nM). The curvilinear dose – response relationship of the glycoconjugate concurred with its binding profile to the ASGPr (K_i = 4.5 nM). Colchicine and its glycoconjugate increased the transfection efficiency in a similar manner when the parenchymal cells were coincubated with the polyplexes and the lysosome disruptive element (LDE; data not shown). Previous studies by Wagner and co-workers[23, 24] and Van Rossenberg et al.[9] demonstrate that (targeted) fusogenic peptides are also very effective in enhancing the transfection efficiency up to 30-fold. Compared to the (targeted) fusogenic peptides, the capacity of the colchicine prodrug to enhance the transfection efficiency was found to be higher.

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Receptor-specific recognition of colchicine – K(GalNAc)$_2$

To establish that colchicine – K(GalNAc)$_2$ promoted the transfection efficacy of DNA/K8 polyplexes in an ASGPr-specific fashion, we first studied the transfection efficiency in the presence of excess *N*-acetyl galactosamine (GalNAc), which blocks ASGPr-mediated uptake. GalNAc did not influence the transfection efficacy of polyplexes that were preincubated with 10 nM colchicine, whereas it markedly reduced that of polyplexes preincubated with colchicine – K(GalNAc)$_2$ (Figure 5 a).

Secondly, the colchicine – K(GalNAc)$_2$ conjugate was not able to stimulate gene transfer to BHK cells, which do not express the ASGPr, whereas colchicine was found to improve the transfection efficiency of the K8 polyplexes in these cells (Figure 5 b). Apparently, colchicine – K(GalNAc)$_2$ must be taken up by the ASGPr to exert its activity and, thus, will only enhance gene delivery in ASGPr-expressing cells. Colchicine is equally potent in the presence of GalNAc and its activity does not require the functional ASGPr, because, as already suggested above, it utilizes a different route of entry. Colchicine may act in a stimulatory manner by facilitating polyplex/plasma membrane fusion, since it is known to interfere with membrane fusion.[13]

Microtubule-disrupting agents were reported to interfere with internalization and recycling of the ASGPr at concentrations of 0.1 – 3.0 μM, which in turn may lead to reduced endocytosis.[12, 25] In this study we show that colchicine already improves the transfection efficiency well below this concentration range (0.01 μM), while the glycoconjugate was found to be effective at concentrations as low as 0.001 μM. This implies that the glycoconjugate would probably be suitable in a dual-step transfection protocol involving preadministration of the glycoconjugate followed by that of the actual (gene) medicine.

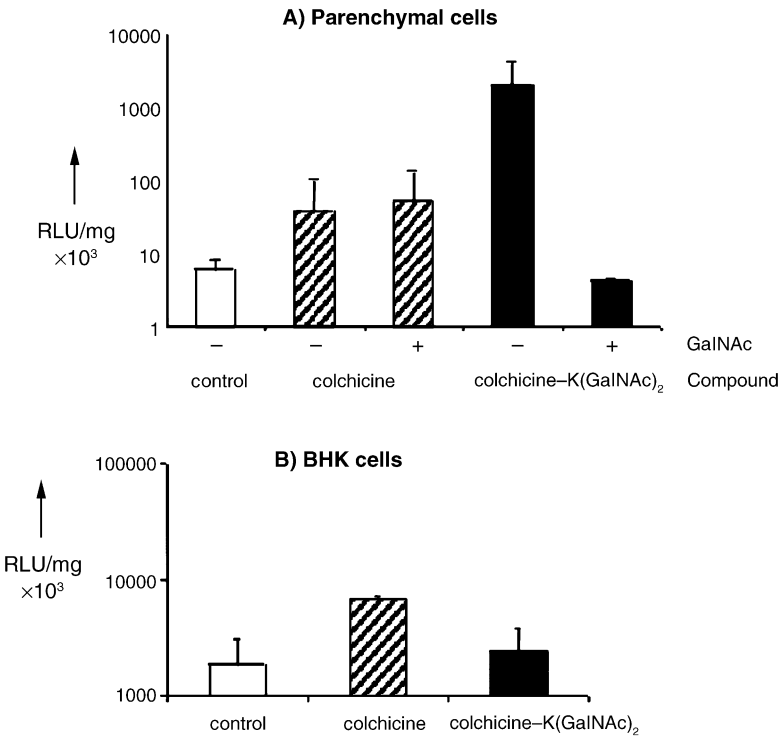

Figure 5. *Effect of colchicine or colchicine – K(GalNac)$_2$ on nonviral gene transfer to mouse parenchymal liver cells. Plasmid DNA (pCMVluc; 1 μg well^{-1}) was condensed in HBS with K8 peptide (N:P ratio of 4:1). A) Parenchymal liver cells were incubated with the polyplexes in the absence (empty bar) or presence of 10 nM colchicine (hatched bar) or 10 nM colchicine – K(GalNAc)$_2$ (solid bar) and with or without 100 mM GalNAc. B) BHK cells were incubated with the polyplexes in the absence (empty bar) or presence of colchicine (hatched bar) or colchicine – K(GalNAc)$_2$ (solid bar) at 100 nM. Data given are the mean values ± the standard deviation of nine results.*

Toxicity

Lysosome leakage and membrane disruption has been reported to promote intracellular release of hydrolytic enzymes including proteases, nucleases, and lipases. Although these enzymes are acid hydrolases, with a catalytic optimum near pH 5.0, leakage of these enzymes into the cytoplasm of the cell may promote apoptosis or necrosis.[26] In addition, prolonged inhibition of tubulin mobility may by itself be toxic.[13] Colchicine and the colchicine glycoconjugate were, however, found to be nontoxic at concentrations up to 100 nM in parenchymal liver cells, as tested by the *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MTT) test (data not shown). Initial toxicity assessment in mice showed that a concentration of 5 μg was well tolerated. It can be anticipated that the doses of colchicine used in this study would probably not be toxic to humans.[13]

In conclusion, we present here the successful synthesis and evaluation of a targeted colchicine prodrug, which was efficiently taken up by ASGPr-expressing cells and appeared to induce rapid lysosomal escape of polyplexed DNA in a receptor-dependent fashion. In addition, with the effective delivery of glycoconjugated colchicine, a second crucial requisite is met towards the application of targeted lysosome disruptive elements. The cell-specific delivery of colchicine to the liver may limit its systemic toxicity. We envisiage that the colchicine – K(GalNAc)$_2$ conjugate will be applicable for improving the therapeutic efficacy of nonviral gene transfer vehicles,[1] of antisense drugs for hepatic genes,[27, 20] and of drugs that accumulate in the lysosomal circuit, by facilitating the escape from the lysosomal pathway.[28]

Experimental Section

Materials: Orosomucoid, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MTT), fluorescein-5-isothiocyanate – dextran (40.000), colchicine, and bovine serum albumin (BSA) were obtained from Sigma Chemicals (St. Louis, MO). Dimethylsulfoxide (DMSO) was obtained from Baker (Deventer, The Netherlands). Percoll was from Fluka (Sigma – Aldrich, St. Louis, MO), and fluorescein-5-isothiocyanate and tetramethylrhodamine-5-isothiocyanate were from Molecular Probes Europe BV (Leiden, The Netherlands). Paraformaldehyde was purchased from Merck (Amsterdam, The Netherlands), and Fluorsave was from Calbiochem-Novabiochem

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Corporation (San Diego, CA, USA). All solvents were of analytical grade. Dry solvents were stored over molecular sieves of 4 Å. Merck Kieselgel 60 F_{254} plates were from Merck (Darmstadt, Germany). Poly(ethyleneglycol) – polystyrene resin was purchased from Perkin-Elmer (Wellesley MA, USA) and Fmoc-protected amino acids were purchased from Nova Biochem (Bad Soden, Germany). Di-N_α,N_ε-(5-(2-acetamido-2-deoxy-β-D-galactopyranosyloxy)pentanomido)lysine ($K(GalNAc)_2$) was synthesized as described by Valentijn et al.[16] and KWK_8AKY (K8) was kindly provided by A. van Keulen (Leiden University, Leiden, The Netherlands).

Analysis: For TLC analysis, compounds were visualized by charring with sulfuric acid/ethanol (1:4, v/v), or by coloring with a solution of ninhydrin (0.3% in acetic acid/*n*-butanol (3:100, v/v)). Column chromatography was performed with Kieselgel 60, 230 – 400 mesh (Merck). 1H NMR spectra (300 MHz) were recorded with a Bruker WM-300 spectrometer. Products were analyzed with a Jasco HPLC system using a LiChrospher 100 RP-18 column (Merck, 5 mm, 4.6 × 250 mm). Electrospray mass spectra were recorded with a Perkin-Elmer SCIEX API 165 Single Quadruple LC/MS instrument. MALDI-TOF mass spectrometry was performed on a Perkin Elmer/PerSeptive Biosystems Voyager-DE-RP MALDI-TOF mass spectrometer.

Synthesis of target conjugate 1: Resin **2** (100 mg, loaded with 0.1 mmol g^{-1} of the divalent galactoside) was deprotected with piperidine (2 mL; 20% in DMF) over 20 min. After several washing steps (3 × DMF, 3 × MeOH, 3 × CH_2Cl_2), *S*-pyridyl-protected mercaptopropionic acid (15 mg; commercially available as the activated *O*-succinimidyl ester SPDP[29]) and an equimolar amount of DMAP were added in a mixture of DMF and CH_2Cl_2 (1:1). The reaction was continued for 3 h and subsequently washed as described above. Next, the resin was taken up in degassed MeOH (2 mL) and DTT (30 mg) was added, followed by 0.03 M triethylamine in MeOH (10 μL). Shaking was continued for 3 h and was followed by extensive washing of the obtained resin **4** with MeOH under an argon atmosphere. Compound **3** (0.1 mmol; prepared by previously described procedures) was then added in a mixture of degassed MeOH and CH_3CN (1:1). Washing of the obtained immobilized conjugate **1** with MeOH was followed by treatment with 0.4 N NaOH solution (1 mL) at 4 °C for 1 h. This procedure yielded conjugate **1**. The crude compound was analyzed by LC/MS (eluting with a 5 – 50% CH_3CN gradient in 0.1% TFA) to give a trace that showed a nearly pure main peak (M_w calcd: 1412; *m/z* found: 1413 $[M+H]^+$ and 1435 $[M+Na]^+$; spectra are included in the Supporting Information). The product was further purified by HPLC and the identity was established by MALDI-TOF MS and NMR spectroscopy: ^{1}H NMR ($CDCl_3$): $\delta = 1.30 - 2.25$ (m, 24 H; 6 × CH_2 Lys, 6 × CH_2 spacer), 1.95 (s, 6 H; CH_3 NHAc), 2.05 – 2.55 (m, 4 H; 2 × CH_2 colchicine), 2.98 – 3.10 (m, 8 H; 4 × CH_2 disulfide spacer), 3.17 (m, 4 H; 2 × CH_2 spacer), 3.45 (m, 4 H; 4 × Hε Lys), 3.65, 3.92, 3.98, 4.15 (s, 12 H; OCH_3 colchicine), 3.81 – 4.56 (m, 15 H; 2 × H1 – 6 GalNAc, Hα Lys), 4.66 (m, 1 H; H7 colchicine), 5.30 (br s, 1 H; NH), 6.29 (br s, 1 H; NH), 6.54 (s, 1 H; H4 colchicine), 6.85 (d, 1 H; H11 colchicine), 6.98 (br s, 1 H; NH), 7.39 (s, 1 H; H8 colchicine), 7.55 (d, 1 H; H12 colchicine), 7.79 (br s, 1 H; NH) ppm; ^{13}C NMR ($CDCl_3$/MeOD): $\delta = 22.7$ (CH_3 NHAc), 22.3, 28.2, 28.5, 29.9, 29.5, 30.6, 32.0, 35.3, 35.7, 35.8, 37.1 (CH_2 spacers, CH_2 Lys, and CH_2 colchicine), 38.5 (Cε Lys), 50.4 (C2 GalNAc), 52.3 (CHNH colchicine), 53.2 (Cα Lys), 56.1, 56.3, 61.4, 61.5 (CH_3 OMe), 60.4, 61.3 (C6 GalNAc), 69.2 (CH_2 spacer), 66.6, 70.3 (C3, C4, C5 GalNAc), 101.0 (C1 GalNac), 107.3, 112.3, 130.3, 135.6 (CH colchicine), 125.5, 134.9, 137.0, 141.5, 150.5, 151.2, 154.4, 163.8, 170.5, 170.6, 171.6, 171.9, 179.4 (C_{arom} colchicine, C(O) amide) ppm; MALDI-TOF MS: *m/z* found: 1435 $[M+Na]^+$ (spectra are included in the Supporting Information).

Synthesis of FITC-labeled colchicine conjugate 5: The preparation of conjugate **5** commenced with coupling of the known deacetylcolchicine **6** (0.25 mmol)[30] and FmocLys(Boc)OH (0.25 mmol) under the influence of HATU (1 equiv) and DIPEA (2 equiv) in CH_2Cl_2 (1 mL). The reaction was terminated after 2 h by pouring the reaction mixture onto water (10 mL). The resulting layers were separated and the organic layer was washed with 1 N $KHSO_4$, 5% $NaHCO_3$, H_2O, and brine. After drying ($MgSO_4$) and concentration in vacuo, the crude oil was purified by column chromatography (EtOAc) and lysine-functionalized colchicine was obtained in 55% yield (LC/MS: M_w calcd: 806; *m/z* found: 807 $[M+H]^+$).

Subsequently formation of compound **7** was accomplished as follows. Removal of the Fmoc group from the colchicine derivative by treatment with piperidine (20% in DMF) was followed by precipitation of the crude deprotected amine from EtOAc/petroleum ether (10:90). Without further purification, the precipitated residue was taken up in a mixture of CH_2Cl_2 (2 mL), SPDP (1 equiv), and DMAP (1 equiv). After 3 h the reaction mixture was concentrated, the residue was taken up in EtOAc, and the organic layer was washed with 5% $NaHCO_3$, H_2O, and brine. After drying ($MgSO_4$) and concentration in vacuo, the crude oil of **7** was purified by column chromatography (EtOAc) and used in the preparation of conjugate **5**.

Resin **4** was treated with compound **7** (instead of compound **3**, which was used for the synthesis of compound **1**) in the above-described procedures. Subsequent removal of the N^ε-Boc group from the obtained resin by addition of a TFA/CH_2Cl_2 (1:1) for 30 min was followed by washing of the resin and addition of FITC (25 equiv) and DIPEA (25 equiv) in DMF (3 mL) to introduce the fluorescent label. The reaction was stopped by washing of the immobilized conjugate (3 × DMF, 3 × MeOH, 3 × CH_2Cl_2), and subsequent treatment with NaOH solution, as described in the preparation of **1**, yielded deprotected **5.** The crude mixture was analyzed by LC/MS (eluting with a 5 – 50% CH_3CN gradient in 0.1% TFA; M_w calcd: 1914; found: 1914 $[M+H]^+$; spectra are included in the Suipporting Information). The conjugate was purified further by HPLC and analyzed with MALDI-TOF spectrometry.

Isolation of mouse parenchymal liver cells: Male C57bl/6 KH mice between 10 and 12 weeks old and weighing 22 – 27 g (Broekman Institute BV, Someren, The Netherlands) were used for parenchymal liver cell isolation. Hepatocytes were isolated from mice by perfusion of the liver with collagenase (type IV; 0.05%, w/v) for 10 min at 37 °C according to the method of Seglen.[31] For transfection studies cells were further purified by a Percoll gradient. Cells were mixed with Percoll in a 1:1 ratio, centrifuged for 10 min at 100 *g*, washed, and resuspended in Dulbecco's modified essential medium (DMEM; Biowhittaker, Verviers, Belgium) containing 10% fetal bovine serum (Biowhittaker). Cells were > 99% pure as judged by light microscopy.

In vitro binding assay: Displacement of ^{125}I-ASOR binding to mouse hepatocytes was determined as follows.[32] Freshly isolated mouse parenchymal liver cells (1×10^6 cells, viability > 90% as determined by 0.2% trypan blue exclusion) were incubated in DMEM containing 2% BSA (0.5 mL) with 5.5 nM ^{125}I-ASOR in the presence or absence of 50 nM-5 μM colchicine – $K(GalNAc)_2$ or $(GalNAc)_2$. After incubation for 2 h at 4 °C under gentle agitation, the medium was removed by aspiration and the cells were washed twice with 0.2% BSA in medium and once with medium lacking BSA. Subsequently cells were counted for radioactivity and assayed for protein content according to the method of Lowry with BSA as a standard. Cell binding was corrected for protein content. Nonspecific binding was measured in the presence of 100 mM GalNAc. Displacement binding data were analyzed according to a single site model by using a computerized nonlinear-fitting program (Prism, graphpad, USA) to calculate the K_i value.

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Confocal laser scan microscopy studies of FITC-labeled colchicine – K(GalNAc)$_2$: Mouse parenchymal liver cells where isolated as described above. Cells were seeded at a density of 2×10^5 per glass cover slip (25 mm diameter, No. 1; Nutacom BV, The Netherlands) in 6-well culture plates. The cells were incubated for 2 h, washed once with DMEM containing 0.2% BSA, and incubated in the presence or absence of 100 nM colchicine – K(GalNAc)$_2$ – FITC and/or 100 mM GalNAc. ASGPr-mediated uptake was measured with TRITC-ASOR. Cells were incubated for 5 min to 1 h at 37 °C (uptake) or 4 °C (binding), subsequently washed three times with DMEM, and fixed in 4% paraformaldehyde for 4 h at 4 °C. Glass cover slips were covered with Fluorsave. Binding and uptake of colchicine – K(GalNAc)$_2$ – FITC by parenchymal cells was detected by confocal laser scan microscopy on a Leica TCS SP spectral confocal microscope.

Transfection of mouse parenchymal cells: Plasmid pCMV-luc, containing the firefly luciferase complementary DNA insert, was kindly provided by Crucell BV (Leiden, The Netherlands). The DNA/K8 polyplexes (N:P charge ratio 4:1; 1 μg of DNA per well in HBS buffer) were prepared as described by Gottschalk et al.[6] Mouse parenchymal liver cells were transfected 3 h after seeding (2×10^5 cells well^{-1}) with K8-condensed DNA. After incubation of the K8/DNA complexes at room temperature for 30 min, colchicine or colchicine – K(GalNAc)$_2$ was added to a final concentration of 1 – 100 nM. After an additional incubation for 30 min at room temperature, the complexes were added to the parenchymal cells in DMEM containing 0.2% BSA (250 μL). After 4 h, further DMEM containing 2% BSA (1 mL) was added, and the cells were incubated for another 44 h. After harvesting of the cells, cell lysates were analyzed for luciferase activity as previously described.[33] Transfection yields were corrected for protein content (as monitored by the bicinchinonic acid(BCA) assay (Pierce, Rockford IL, USA) with BSA as the reference). In analogy, BHK cells (seeded at 2×10^6 cells well^{-1}) were transfected with preformed K8/DNA condensates in the absence or presence of colchicine or the glycoconjugated colchicine.

MTT cytotoxicity test: Parenchymal liver cells were transfected with the DNA/K8 polyplexes as described above. After 48-hour incubation the medium was replaced by fresh medium and MTT was added to a final concentration of 0.5 mg mL^{-1}. Cells were incubated for 30 min at 37 °C, medium was removed, and DMSO was added to the cells. The extinction of the supernatant was measured at 550 nm.

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