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Rostislav A. Petrov^a, Svetlana Yu. Maklakova^a, Yan A. Ivanenkov^{a-c,g,*}, Stanislav A. Petrov^a, Olga V. Sergeeva^{a,d}, Emil Yu. Yamansarov^a, Irina V. Saltykova^a, Igor I. Kireev^f, Irina B. Alieva^f, Ekaterina V. Deyneka^b, Alina A. Sofronova^h, Anastasiia V. Aladinskaia^b, Alexandre V. Trofimenko^b, Renat S. Yamidanov^g, Sergey V. Kovalev^a, Victor E. Kotelianski^d, Timofey S. Zatsepin^{a,d}, Elena K. Beloglazkina^a, Alexander G. Majouga^{a,c,e}.

^a Lomonosov Moscow State University, Chemistry Dept, Leninskie gory, Building 1/3, GSP-1, Moscow, 119991, Russian Federation.

- ^b Moscow Institute of Physics and Technology (State University), 9 Institutskiy lane, Dolgoprudny City, Moscow Region, 141700, Russian Federation.
- ^c National University of Science and Technology MISiS, 9 Leninskiy pr, Moscow, 119049, Russian Federation.

^d Skolkovo Institute of Science and Technology, 100 Novaya st., 143025 Skolkovo, Russian Federation.

^e Dmitry Mendeleev University of Chemical Technology of Russia, Miusskaya sq. 9, Moscow, 125047, Russian Federation.

^f Lomonosov Moscow State University, A.N. Belozersky Institute of Physico-Chemical Biology, Leninskye gory, house 1, building 40, Moscow, 119992, Russian Federation

⁸ Institute of Biochemistry and Genetics Ufa Science Centre Russian Academy of Sciences (IBG RAS), Prosp. Oktybrya 71, Ufa, Bashkortostan, 450054, Russian Federation.

^h Lomonosov Moscow State University, Faculty of Bioengineering and Bioinformatics, Moscow, Russia.

* corresponding author, e-mail: yai@chemdiv.com

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Asialoglycoprotein receptor (ASGP-R) is a promising biological target for drug delivery into hepatoma cells. Nevertheless, there are only few examples of small-molecule conjugates of ASGP-R selective ligand equipped by a therapeutic agent for the treatment of hepatocellular carcinoma (HCC). In the present work, we describe a convenient and versatile synthetic approach to novel mono- and multivalent drug-conjugates containing *N*-acetyl-2-deoxy-2-aminogalactopyranose and anticancer drug – paclitaxel (PTX). Several molecules have demonstrated high affinity towards ASGP-R and good stability under physiological conditions, significant *in vitro* anticancer activity comparable to PTX, as well as good internalization via ASGP-R-mediated endocytosis. Therefore, the conjugates with the highest potency can be regarded as a promising therapeutic option against HCC.

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Cancer is the second leading cause of death. In 2015, it was responsible for 8.8 million deaths worldwide. Generally, about 1 in 6 deaths is due to cancer. Hepatocellular carcinoma (HCC, liver cancer) is the most common type of primary liver cancer and accounts for 90% of all liver cancers (~788,000 deaths are associated with HCC¹). Many therapeutic options including small-molecule drugs are available to date. However, they have a number of disadvantages such as low selectivity and high toxicity rate with a wide range of adverse side effects. Targeted drug delivery (TDD) can be reasonably regarded as a promising route for the modification of medicinal agents, which allows scientists to improve the pharmacological profile of anticancer drugs, especially highly cytotoxic molecules. Using this approach, therapeutics can be selectively localized (or targeted) in the desired tissue, organ or cell, to improve their therapeutic index and efficiency by increasing its active concentration and reducing the total amount within the organism².

Asialglycoprotein receptor (ASGP-R, C-type lectin family) is one of the most attractive targets for the TDD of medications in liver cells³. This is due to several key reasons: 1) an outstanding receptor selectivity toward galactose derivatives, 2) elevated expression level in hepatocytes and predominant localization on the surface, 3) a relatively high exposition (over 500K receptors per hepatocyte), and 4) receptor internalization into the cells via clathrin-mediated endocytosis (turnover approx. 15-20 min with or without ligands)⁴⁵. Several comprehensive reviews describing the architecture and functions of ASGP-R as well as ligand properties and selectivity have recently been described^{3,6-9}. The receptor recognizes hydroxyl groups in 3rd and 4th positions of galactose. The outer part of the receptor consists of three subunits: 2×H1 (46 kDa) and H2 (50 kDa), each of them is able

to selectively bind galactose. The core structure of the subunits includes a 40-amino-acid N-terminal cytoplasmic domain, a ~20amino-acid single-pass transmembrane domain, an ~80-aminoacid extracellular stalk region, and an ~140-amino-acid functional calcium-dependent carbohydrate recognition domain (CRD, C-type Ca2+-dependent superfamily) anchored via intervening neck region. The monosaccharide ligands, e.g. Dmannose, D-glucose, D-galactose and their derivatives, interact with CRDs by direct calcium coordination. The trimeric ensemble of the ASGP-R extracellular subunits is the most abundant configuration observed among mammalian species and demonstrates the highest binding affinity to endogenous ligand asialoorosomucoid (ASOR), whereas single CRD exhibits low binding affinity. Therefore, it is not surprising that in contrast to monovalent ligands, bivalent and especially trivalent analogues show dramatically enhanced binding potency toward ASGP-R¹⁰. However, several bivalent ligands with a comparatively high target affinity have been reported as well^{11–14}. The variation in the number and structure of the galactose fragments may increase the binding affinity and in vitro activity, which has been the subject of many studies. However, in accordance with the results by Prakash et al.,¹⁵ the number of galactose moieties did not have a significant effect on activity in vivo.

There are many examples of ASGP-R-targeted conjugates (including functionalized nanoparticles) equipped by siRNA or different small-molecule drugs targeted against several liverrelated disorders¹⁶. For example, PK2 by Pfizer was evaluated in Phase 1/2 clinical trial in patients with primary or metastatic liver cancer¹⁷. Doxorubicin (Dox) was attached via a lysosomally degradable tetrapeptide sequence N-(2to hydroxypropyl)methacrylamide copolymers bearing the GalNAc core-head. As a result, it was clearly demonstrated that the liverspecific Dox delivery was effective, and a dose schedule was recommended for Phase 2 studies with subjects suffering from primary hepatocellular tumors. However, no additional information has been published for this trial. Alnylam Pharmaceuticals is the leading company within the title field¹⁸. Several clinical trials have been proceeded, for instance against TTR-related amyloidosis^{19–24}, haemophilia $A/B^{25,26}$, hypercholesterolemia²⁷, HBV²⁸, primary hyperoxaluria type I²⁹, etc. Recently, the company has initiated Phase 2 clinical trial (ORION 30) and Phase 1 study 27 with Inclisiran (ALN-PCSSC) against elevated low density lipoprotein cholesterol (LDL-C) and subjects diagnosed with atherosclerotic cardiovascular disease (ASCVD) or ASCVD-risk equivalents (e.g., diabetes and familial hypercholesterolemia). ALN-GO1 has been entered in Phase 1/2 clinical investigation in healthy adult subjects and patients with primary hyperoxaluria Type 1 (PH1)²⁹. Amgen has developed AMG-529, an ASGP-R antagonist, which is currently ongoing Phase I clinical evaluation for the treatment of cardiovascular diseases³¹. However, there are no ASGP-R-targeted drug conjugates in clinics containing Dox or PTX attached to the GalNAc warhead through a simple linker. This is the first report describing a direct conjugation of PTX with the ASGP-R-specific ligand via a relatively short and spacer of an appropriate length.

The [3+2] azide-alkyne cycloaddition is a convenient and simple method for laboratory synthesis which does not affect other functional groups of reactants. In addition, the resulting triazole cycle can serve as a bioisosteric replacement for peptide bond. Therefore, we introduced azido group in the galactose moiety and alkyne fragment in PTX for their subsequent conjugation. This arrangement was based on the fact that galactose fragment linked via position 4 by triazole fragment has lower affinity as compared with position 1^{32} .

As a promising galactose moiety, we have selected ligand **3** (Fig. 1), previously described by Mamidyala³², Sanhueza³³ and co-authors, that has demonstrated high binding affinity towards the receptor. In addition, warheads **1** and **2** were proposed as alternative cores in order to assess the effect of allyl group and attachment point on binding.



Fig. 1. GalNAc core-heads used in this study.

Initially, the selected core-heads have been synthesized by analogy to the approaches described previously³⁴⁻³⁶. Thus, GalNAc building block **1**, containing the azide attachment point at C1 position, was prepared in full accordance with the synthetic route published by Salunke and co-workers³⁴, while sugars **2** and **3** were synthesized following a slightly modified procedure (Scheme 1).



Scheme 1. Synthesis of building blocks 2 and 3. (i) Ac₂O/Py, rt, 2h; (ii) TMSOTf/DCE, 50°C, 12h; (iii) TMSOTf, AlOH (4.5 eq.)/DCE, rt, 48h; (iv) 1M MeONa/MeOH, rt, 3h; (v) TsCl/Py, 0°C, 1h, then stirring at rt, 12h; (vi) NaN₃/DMF, 50°C, 6h; (vii) 2-azidoethanol, TMSOTf/DCE, rt, 48h; (viii) 1M MeONa/MeOH, rt, 3h.

Thus, the initial cyclic D-galactosamine **4** was treated with Ac_2O in pyridine to furnish fully acetylated derivative **5** in good yield (89%). The obtained intermediate was then readily converted into the oxazoline derivative **6** upon the treatment with TMSOTf at 50°C (yield 92%). The subsequent ring opening in the presence of allyl alcohol or 2-azidoethanol proceeded smoothly and yielded compound **7** or **10**, respectively. Alkaline alkoholysis of the synthesized intermediates using MeONa provided O-deacetylated compound **2** equipped by the extended azide attachment point at position C1 or intermediate **8**. The latter was then easily converted into compound **9** upon the treatment with TsCl in Py (yield 63%). The desired product **3**, containing the attachment point at position C6, was obtained from compound **9** through the reaction with NaN₃ in DMF.

PTX can be modified at positions C2', C7 and C1. The functionalization of C1-OH reduces the activity, but not significantly, while the modification of C7-OH does not influence the binding potency. The introduction of substituents at position C2'-OH, e.g. fluorine or alkyl, is unfavorable, while esterification results in loss of microtubule disassembly activity *in vitro*, but not cytotoxicity³⁷. It has been reported that PTX esters are instable under the conditions of cellular endocytosis and susceptible to esterase-mediated hydrolysis³⁸. Acylation of C2'-OH proceeds readily and rapidly as compared to acylation of C7-OH and especially C1-OH.

The general synthetic approach to the desired TDD system is depicted in Scheme 2. PTX was modified at the most convenient C2' and C7 diversity points. It should be noted that in PTX structure the most reactive hydroxyl group is attached to C2' atom, while -OH moiety at position C7 is much less pliable. This allowed us to carry out the selective modification of C2'-OH group keeping position C7 unsubstituted or obtain C2', C7disubstituted analogues. We have synthesized six novel conjugates of the molecule with selective ASGP-R ligands described above. Thus, the initial acylation of PTX was performed with 5-hexynoic acid in CH₂Cl₂ in the presence of EDC and DMAP following the procedure described by Pilkington-Miksa and colleague³⁹. As a result, monovalent (C2' modification) and bivalent (C2' and C7 attachment) precursors 11 and 12 were obtained in good yields. Monovalent building block was isolated using 1 eq. of the acid, while bivalent analogue was synthesized in the presence of 2-fold excess (2 eq.) of the reactant. Purification was performed using a routine column chromatography in CH₂Cl₂:MeOH (40:1).



Scheme 2. Synthesis of monovalent (13a-c) and bivalent (14a-c) ASGP-R-targeted conjugates bearing PTX

During the next step, intermediates 1-3 were introduced into the click reaction ([3+2]-azide-alkyne cycloaddition) with building block 11 or 12 using CuI and Et₃N (0.2 and 0.4 eq., respectively). The desired novel conjugates 13a-c and 14a-c were obtained in moderate to good yields without any complications. The final products were then purified using reversed-phase HPLC (H₂O/MeCN). The structures of the synthesized compounds are in excellent agreement with the obtained ¹H



Scheme 3. Synthesis of GalNAc "trident" containing azide attachment point

NMR and HRMS spectra (*see SI*). Thus, one (for monovalent conjugates) or two (for bivalent ligands) characteristic singlet (or sometimes overlapped with other signals) proton at C5 atom of 1,4-substituted 1,2,3-triazole ring is seen in ¹H NMR spectra at \sim 7.88 ppm.

As briefly described above, trivalent ligands possess the highest binding affinity for ASGP-R vs. mono- and, in general, bivalent analogues. To perform a comparative biological evaluation, we have synthesized a conjugate of PTX and triantennary GalNAc vector. For this purpose we equipped the GalNAc cluster designed by Prakash and colleagues¹⁵ with azido group following the synthetic protocol depicted in Scheme 3.

We have prepared the desired ligand using two basic units: the GalNAc moiety 16 and the tris-tricarboxylic acid 17. The first was obtained by glycosylation of benzyl (6-hydroxyhexyl)carbamate with oxazoline 6^{15} and subsequent removal of the protecting group. The second was prepared according with the protocol described previously². The activation of carboxylic function of the initial compound 17 was carried out by pentafluorophenyl trifluoroacetate (Pfp-TFA). Compound 18 was obtained with good yield and high purity. The activated ester 18 (1 eq.) was treated with amine 16 (3.5 eq.) in DMF in the presence of HOBT and DIPEA as a base. The product was isolated with 54% yield and was subsequently subjected to alkaline alcoholysis with MeONa/MeOH to furnish the deacetylated product 20 (94%). The conversion was complete and quantitative. As a result, we have obtained the ASGP-R ligand containing azido group, which can be used for further derivatization with small-molecule drug compounds or molecular diagnostic tools. The final product 21 was synthesized using Cucatalyzed [3+2] azide-alkyne cycloaddition of intermediate 11 with azide 20 under the standard conditions (sodium ascorbate, CuSO₄, DMF/H₂O). Conjugate **21** was isolated with good yield (66%) using HPLC (H₂O:MeCN) and fully characterized (Scheme 4).

At the final stage, we examined the cytotoxicity of the resulting conjugates using the culture of human HCC cells HepG2. It is well documented that, after the isolation of hepatocytes from liver, the amount of ASGP-R on surface decreases⁴⁰. Accordingly, we have selected a stable, proliferating HepG2 cell line containing 76K receptors per cell⁴¹. The HepG2 cell line has been considered as an appropriate system to evaluate efficiency of TDD drug conjugates with selective ASGP-R-



Scheme 4. Synthesis of trivalent drug conjugate 21

targeted ligands⁴². To assess the cytotoxic effect of the synthesized conjugates, a standard MTS test was performed. The obtained results are summarized in Table 1. Cytotoxicity of the unmodified PTX against HepG2 cells was reported by Luo and

co-authors 43 and addressed in the current work for a comparative assessment.

 Table 1. Cytotoxicity of the obtained conjugates and PTX against HepG2 cells

1	CC_{50} (µmoi)	Compound	СС ₅₀ (µmol)
13a	0.092±0.016	14c	na [*]
13b	0.82±0.27	21	0.11±0.12
13c	3.25±0.10	РТХ	0.12±0.01
14a	22.9±0.1	PTX ⁴³	0.21±0.03
14b	na [*]		

na – CC₅₀ > 50 μ mol (CC₅₀ – cellular cytotoxicity)

The obtained conjugates were relatively stable under pH=5.0 and 7.4. HPLC-MS analysis did not reveal hydrolysis even after 24h (8 time points). The compounds were tested for binding to ASGP-R using surface plasmon resonance (SPR), with results reported as dissociation constants (K_d). The synthesized compounds showed high affinity towards the receptor (K_d for the conjugates are close to 10⁻⁹ while for the unmodified GalNAc $K_d \sim 10^{-3}$). The main results for all the compounds evaluated and detailed experimental protocol are presented in SI.

Nuclear and cytoskeletal morphology was analyzed by immuno-fluorescence microscopy (*see SI*). The untreated cells showed typical nuclear and cytoskeleton structures, with formation of the normal mitotic spindles. Microtubules organized as diffuse in cytoplasm (Fig. 2). After the model cells were treated with PTX, characteristic morphology changes were observed: mitoses with more than two poles; many abnormal nuclei (micronuclei or multinuclear), altered microtubule structure (thicker and denser microtubule bundles) (Fig. 3). The similar cellular morphological changes were observed upon the treatment using PTX-contained conjugates. Since esterification at position C2' resulted in loss of microtubule disassembly activity *in vitro*, we tentatively speculate that the conjugates release PTX presumably *via* esterase-mediated hydrolysis³⁷.



Fig. 2. The untreated HepG2 cells were fixed, permeabilized, and immunostained with antibody against α-tubulin (*Green*). Nuclei were stained with DAPI (*in red*).

The active site of the CRD was firstly mutated from a Manbinding protein (MBP) to that of ASGP-R⁴⁴. The mutated protein termed QPDWG was subsequently modified to another variant – QPDWGH – with higher binding affinity towards GalNAc compared to Gal. Thus, the crystal structure of QPDWGH complexed with GalNAc [PDB: 1BCH⁴⁵] has been considered as an appropriate computational model to predict the binding to ASGP-R. The first crystal structure of the H1 subunit was determined by Meier et al.⁴⁶. Key supramolecular interactions in QPDWGH crystal are described in 47 ,⁴⁸ and in *SI*.



Fig. 3. Morphological changes observed in HepG2 cells treated with the selected conjugates and PTX. Cells were fixed, permeabilized, and immunostained with antibody against αtubulin (green); nuclei were stained with DAPI (red).

Recently, two novel X-Ray crystallographic structures of *N*-[(1S,2R,3R,4R,5S)-2,3-dihydroxy-1-(hydroxymethyl)-6,8-dioxabicyclo[3.2.1]octan-4-yl]acetamide (I) and α -lactose in complex with ASGP-R have been published³². We used this crystallographic data to construct our 3D computational model of the ASGP-R binding site. The model was developed and internally validated in ICM-Pro Software v.3.8-5 (MolSoft⁴⁹). Then, three structures **II-IV** (Fig. 5) containing triazole fragment were docked into the site to assess their binding affinity. As a result, relatively good scorings were calculated for all the structures docked. Structures **II** and **III** were predicted with a similar binding affinity, in contrast to structure **IV**. Docking details is presented in *SI*.



Fig. 4. 3D *in silico* model of the ASGP-R Gal binding site (PDB: 5JQ1).



Fig. 5. The most similar to the template molecule I (*yellow*) binding modes predicted for structures **II-IV** (*superposition is shown*). The corresponding energetic score values (*K*cal/mol) are: -30, -32, and -26 for structures **II-IV**, respectively.

It is not surprising that chemotherapy with anticancer drugs, e.g. PTX, Dox or Cisplatin, is accompanied with a relatively low specificity, non-selective biodistribution and a plethora of severe side-effects that significantly restrict the applicability of these drugs. One of the most promising ways to improve the efficiency and safety of HCC chemotherapy is the TDD of drug molecules selectively into hepatocytes through the ASGP-R-mediated endocytosis. Thus, GalNAc-containing carriers of different types and architectures have achieved notable successes in recent years, especially in development of drug-ligand conjugates, ligandanchored nanocarriers, and nucleic acid therapeutics (gene delivery systems). Moreover, ASGP-R-mediated targeting is currently used for diagnostics. This includes imaging with positron emission tomography & magnetic resonance imaging, intracellular uptake by nuclear and magnetic resonance imaging, identification of circulating tumor cells in HCC patients, etc. It can also be used for assessing disorders in pre-operative $^{\rm 50-52}$ and post-operative liver functions^{53,54}. Based on the data on the activity of ASGP-R ligands in vivo¹⁶, we suggest more simple and convenient route to bivalent conjugates in order to avoid laborious synthetic pathways to trivalent ligands. We have synthesized a small series of conjugates containing PTX as an anticancer drug and mono- (13a-c), bi- (14a-c) and trivalent (20) GalNAc-equipped ASGP-R ligands using click reaction. As mentioned above, according to the available data³⁷, the most convenient points suitable for modification in the structure of PTX are position C2' and position C7. There are many examples of the conjugation of PTX through these atoms (see the references above). Ester group has been considered as one of the most felicitous esterase-cleavable trigger to release the drug inside the target cells³⁸. It should also be noted that, as a rule, an increase in the number of anchoring moieties in the structure of drug-conjugates corresponds to higher binding affinity³³. The performed biological evaluation with ASGP-R-expressing HepG2 cell line has revealed monovalent conjugates 13a and 13b as the most effective cytotoxic agents within the novel series. Thus, compound 13a showed cytotoxic potency (CC₅₀=0.092 µmol) comparative to a "virgin" PTX (CC₅₀=0.12 µmol) and trivalent conjugate 21 ($CC_{50}=0.11 \mu mol$), while compound 13b was slightly less active. Computational study described above predicted that the core-heads of compounds 13a,b and 14a,b possessed superior binding affinity vs. the core-head of compound 14c. However, the comparison of CC50 values, measured in the performed cell-based assay, with the calculated in silico scorings is valuable rather for a rough estimation. For more exact prognosis a protein-based binding assay is urgently needed, and we are working on it. It is well known that drugconjugates containing selective ligands targeted on receptors abundantly expressed in different types of cancer cells usually demonstrate lower systemic toxicity and side-effects in contrast to parent drug molecule. This benefit can be achieved only in the case of an effective endocytosis, a sufficient drug release capacity, and an appropriate pharmacokinetic profile of such conjugates. Anyway, the presence of GalNAc-containing fragments in the structure of a hybrid drug molecule increases the chances to deliver a drug specifically into the HCC nest. Indeed, in many cases, a promising activity observed in vitro is not displayed in vivo and vice versa. However, it should be expected that during the subsequent in vivo trials bivalent conjugate 14a will show lower off-target toxicity, appropriate selectivity and high anticancer potency as compared to PTX and trivalent analogue 20.

Summarizing, we have synthesized a series of novel monoand bivalent small-molecule conjugates of the selective ASGP-R ligands with PTX. We have validated a convenient and versatile

synthetic route including an optimized amide synthesis followed by click reaction to obtain the desired molecules with good yields. All the synthesized compounds were then evaluated on their cytotoxic activity against HepG2 cells. As a result, two monovalent conjugates 13a and 13b showed high efficiency comparable with PTX and trivalent derivative. Moreover, conjugates 13a-c have higher lipophilicity, lower molecular weight and flexibility than bivalent analogues and are more appropriate for a passive transport. Molecular docking study has predicted the sugar moieties of the compounds (series **a**,**b**) as the most attractive core-heads for good ASGP-R binding. During SPR study, the conjugates demonstrated high affinity towards the target receptor. The synthesized hybrid molecules were quite stable and caused the similar to PTX morphological changes in HepG2 cells. The compounds with the best anticancer activity are now planned to advance further in in vivo trials.

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References and notes

- 1. World Health Organization: Cancer. Fact sheet.
- http://www.who.int/mediacentre/factsheets/fs297/en/. 2017.
 Maklakova SY, Kucherov FA, Petrov RA, et al. A new approach to the synthesis of ligands of asialoglycoprotein receptor for targeted delivery of oligonucleotides to hepatocytes. *Russ Chem Bull.* 2015;64(7):1655-1662. doi:10.1007/s11172-015-1056-6.
- D'Souza AA, Devarajan PV. Asialoglycoprotein receptor mediated hepatocyte targeting - strategies and applications. *J Control Release*. 2015;203:126-139. doi:10.1016/j.jconrel.2015.02.022.
- Spiess M. The asialoglycoprotein receptor: a model for endocytic transport receptors. *Biochemistry*. 1990;29(43):10009-10018. doi:10.1021/bi00495a001.
- Stockert J. The asialoglycoprotein receptor : relationships between structure, function, and expression. *Physiol Rev.* 1995;75(3):591-609.
- Huang X, Leroux JC, Castagner B. Well-defined multivalent ligands for hepatocytes targeting via asialoglycoprotein receptor. *Bioconjug Chem.* 2017;28(2):283-295. doi:10.1021/acs.bioconjchem.6b00651.
- Hu J, Liu J, Yang D, Lu M, Yin J. Physiological roles of asialoglycoprotein receptors (ASGPRs) variants and recent advances in hepatic-targeted delivery of therapeutic molecules via ASGPRs. *Protein Pept Lett.* 2014;21(10):1025-1030.
- Wang Y, Du H, Zhai G. Recent advances in active hepatic targeting drug delivery system. *Curr Drug Targets*. 2014;15(6):573-599.
- Roggenbuck D, Mytilinaiou MG, Lapin S V., Reinhold D, Conrad K. Asialoglycoprotein receptor (ASGPR): a peculiar target of liverspecific autoimmunity. *Autoimmun Highlights*. 2012;3(3):119-125. doi:10.1007/s13317-012-0041-4.
- Biessen EA, Beuting DM, van Berkel TJC, Roelen HCPF, van de Marel GA, van Boom JH. Synthesis of cluster galactosides with high affinity for the hepatic asialoglycoprotein receptor. *J Med Chem.* 1995;38(9):1538-1546. doi:10.1021/jm00009a014.
- Lee K, Rafi M, Wang X, et al. In vivo delivery of transcription factors with multifunctional oligonucleotides. *Nat Mater*. 2015;14(7):701-706. doi:10.1038/nmat4269.
- Chang WY, Kao HW, Wang HE, et al. Synthesis and biological evaluation of technetium-99m labeled galactose derivatives as potential asialoglycoprotein receptor probes in a hepatic fibrosis

mouse model. Bioorganic Med Chem Lett. 2013;23(23):6486-6491. doi:10.1016/j.bmcl.2013.09.012.

- Valentijn ARPM, Van Der Marel GA, Sliedregt LAJM, Van Berkel 13. TJC, Biessen EA, Van Boom JH. Solid-phase synthesis of lysinebased cluster galactosides with high affinity for the asialoglycoprotein receptor. Tetrahedron. 1997;53(2):759-770. doi:10.1016/S0040-4020(96)01018-6.
- 14. Biessen EA, Valentijn AR, De Vrueh RL, et al. Novel hepatotrophic prodrugs of the antiviral nucleoside 9-(2phosphonylmethoxyethyl)adenine with improved pharmacokinetics and antiviral activity. Faseb J. 2000;14(12):1784-1792. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db= PubMed&dopt=Citation&list uids=10973928.
- 15. Prakash TP, Yu J, Migawa MT, et al. Comprehensive structureactivity relationship of triantennary N-acetylgalactosamine conjugated antisense oligonucleotides for targeted delivery to hepatocytes. J Med Chem. 2016;59(6):2718-2733. doi:10.1021/acs.jmedchem.5b01948.
- Ivanenkov YA, Maklakova SY, Beloglazkina EK, et al. 16. Development of liver cell-targeted drug delivery systems: experimental approaches. Russ Chem Rev. 2017;86(8):750-776. doi:10.1070/RCR4707.
- 17. Seymour BLW, Ferry DR, Anderson D, et al. Hepatic drug targeting: Phase I evaluation of polymer-bound doxorubicin. J Clin Oncol. 2009;20(6):1668-1676. doi:10.1200/jco.20.6.1668.
- 18. Alnylam Pharmaceuticals. http://www.alnylam.com/.
- ClinicalTrials.gov identifier: NCT02595983. The study of an 19. investigational drug, revusiran (ALN-TTRSC), for the treatment of transthyretin (TTR)-mediated amyloidosis in patients whose disease has continued to worsen following liver transplant. First received: October 30, 2015. Last updated: July 27, 2016. Current status: ongoing, but not recruiting participants.
- 20. ClinicalTrials.gov identifier: NCT02292186. A extension study to evaluate revusiran (ALN-TTRSC) in patients with transthyretin (TTR) cardiac amyloidosis. First received: November 10, 2014. Last updated: July 18, 2016. Current status: ongoing, but not recruiting participants.
- 21. ClinicalTrials.gov identifier: NCT01981837. Phase 2 study to evaluate ALN-TTRSC (revusiran) in patients with transthyretin (TTR) cardiac amyloidosis. First received: November 6, 2013. Last updated: February 4, 2016. Current status: has been completed.
- 22. ClinicalTrials.gov identifier: NCT01814839. A Phase 1, single- and multi-dose, dose escalation study to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics of subcutaneously administered ALN-TTRSC (Revusiran) in healthy volunteers. First received: March 18, 2013. Last updated: February 4, 2016. Current status: has been completed.
- ClinicalTrials.gov identifier: NCT02319005. ENDEAVOUR: Phase 23. 3 multicenter study of revusiran (ALN-TTRSC) in patients with transthyretin (TTR) mediated familial amyloidotic cardiomyopathy (FAC). First received: December 12, 2014. Last updated: August 12, 2016. Current status: ongoing, but not recruiting participants.
- ClinicalTrials.gov identifier: NCT02797847. A safety and 24. tolerability study of an investigational drug, ALN-TTRSC02, in healthy subjects. First received: May 18, 2016. Last updated: June 8, 2016. Current status: recruiting participants.
- ClinicalTrials.gov identifier: NCT02554773. An open-label 25. extension study of an investigational drug, ALN-AT3SC, in patients with moderate or severe hemophilia A or B. First received: September 15, 2015. Last updated: July 19, 2016. Current status: recruiting participants.
- ClinicalTrials.gov identifier: NCT02035605. A Phase 1 study of an 26. investigational drug, ALN-AT3SC, in healthy volunteers and hemophilia A or B patients. First received: January 13, 2014. Last updated: February 12, 2016. Current status: recruiting participants.
- 27. ClinicalTrials.gov identifier: NCT02314442. A Phase 1 study of an investigational drug, ALN-PCSSC, in subjects with elevated low density lipoprotein cholesterol (LDL-C). First received: December 5, 2014. Last updated: December 16, 2015. Current status: has been completed.
- 28. ClinicalTrials.gov identifier: NCT02826018. A study of ALN-HBV in healthy adult volunteers and non-cirrhotic patients with chronic hepatitis B virus (HBV) infection. First received: July 5, 2016. Last updated: July 6, 2016. Current status: recruiting participants.
- ClinicalTrials.gov identifier: NCT02706886. Study of ALN-GO1 in 29. healthy adult subjects and patients with primary hyperoxaluria Type 1. First received: March 3, 2016. Last updated: March 8, 2016. Current status: recruiting participants.
- ClinicalTrials.gov identifier: NCT02597127. Trial to Evaluate the 30.

Effect of ALN-PCSSC Treatment on Low Density Lipoprotein Cholesterol (LDL-C) (ORION). First received: November 3, 2015. Last updated: May 5, 2017. Current status: This study is ongoing, but not recruiting participants.

- ClinicalTrials.gov identifier: NCT03170193. AMG 529 20160338 31. First in Human Study. First received: May 25, 2017. Last updated: NA. Current status: this study is currently recruiting participants.
- Mamidyala SK, Dutta S, Chrunyk BA, et al. Glycomimetic ligands 32. for the human asialoglycoprotein receptor. J Am Chem Soc. 2012;134(4):1978-1981. doi:10.1021/ja2104679.
- 33. Sanhueza CA, Baksh MM, Thuma B, et al. Efficient liver targeting by polyvalent display of a compact ligand for the asialoglycoprotein receptor. J Am Chem Soc. 2017;139(9):3528-3536. doi:10.1021/jacs.6b12964.
- Salunke SB, Babu NS, Chen C-T. Iron(III) chloride as an efficient 34. catalyst for stereoselective synthesis of glycosyl azides and a cocatalyst with Cu(0) for the subsequent click chemistry. Chem Commun. 2011;47(37):10440. doi:10.1039/c1cc13370e.
- Wang Q, Ekanayaka SA, Wu J, Zhang J, Guo Z. Synthetic and 35. immunological studies of 5'-N-phenylacetyl sTN to develop carbohydrate-based cancer vaccines and to explore the impacts of linkage between carbohydrate antigens and carrier proteins. Bioconjug Chem. 2008;19(10):2060-2067. doi:10.1021/bc800243f.
- 36. Wong CH, Hendrix M, Manning DD, Rosenbohm C, Greenberg WA. A library approach to the discovery of small molecules that recognize RNA: use of a 1,3-hydroxyamine motif as core. J Am Chem Soc. 1998;120(33):8319-8327. doi:10.1021/ja980826p.
- Fu Y, Li S, Zu Y, et al. Medicinal chemistry of paclitaxel and its 37. analogues. Curr Med Chem. 2009;16(30):3966-3985. doi:10.2174/092986709789352277.
- 38. Böhme D, Beck-Sickinger AG. Controlling toxicity of peptide-drug conjugates by different chemical linker structures. ChemMedChem. 2015;10(5):804-814. doi:10.1002/cmdc.201402514.
- 39. Pilkington-Miksa M, Arosio D, Battistini L, et al. Design, synthesis, and biological evaluation of novel cRGD-paclitaxel conjugates for integrin-assisted drug delivery. Bioconjug Chem. 2012;23(8):1610-1622. doi:10.1021/bc300164t. 40.
 - Schwartz AL, Ashwell G. The hepatic asialoglycoprotein receptor. Crit Rev Biochem 1984.16(3):207-233 doi:10.3109/10409238409108716.
- Li Y, Huang G, Diakur J, Wiebe L. Targeted delivery of 41. macromolecular drugs: asialoglycoprotein receptor (ASGPR) expression by selected hepatoma cell lines used in antiviral drug development. Curr Drug Deliv. 2008;5(4):299-302. doi:10.2174/156720108785915069.
- Zhang X, Ng HLH, Lu A, et al. Drug delivery system targeting 42. advanced hepatocellular carcinoma: current and future. Nanomedicine Nanotechnology, Biol Med. 2016;12(4):853-869. doi:10.1016/j.nano.2015.12.381.
- Luo D, Cheng SC-S, Xie H, Xie Y. Effects of Bcl-2 and Bcl-X L 43. protein levels on chemoresistance of hepatoblastoma HepG2 cell line. Biochem Cell Biol. 2000;78(2):119-126. doi:10.1139/o00-008.
- Kolatkar AR, Weis WI. Structural basis of galactose recognition by 44. C-type animal lectins. J Biol Chem. 1996;271(12):6679-6685. doi:10.1074/jbc.271.12.6679.
- Kolatkar AR, Leung AK, Isecke R, Brossmer R, Drickamer K, 45. Weis WI. Mechanism of N -acetylgalactosamine binding to a Ctype animal lectin carbohydrate-recognition domain. J Biol Chem. 1998;273(31):19502-19508. doi:10.1074/jbc.273.31.19502.
- Meier M, Bider MD, Malashkevich VN, Spiess M, Burkhard P. 46. Crystal structure of the carbohydrate recognition domain of the H1 subunit of the asialoglycoprotein receptor. J Mol Biol. 2000;300(4):857-865. doi:10.1006/jmbi.2000.3853.
- 47. Wu J. Targeting hepatocytes for drug and gene delivery emerging novel approaches and applications. Front Biosci. 2002;7(4):A806. doi:10.2741/A806.
- D'Souza AA, Jain P, Galdhar CN, Samad A, Degani MS, 48. Devarajan P V. Comparative in silico-in vivo evaluation of ASGP-R ligands for hepatic targeting of curcumin gantrez nanoparticles. AAPS J. 2013;15(3):696-706. doi:10.1208/s12248-013-9474-6. 49.
- Molsoft, www.molsoft.com, 2017.
- 50. Hwang EH, Taki J, Shuke N, et al. Preoperative assessment of residual hepatic functional reserve using 99mTc-DTPA-galactosylhuman serum albumin dynamic SPECT. J Nucl Med. 1999:40(10):1644-1651.

http://www.ncbi.nlm.nih.gov/pubmed/10520704.

51. Iimuro Y, Kashiwagi T, Yamanaka J, et al. Preoperative estimation of asialoglycoprotein receptor expression in the remnant liver from CT/ 99m Tc-GSA SPECT fusion images correlates well with

postoperative liver function parameters. J Hepatobiliary Pancreat Sci. 2010;17(5):673-681. doi:10.1007/s00534-010-0264-6.

- 52. Yumoto Y, Yagi T, Sato S, et al. Preoperative estimation of remnant hepatic function using fusion images obtained by 99mTclabelled galactosyl-human serum albumin liver scintigraphy and computed tomography. Br J Surg. 2010;97(6):934-944. doi:10.1002/bjs.7025.
- Acceleration 53. Yoshida M, Shiraishi S, Sakaguchi F, et al. Fused 99m-Tc-GSA SPECT/CT imaging for the preoperative evaluation of postoperative liver function: can the liver uptake index predict

Graphical Abstract

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