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Development of liver cell-targeted drug delivery systems: experimental approaches

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The review is devoted to liver cell-targeted drug delivery systems. Primary attention is paid to the therapy of hepatocellular carcinoma, liver fibrosis, liver cirrhosis, viral hepatitis (A – G), cholangiocarcinoma, autoimmune liver diseases and some metabolic disorders. A general overview, modern classification and specific features of drug delivery systems are presented. The key characteristics and parameters of these systems and their advantages and restrictions for clinical applications are discussed. A considerable body of information is presented as summary tables convenient for perception and comparison. The data presented are critically analyzed and an expert evaluation of the therapeutic potential of the drug delivery systems in question is given. Discussion of invasive local delivery approaches, transmembrane systems and implants, as well as prodrugs is beyond the scope of the review, except for a few examples.
The bibliography includes 344 references.

Contents

I. Introduction 750
II. Targeted delivery systems 752
III. Drug delivery to liver 753
IV. Conclusion. An expert evaluation 768

I. Introduction

In the early 20 century, Paul Ehrlich formulated a fundamental concept of 'magic bullet', *i.e.*, targeted transport of drug molecules (DM). According to this concept, the most significant and safe therapeutic effect can be achieved using specific drug delivery to the focus of disease (tissue, organ, cell, organelle) or directly to pathogens (parasitic microorganisms).[1] Further evolution of P.Ehrlich's concept led to the emergence of particular avenues of scientific research in the field of design of novel drugs and diagnostic agents and to the development of classification and terminology to describe the field of targeted drug delivery. For instance, the term 'targeted drug delivery' (or 'drug targeting'), which is used in field of design of targeted delivery systems, differs from the term 'targeted therapy' (or 'targeting therapy', 'targeted medicine'), which is often used in the field of novel drug design. The former term implies preferential accumulation of the substance being transported at the focus of disease irrespective of the method of administration.[2] The latter one characterizes a specific interaction between DM

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and a receptor on the molecular level.[3, 4] Main goals and principles of targeted drug delivery are as follows:[5, 6]

— more efficient transport of DM from the administration site to the focus of disease;

— improvement of pharmacokinetic parameters and physicochemical properties of drugs, including metabolic stability, bioavailability and solubility;

— release of the active substance in a necessary therapeutic dose (or its derivative[7]) at or in the immediate vicinity of the focus of disease;

— protection of a therapeutic agent (small molecule, nucleic acid, peptide) from intracellular and extracellular factors (medium pH, enzymes, oxidative stress);

— reduction of side (toxic) effects on normal cells mainly due to administration of lower drug doses, which corresponds to a higher therapeutic index and optimal therapeutic window.

However, a number of major pharmaceutical companies make use of specific decision-making framework (extended rules), such as AstraZeneca's 5R strategy.[8] The goals listed above are achieved using a number of approaches and methods including 'mechanical' delivery (topical creams and gels, transdermal systems and implants, invasive local delivery approaches), transporters (carriers, vectors) including liposomes, polymeric micelles, virosomes and bacteriophages, various types of nanoparticles and nanogels, dendrimers and their functionalized derivatives, external factors (magnetic field and sonication), as well as chemical modification of the active substance including prodrugs.[9, 10] According to commonly accepted classification, there are two main types of transport, *viz*., passive and active. However, these terms do not exactly reflect the essence of the interactions and phenomena occurring *in vivo*. In particular, passive transport is characteristic of almost all targeted delivery systems (particles) irrespective of whether the drug is organ- or tissue-specific or not. Unlike passive transport, active transport is first of all due to the interaction between modified transporters with specific proteins (in most cases, transmembrane receptors) that are overexpressed in the target cells. Stable supramolecular interactions occur at distances shorter than 0.5 nm. Most targeted delivery systems cannot target without assistance. For instance, in the case of tumour diseases they reach the focus of disease through general circulation and passive absorption (systemic targeting). Therefore, active delivery means solely ligand – receptor interaction with target cells that follows drug delivery to the necessary tissue or organ. In this connection, poly(ethylene glycol) (PEG)-modified targeted drug delivery systems with prolonged plasma half-life ($t_{1/2}$) are widely used. Below we will discuss both passive and active liver cell-targeted drug delivery systems taking account of the limitations mentioned above.

The abbreviations used in this review are as follows:

DM — Drug Molecules;

ASGPR — Asialoglycoprotein Receptor;

AUC — Area Under pharmacokinetic Curve;

BSA — Bovine Serum Albumin;

CAIX — Carbonic Anhydrase 9;

CD44 — Cell-surface glycoprotein CD44;

Chol — Cholesterol;

Coll-VIR — Collagen type VI Receptor;

CS — Chitosan;

DNA — Deoxyribonucleic Acid, DNA;

DOPE — Dioleoylphosphatidylethanolamine;

Dox — Doxorubicin;

EDC — 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide;

EGF — Epidermal Growth Factor;

EGFR — Epidermal Growth Factor Receptor;

EPR effect — Enhanced Permeability and Retention effect;

FAK — Focal Adhesion Kinase;

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FAR — Fatty Acid Receptors;
FR — Folate Receptor;
5-FU — 5-Fluorouracil;
GA — Glycyrrhetinic Acid;
Gal — Galactose;
GalNAc — *N*-Acetylgalactosamine;
GalR — Galactose-specific Receptors;
GAR — Glycyrrhetinic Acid Receptor;
GL — Glycyrrhizic Acid;
GLR — Glycyrrhizin Receptor;
Glc — Glucose;
GLP-1 — Glucagon-like Peptide-1;
GLP-1R — Glucagon-like Peptide-1 Receptors;
GFP — Green Fluorescent Protein;
HA — Hyaluronic Acid;
HAR — Hyaluronic Acid Receptor;
HAV, HBV, HCV, HDV, HEV — Hepatitis A (B, C, D, E) Virus;
HCC — Hepatocellular Carcinoma;
HDL — High Density Lipoproteins;
HDLR — High Density Lipoprotein Receptors;
HSA — Human Serum Albumin;
HSC — Hepatic Stellate Cells (Ito cells);
HSP — Heat Shock Proteins;
IFN — Interferon;
IFP — Interstitial Fluid Pressure;
Ig — Immunoglobulin;
LA — Lactobionic Acid;
Lac — Lactose;
LAPTM4B — Lysosomal Protein Transmembrane 4 Beta;
LDL — Low Density Lipoproteins;
LDLR — Low Density Lipoprotein Receptors;
LPS — Lipopolysaccharides;
Man — Mannose;
M6P — Mannose-6-phosphate;
M6P/IGF-IIR — Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor;
MR — Mannose Receptor;
MRT — Mean Residence Time (for drug molecules);
NCS — Neocarzinostatin;
PAMAM — Poly(amidoamine);
PAMA — Poly(2-dimethylaminoethyl Methacrylate);
PAR 1, 3, 4 — Thrombin Receptors;
PBLG — Poly(γ-benzyl-L-glutamate);
PCL — Polycaprolactone;
PDGFR — Platelet-Derived Growth Factor Receptors;
pDNA — plasmid DNA;
PEI — Polyethyleneimine;
PEG — Poly(ethylene glycol);
Pgp — P-glycoprotein;
PK2 — a system for targeted delivery of Dox based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) functionalized with D-galactosamine;
PLGA — Poly(L,D-lactic acid-*co*-glycolic acid) copolymer;
PLL — Poly(L-lysine);
pPB — PDGF-receptor Recognizing Peptides;
PTX — Paclitaxel (Taxol);
Pu — Pullulan;
PVA — Polyvinyl Alcohol;
PVLA — Poly(*N*-*p*-vinylbenzyl-*O*-β-D-galactopyranosyl-(1 → 4)-D-gluconamide);
RBP — Retinol Binding Protein;
RES — Reticuloendothelial System;
SCTS — Sulfated Chitosan;
SEC — Sinusoidal Endothelial Cells;
Sia — Sialic acid;
siRNA — small interfering RNA;
sito-G — β-sitosterol-β-D-glucoside;
SMA — poly(styrene-*co*-maleic anhydride) copolymer;
SMANCS — Lipid transport system based on SMA and NCS;
SOD — Superoxide Dismutase;
SR — Scavenger Receptors;
SST — Somatostatin;
SSTR 2, 3, 5 — Somatostatin Receptors;
StabR — Stabilin Receptors;
$t_{1/2}$ — Plasma half-life;
TfR 1, 2 — Transferrin Receptors 1, 2;
TTR — Transthyretin;
VEGF-A — Vascular Endothelial Growth Factor A.

II. Targeted delivery systems

Irrespective of how they were administered (orally or intravenously), drugs are eliminated from systemic circulation by Kupffer cells in the course of phase 1 metabolic detoxification of xenobiotics.[11] Covalent binding to a liver cell-specific transport system provides targeted drug delivery to the target organ and increases the plasma half-life $t_{1/2}$.[12] Galactosylation of many polymers (*e.g.*, PLGA) leads to significant improvement of their biodistribution parameters and selectivity.[13] At present, the most widely used method for modification of drugs and delivery systems is PEGylation (see below). PEGylated conjugates are used for therapy of various diseases including hepatitis C.[14] Chemical modifications using asialofetuin (endogenous ligand ASGPR)[15] and PLL (oligonucleotide complexes)[16] were reported. Nanoparticles are eliminated from systemic circulation by RES macrophages, Kupffer cells representing > 30% of the total liver cell population (~ 7% of the volume of the liver) and being a major deposition site.

II.1. Nanoparticle size and stability

Particle size belongs to key parameters responsible for the properties and transport characteristics. In one of the first milestone publications particle Matsumura and Maeda[17] showed that ^{51}Cr-labelled proteins (including SMANCS) with molecular masses in the range from 15 to 70 kDa can effectively accumulate in sarcoma S-180 cells injected into mice. The rate of specific deposition of smaller particles was higher than that of larger particles; however, the latter were characterized by prolonged residence time at the site of tumour. It was also demonstrated[18] that liposomes 90 nm in diameter can effectively penetrate vascular endothelial walls in the region of solid tumours and reside there for a relatively long time (more than a week). Particles 50 – 300 nm in size have longer plasma circulation times than larger-size particles.[19, 20]

Taking account of high heterogeneity of tumours[21, 22] and different tumour growth rates, it seems difficult to unambiguously specify the nanoparticle size that meets optimal transport conditions. However, it is believed that in most cases it should be of the order of 400 nm. Stability of nanoparticles is at least equally important factor influencing their plasma half-life $t_{1/2}$, clearance and distribution volume. Usually, the size of drug-loaded polymeric micelles

is in the range of 100 – 150 nm; however, *in vivo* studies revealed their instability in blood plasma and relatively fast degradation.[23, 24] Also, most intravenously administered micellar delivery systems release the active substance within 15 – 20 min, which is first of all due to their interaction with blood components.[25, 26]

II.2. PEGylation

The efficiency of transporters depends strongly on their plasma half-life. Usually, the longer the $t_{1/2}$ times of particles the more probable their accumulation in the effective dose in the target organs including tumours. One of the most widely used methods for optimizing the pharmacokinetic parameters of DM and nanoparticles is PEGylation.[27] PEGylated nanoparticles have prolonged circulation times $t_{1/2}$ that are significantly influenced by the molecular mass of PEG. For instance, the plasma half-lives of PEG-6000 and PEG-190 000 intravenously administered in mice were 30 min and > 24 h, respectively.[28] Similar results were obtained for PVA-14 800 and PVA-434 000 (90 min and 23 h, respectively) in another study.[29] It should be noted that the liver clearance increases for PEG with a molecular mass of 50 000.[28] The structure of PEG-modified macromolecules also influences the pharmacokinetic properties of nanoparticles, but is quite rarely discussed in the scientific literature. It should be noted that PEGylation does not ensure improved penetration into the target organ and the maximum concentrations of PEGylated formulations in, *e.g.*, tumours often remain insufficient to achieve the desired effect and simultaneously maintain an optimal therapeutic window. Usually, more than 80% of the administered dose is eliminated from systemic circulation within the first few hours and only ~5% of the dose circulates for 12 h after administration.[30] Many authors state that the concentration of nanoparticles in tumours can be increased by more than 100% upon PEGylation.[30, 31] However, an intravenously administered dose is mainly accumulated in liver and spleen, while the remaining proportion of nanoparticles is deposited in lungs and kidneys. As a result, only ~2% of the administered dose reaches the target 4 h after administration. From the aforesaid it follows that a 100% 'improvement' actually means that concentration of nanoparticles in the tumour increases from 1% to 2%.[32] In this connection, the issue arises of the therapeutic dose of PEGylated drugs and a number of problems are still to be solved despite clearly seen benefits of PEGylation.[32]

III. Drug delivery to liver

Liver diseases, in particular, liver cirrhosis and liver fibrosis, viral hepatitis (A – G), cholangiocarcinoma and hepatocellular carcinoma (HCC), autoimmune diseases leading to uncontrollable inflammatory processes, as well as metabolic disorders belong to the most dangerous liver diseases that cause death.[33] For instance, HCC is the fifth most common malignancy worldwide and the third leading cause of cancer related deaths.[34] In 2013, HCC is prevalent in East Asia and Africa (> 80% of cases), which corresponds to an incidence rate of 20 per 100 000 (*cf.* at most 5 per 100 000 for North and South America, Northern Europe and Oceania).[35] Russian Federation ranks 9th in Europe and 31st in the

Table 1. Liver-cell specific drug delivery systems.

Disease	Liver cell type	Drug delivery system or mechanism
HAV, HBV, HCV, alcoholic and nonalcoholic steatohepatitis, genetic pathologies including Wilson's disease, hemochromatosis, α1-antitrypsin deficiency, metabolic disorders, liver cancer, hemophilia A, hemophilia B, liver fibrosis and liver cirrhosis	Hepatocytes	ASGPR-mediated targeted transport and apolipoproteins, delivery systems based on viral particles (virosomes, gene therapy): Coxsackie virus receptors, adenovirus receptors, retrovirus receptors, Sendai virus receptors, lentivirus receptors, HBV receptors and HIV receptors. Integrin receptors and PEGylated interferons
Acute and chronic inflammatory processes in liver, alcoholic and nonalcoholic steatohepatitis, liver fibrosis	Kupffer cells and SEC	Inactivated virosomes, liposomes and micelles. RES-mediated transport if particles are larger than 100 nm in size. Cells mainly bind negatively charged molecules (modified liposomes, polymers, oxidized LDL and proteins) through the interaction with SR. Endocytosis mediated by the interaction of the delivery system with M6P/IGF-IIR, FR and HAR
Liver fibrosis and liver cirrhosis	HSC	Targeted delivery systems capable of binding the receptors in HSC endoplasmic membrane: M6P/IGF-IIR, Coll-VIR, PDGFR. Albumin molecules modified with M6P or peptide ligands to PDGFR or Coll-VIR. Antibodies (through the interaction with synaptophysin, or p38 — a marker protein), liposomes (M6P-HSA or ligands to Coll-VIR), virosomes (nonselective adenoviral vectors) bearing specific promoters (CSRP2, SM22α or TIMP-1). Oligonucleotide – cholesterol conjugates
Primary biliary liver cirrhosis	Cholangiocytes	Endocytosis mediated by the interaction with secretin receptor and integrin αvβ6
HCC	Liver tumour cells	Endocytosis mediated by the interaction with ASPGR. Modified monoclonal antibodies (AF-20 — against surface glycoprotein gp180, which is overexpressed in HCC), oligopeptides — ligands to EGFR — and Lipiodol

world for primary hepatic cancer related deaths (mortality rate ~1%). The highest incidence rate (4.6 per 100 000) is observed in Tyumen region, Yakutia and southern regions of the North Caucasus. Usually, liver cancer dominantly affects males than females (male : female ratio is 4 : 1). The disease can occur at any age (most often, after 40 years). Patients with HCC demonstrate the lowest survival (less than a year) compared to other types of cancer.[36] The incidence rate increases (more than 500 000 new cases are diagnosed annually). Surgical removal of a tumour is an efficient method used in the early stages of cancer. Chemotherapy including targeted drug delivery systems is utilized in the post-operative period and at later stages of cancer development.[37]

Although the concentration of most drugs in liver after administration is rather high, in some cases the desired therapeutic values are not reached. Considering therapy of liver cancer, one deals with a relatively high recurrence rate. Usually, increasing the therapeutic dose leads to the development of drug resistance, mainly due to overexpression of ABC transporter (*e.g.*, Pgp) genes and to higher probability of off-target effects.[38]

When treating liver diseases, one should take account of the type of the target cells. These can be hepatocytes (>80% of the volume of the liver), Kupffer cells, sinusoidal endothelial cells and HSC. Morphological features of the liver have been considered in detail elsewhere.[6, 39, 40] Table 1 lists the main liver diseases and liver cell-specific targeted drug delivery systems. An analysis of the available liver cell-specific targeted drug delivery systems can be found in a number of reviews [39, 41, 42] including specialized publications on HCC.[34, 43, 44]

It is believed that efficient drug delivery to the liver is possible if the particle size is in the range of 50–250 nm.[45] In this case, nanoparticles arrive at the liver parenchyma where receptor-mediated endocytosis can occur, whereas particles larger than 250 nm in size are non-specifically deposited in other types of cells, in particular, monocytes, being stronger influenced by the RES.[46, 47] For instance, a study of spheroid models for liver cancer [48] showed that less than 5% of the targeted delivery systems 100 nm and more in size can penetrate, *i.e.*, the extracellular matrix represents a transport barrier that restricts nanoparticle penetration, and that partial degradation of the matrix components under the action of, *e.g.*, collagenases, in some cases facilitates better penetration of nanoparticles of specified size into the target cells.[49]

III.1. Passive delivery systems

The course of certain diseases, *e.g.*, uncontrollable inflammatory visceral diseases, in particular, rheumatoid arthritis and cancer, is accompanied by an increase in the lumen between vascular endothelial cells and by relative underdevelopment of the lymphatic system. This significantly facilitates passive delivery of nanoparticles of appropriate size and shape to the focus of disease. Accumulation of nanoparticles in the liver proceeds by a similar mechanism. This phenomenon was termed the Enhanced Permeability and Retention (EPR) effect [50, 51] and for the first time reported by Professor Maeda.[17, 52] The first successful examples of systems for targeted drug delivery driven by the EPR effect include SMANCS/ethiodol (see below) [53] and a liposomal form of doxorubicin, that is, Doxil™/Caelyx™ (Ref. 54). The latter was approved by the US Food and Drug Administration (FDA) in 1995 as the first nanoformulation of doxorubicin.[55] Unlike the original drug, Doxil™/Caelyx™ demonstrated specific biodistribution and was introduced to the pharmaceutical market for a number of therapeutic indications.[56] It should be noted that the EPR effect can vary over a wide range depending on the *in vivo* xenograft model.[57] The morphology and dynamics of angiogenesis in animal models differ significantly from those observed in humans in the course of clinical trials. In particular, the normalized accumulation of PEGylated liposomes bearing ^{111}In-labelled diethylenetriaminepentaacetic acid in breast cancer cells, cervix cancer and bronchus cancer lie in the range of 2.7%–53% ID kg^{-1} (ID is the injected dose),[58] thus indicating high heterogeneity of the EPR effect under the clinical trial conditions. In most cases, the nanoparticle clearance is due to the RES which contains macrophages (~80% of the macrophage population) and phagocytes. This in turn can be used for passive delivery directly to macrophages, lymph nodes and spleen. This type of passive delivery is of particular importance for, *e.g.*, therapy of leishmaniasis and malaria.[59] Recall that the liver is the main organ of the RES. Using simple types of modification, *e.g.*, PEGylation of nanoparticles, one can reduce the loss of conjugates in the RES and thus increase $t_{1/2}$ and prolong the therapeutic action.[60] Passive drug delivery can be facilitated using other factors including pH of the medium (in some cases, reduced pH in the vicinity of tumour cells) and redox systems (glutathione). However, in some cases passive targeting does not guarantee a significant therapeutic effect.[61] Table 2 presents a comprehensive list of passive liver cell-specified drug delivery systems whose action was tested in clinical trials.

For instance, ALN-VSP liposomes containing two types of siRNA (1 : 1) complementary to kinesin mRNA and VEGF-A are studied in a Phase 1 clinical trial against HCC. The lipid transport system SMANCS based on SMA and NCS (an antitumour protein) (Fig. 1) has been

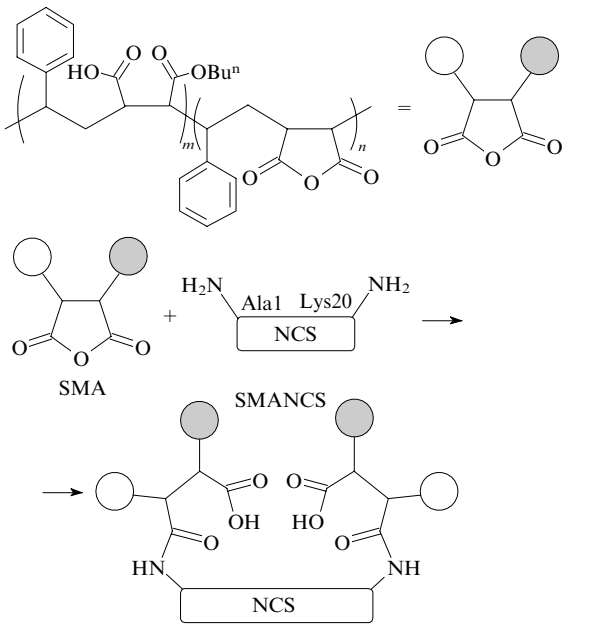


Figure 1. Structure of SMANCS drug delivery system: a schematic.[116]

Table 2. Passive drug delivery systems for therapy of liver diseases.

Drug delivery system	Company [a]	Description of drug delivery system or the active substance	Phase of development [b] or year of introduction to the market	Disease	Ref.
DC-Bead E-7040	Eisai	Delivery system for transcatheter arterial chemoembolisation, containing polyvinyl alcohol hydrogel microbeads	2014	HCC	62
SM-11355 (DACHPM)	Bristol-Myers Squibb and Dainippon Sumimoto Pharma	Miriplatin	2010	HCC	63
SMANCS (YM-881)	Astellas Pharma and Kuraray	Zinostatin	1994	HCC	64
ThermoDox™ (Dox-LTSL, YHI-801)	Duke University and The University of Hong Kong	Combination of thermally sensitive liposomes (Dox) and radiofrequency ablation	Phase 3 (orphan drug status)	HCC	65
Livatag® (BA-003)	Onxeo	Dox-modified polyisohexylcyan-acrylate nanoparticles (Transdrug™ technology)	Phase 3	HCC	66
ADI-PEG-20 (pegargiminase, ADI-PEG)	Polaris Pharmaceuticals	PEGylated arginine deiminase	Phase 3	HCC	67
MagneTarg™ (MTC-Dox)	FeRx	Dox	Phase 2/3 (discontinued)	HCC	68
CDDP-TI (MPI-5010)	Matrix Pharmaceutical and Novartis	Injectable gel containing collagen as a carrier matrix, epinephrin (0.1 mg ml^{-1}) and cisplatin (4 mg ml^{-1})	Phase 2	HCC	69
PLK1-SNALP (TKM-PLK1, TKM-080301)	Arbutus Biopharma and Alnylam Pharmaceuticals	Stable nucleic acid lipid nanoparticles (SNALP) containing PLK1-specific siRNA (PLK1 is serine/threonine-protein kinase)	Phase 2	HCC	70
IntraDose-FU (SinoFuan, 5-FU-TI, FU-TI, MPI-5011)	Matrix Pharmaceutical and Simcere Pharmaceutical Group	Therapeutic implant containing epinephrin (0.1 mg ml^{-1}) and 5-FU (30 mg ml^{-1})	Phase 2	HCC	71
BrachySil (^{32}P BioSilicon)	pSivida and The Singapore General Hospital	Percutaneous implant; ^{32}P-labelled 20-μm BioSilicon™ microparticles (material purity of more than 99.9%)	Phase 2	HCC	72
BCT-100 (PEG-BCT-100)	Bio-Cancer Treatment International and The University of Hong Kong	PEGylated recombinant human arginase	Phase 2	HCC	73
Mipsagargin (G-202)	The Johns Hopkins University and GenSpera	Thapsigargin-based prodrug	Phase 2	HCC	74
Epirubicin-loaded nanoparticles	BTG International	Nanoparticles containing epirubicin	Phase 2	HCC	75
NBTXR-3 (PEP-503)	Nanobiotix	Hafnium dioxide (HfO_2) nanocrystallites ($\sim$50 nm in size) with amphoteric biocompatible coating	Phase 1/2	HCC	76
DCR-M1711 (DCR-MYC)	Dicerna Pharmaceuticals	Lipid nanoparticles containing a siRNA that targets the oncogene MYC	Phase 1/2	HCC	77

Table 2 (continued).

Drug delivery system	Company [a]	Description of drug delivery system or the active substance	Phase of development [b] or year of introduction to the market	Disease	Ref.
ALN-VSP (ALN-VSP01 and ALN-VSP02)	Alnylam Pharmaceuticals and Arbutus Biopharma	Liposomal siRNA targeting VEGF-A and KSP (kinesin spindle protein participating in the formation and maintenance of the mitotic spindle structure)	Phase 1 (completed)	HCC	78
MRX-34	Mirna Therapeutics	Liposome-encapsulated mimic of miR-34 tumour suppressor	Phase 1	HCC	79
EC-1456	Endocyte	Folic acid – tubulysin conjugate	Phase 1	HCC	80
MTL-CEBPA	MiNA Therapeutics	A nanoparticle formulation of a double-stranded small activating RNA targeting the CEBPA gene (SMARTICLES)	Phase 1	HCC	81
LIC-pIC complex (NS-9)	Nippon Shinyaku	Complex of a synthetic double-stranded RNA with positively charged liposomes containing 2-*O*-(2-diethylaminoethyl)-carbamoyl-1,3-*O*-dioleoylglycerol	Cancelled	HCC	82
ND-L02-s0201	Nitto Denko	A lipid formulation containing vitamin A and a siRNA complementary to Hsp47	Phase 1/2	Liver fibrosis	83
Algeron (CePEG-IFN, Cepeginterferon alfa-2b)	BIOCAD biotechnological company	'Next-in-Class' PEGylated interferon-α2b with a covalent bond between PEG (20 kDa) and $N^{2.1}$-{4-[ω-methoxypoly-(oxyethylene]butyl}interferon-α2b	2013, phase 3	HCV	84
Hecolin (HEV-239)	Xiamen Innovax Biotech	A vaccine based on aluminium hydroxide nanoparticles and virus-like particles assembled from the structural protein aa376-aa606 encoded by the gene ORF-2 and produced in *Escherichia coli*	2012	HEV	85
EPAXAL (vaccine)	Crucell N.V.	Virosomes (IRIV) modified with deactivated HAV isolated from RG-SBXA112	2008	HAV	86
Reiferon Retard (PEG-IFN-α2a)	Minapharm Pharmaceuticals	PEGylated interferon-α2a	2007	HCV	87
Pegasys® (PEG-IFN2α, R-420)	Roche	PEGylated interferon-α2a	2001	HCV, HBV, liver cirrhosis	88
PEG Intron® (PegIntron, MK-4031)	Merck Sharp& Dohme	PEGylated interferon-α2b	2000	HCV, HBV	89
TGP-943	Takeda Chemical Industries	Third-generation recombinant pre-S2 + S-containing HBV vaccine based on HBsAg-M nanoparticles	1996	HBV	90
ButaNG	Instituto Butantan	Vaccine based on aluminium hydroxide nanoparticles and recombinant HBsAg produced in yeast *Hansenula polymorpha*	Launched on the market	HBV	91
GSK-257049 (AS01E) Mosquirix	GlaxoSmithKline (GSK)	Pre-erythrocytic recombinant vaccine based on a protein isolated from *Plasmodium falciparum* and modified with the surface antigen (RTS,S), also in the form of non-infectious virus-like particles (VLPS) produced in *Saccharomyces cerevisiae*, combined with AS01 adjuvant	Registration	HBV	92

Table 2 (continued).

Drug delivery system	Company [a]	Description of drug delivery system or the active substance	Phase of development [b] or year of introduction to the market	Disease	Ref.
BMS-914143-λ	Bristol-Myers Squibb	PEGylated recombinant interferon-λ_1 (20 kDa, IFN- λ_1/IL29). Conjugate: *N*-{3-[α-methylpoly(oxyethylene)oxy]propyl}-L-methionyl-{[171-serine]-human interleukin-29 (IL29)-(7 – 181)-peptide}	Phase 3	HCV, HBV, HDV	93
ELAD (implant)	Vital Therapies	Extracorporeal implant containing immobilized human hepatocytes and hollow fibres	Phase 3	Acute alcoholic hepatitis	94
YPEG-IFN-α2b	Xiamen Amoytop Biotech	Recombinant Y-shaped PEGylated interferon-α2b (40 kDa)	Phase 3	HBV, HCV	95, 96
PEG-IFN-octadecylamine	The First Hospital of Jilin University (China)	Interferon IFN-octadecylamine (aa-171) containing $Arg^{126}Asp$ mutation and modified with monomethoxy-PEG (20 kDa, N-terminal)	Phase 3	HCV	97
BIP-48 PEG-IFα2b	FIOCRUZ	PEG (48 kDa) covalently bound to interferon-α2b	Phase 2/3	HCV	98
Locteron CR2b	OctoPlus' and Biolex Therapeutics	Microbeads based on PolyActive™ targeted delivery technology, containing BLX-883 (recombinant interferon-α2b)	Phase 2	HCV	99
Oral HVB vaccine	Microscience	Oral vaccine based on spi-VEC delivery system	Phase 2 (terminated)	HBV	100
IFN-α2b XL	Flamel Technologies	Interferon-α2b based on Medusa® — self-organizing amino acid-based polymeric nanoparticles	Phase 2 (no significant activity, terminated)	HCV, HBV	101
OTx-287 (OncoLipin-2)	Oncodigm BioPharma and The UNiversity of Minnesota	Liposomal recombinant interleukin-2 combined with ribavirin	Phase 2 (withdrawn)	HCV	102
HCV-IET	Transition Therapeutics	PEGylated interferon-α combined with ribavirin and EMZ702	Phase 1/2 (terminated)	HCV	103
AOP2014/ P-1101	AOP Orphan Pharmaceuticals	Long-acting third-generation recombinant interferon-α2b modified with PEG-proline (N-terminal): {1-[3-(*R*,*S*)-3,7-bis{[ω-methoxypoly(oxyethylene)carbonyl]amino}-heptyl]-L-prolyl}interferon	Investigational New Drug (IND), status not confirmed, phase 1 phase 2	HBV, HCV	104
PEG-Infergen (PEG-interferon alfacon-1)	Nektar Therapeutics and Roche	PEGylated interferon-α	Phase 1	HCV	105
DA-3021	Dong-A Pharmaceutical	PEGylated interferon-α2a	Phase 1	HCV, HBV	106
TRK-560	Toray Industries	PEGylated interferon-β	Phase 1	HCV	107
Peglambda (PEG-IFN-λ_1)	Nanogen Pharmaceutical Biotechnology	Recombinant interferon-λ_1 conjugated to α-methoxy-ω-(4-nitrophenoxy-carbonyl)-PEG (40 kDa, PEG-pNC)	Phase 1	HCV	108

Table 2 (continued).

Drug delivery system	Company [a]	Description of drug delivery system or the active substance	Phase of development [b] or year of introduction to the market	Disease	Ref.
ARB-1598 (CMP-001)	Cytos Biotechnology	Vaccine based on Immunodrug™QbG10 targeted delivery system (virosomes containing a G10 immunostimulatory DNA sequence encoding a TLR9 agonist)	Phase 1	HBV	109
AIC-649	AiCuris	Inactivated Parapox viral nanoparticles	Phase 1	HBV	110
MAXY-α (RO-5014583)	Maxygen and Roche	Optimized PEGylated interferon-α	Phase 1 (terminated)	HCV, HBV	111
PEV2A/PEV2B (PeviTER™/PeviPRO™)	Pevion Biotech	Vaccine based on (PeviTER/PeviPRO, virosome) technology using synthetic peptide antigens	Phase 1 (terminated)	HCV	112
DNA-Vaccine	GlaxoSmithKline (GSK)	DNA-Vaccine based on PowderJect® targeted delivery system	Phase 1 (terminated)	HBV	113
NUC-B-1000	Nucleonics	Cationic lipid delivery system containing a hairpin siRNA plasmid bearing a RNA polymerase III promoter	Phase 1 (terminated)	HBV	114
Cliavist® (Ferucarbotran, SHU-555A, ZK-132281)	Bayer and Meito Sangyo	Carboxydextran-coated Fe_3O_4/maghemite-γFe_2O_3 superparamagnetic nanoparticles (~60 nm in size)	2001	Diagnosis of liver diseases	115

[a] Listed are the drug development company and the company conducting clinical trials for this indication; [b] The latest phase of clinical trials associated with therapy of liver diseases.

used in Japan for therapy of hepatitis C since 1993. Besides, SMANCS is used for the treatment of HCC and liver cirrhosis. This transport system exhibits no significant off-target-toxicity and is suitable for X-ray computed tomography.[116]

Structurally, NCS is a seven-stranded antiparallel β-sandwich. The protein isolated from *Streptomyces neocarzinostaticus* demonstrates pronounced antitumour activity and rather high general cytotoxicity. The molecular mass of SMANCS is 16 kDa, of which 2 kDa correspond to SMA while the molecular mass of NCS is 12 kDa. Ethiodol is an injectable radio-opaque contrast agent containing iodine (37%) and fatty acid ethyl esters isolated from poppyseed oil. The SMANCS/ethiodol system can selectively accumulate in tumour cells, which has led to a decrease in the volume of tumours by 10% – 99% in 90% patients under clinical trial conditions. The therapeutic effect of this system is mainly due to unique characteristics of the vascular and lymphatic systems in tumours. The ratio of SMANCS concentrations in the tumour and in systemic circulation estimated using a ^{51}Cr-labelled analogue was 1 : 1 after 3.2 h and 5 : 1 after 19 h (*cf.*, 9.3 and 56.7 h for BSA, and 15.3 and 72 h for mIgG). The SMANCS transport system preferentially accumulates in the liver (42% – 50% of intravenously administered dose), spleen (24% – 40%) and tumour. Less than 5% of the dose accumulated at the focus of disease 72 h after administration, whereas the concentration of the substance in blood plasma 24 h after administration was at most 1%.

Now we will dwell on opsonization and phagocytosis, as applied to passive delivery systems. In a broad sense, by opsonization is meant adsorption of opsonins (antibodies, complement factors, serum proteins including fibrinogens and fibronectin, lipoproteins) on the surface of alien microorganisms and other alien particles, in particular, nanotransporters.[117] This modification promotes phagocytosis of alien particles by macrophages. Opsonization by complement factors C3 – C5 and immunoglobulins targets nanoparticles to Kupffer cells and subsequent formation of a phagosome which undergoes transformation to the late phagolysosome in the course of 'ripening'. Degradation of biomolecules occurs in phagolysosomes at a reduced pH. From this point of view phagocytosis should be treated as nonspecific mechanism of uptake of alien particles which depends on the particle size, shape, charge and hardness.[118] For instance, optimal phagocytosis occurs if particles are 1 – 3 μm in size. Small hydrophilic particles (< 35 nm in diameter) do not interact with Kupffer cells but are readily excreted by kidneys and can be more effectively deposited in hepatocytes.[119] It is believed that targeting hepatocytes requires that the particle size be at most 80 – 100 nm.[120] Usually, nanoparticles less than 100 nm in diameter can penetrate vessel walls in liver endothelium with relative ease and interact with parenchymal cells. Large hard particles (~20 μm and more in size) are eliminated by the RES, in particular, by the liver and spleen macrophages;[121, 122] however, often the process requires an extensive cytoskeleton remodelling.[123] Liu and co-workers[124] studied the specific features of biodistribution of liposomal nanoparticles of different size (30 – 400 nm) in mice and established that particle larger than 250 nm in size preferentially accumulate in spleen rather than liver irrespective of surface modification with PEG. Generally, cationic nanoparticles with ζ-potential > 25 mV are more intensely activated by complement factors compared to particles with ζ-potential < 15 mV.[125] Self-aggregation of charged nanoparticles and

opsonization by serum proteins promote their accumulation in the RES, whereas neutral nanoparticles show little uptake.[126] In a general case, positively charged nanoparticles are more prone to cellular uptake compared to hydrophilic neutral or negatively charged nanoparticles.[127] Longer half-lives $t_{1/2}$ in the case of anionic delivery systems are due to low absorption of opsonins. In particular, intravenous administration of polycationic liposomes containing the SOD plasmid was followed by a noticeable decrease in the level of lipid peroxidation and an increase in the level of the reduced form of glutathione.[128] Nucleic acids can be effectively delivered to hepatocytes by cationic and PEGylated liposomes 80 – 100 nm in size; an example is provided by gene therapy of hepatitis B.[129] Other factors of interest influencing phagocytosis of nanoparticles include their shape,[130, 131] mechanical properties [132, 133] and hydrophilicity.[134, 135]

Charged DM and nanoparticles interact with tumour cell membranes (including HCC cells) more efficiently than PEGylated substances; this is associated with steric effects.[136, 137] Nevertheless, examples of effective utilization of PEGylated conjugates are also available.[138] In some cases, the focus of tumour has a lower pH compared to normal cells and thus reduces the therapeutic potential of weakly basic drugs in contrast to low-pK_a antitumour drugs. Weakly basic drugs can be delivered to tumour cells using pH-responsive carriers,[139] *e.g.*, polymethacrylate nanoparticles.[140, 141] Yet another feature of tumour cells is increased interstitial fluid pressure (IFP) ($\sim$5 – 40 mm Hg depending on the size of the tumour),[142] which in turn presents a barrier to passive diffusion of delivery systems. The transport properties can be improved by using specific molecules, *e.g.*, angiotensin II receptor antagonists.[143] Usually, the IFP in normal tissues is at most 3 mm Hg. Nanoparticle transport through convective flow depends on the difference between the IFP and the hydrostatic pressure in capillaries (10 – 30 mm Hg) and on the oncotic pressure (see p. 192 in Ref. 144). *In vivo* studies showed that many liposomes cannot penetrate deeply into tumour tissues [18] and are mainly deposited near the vessel walls at distances of the order of $\sim$50 μm within 48 h after systemic administration. The penetration into solid tumours remains limited even taking account of its dependence on the liposome size.[145] The interaction of nanoparticles with components of the extracellular matrix reduces their mobility and creates higher barriers to targeted transport. Note that if the nanoparticle size is comparable with the size of pores in tumour vascular endothelium, the EPR effect can be noticeably suppressed as a result of steric hindrances to transport.

III.2. Active delivery systems

In spite of considerable advances in the field of passive drug delivery, there has been high activity in the design of active targeted delivery systems with a more specific volume of distribution. Chemical modification of nanotransporters or functionalization of the active substances using 'anchor' molecules (sugar analogues, peptides, vitamins, metabolites, fatty acids and lectins, hormones, aptamers, antibodies and neuromediators) with high affinity to specific proteins (mostly, receptors) enables better drug targeting.[146] According to the optimal scenario, specific recognition of DM by unique receptors, *e.g.*, on the surface of tumour cells should promote endocytosis and subsequent intracellular release of DM. In this case, the limiting factors include the concentration of receptors on the surface of the target cell compared to normal cells, effective endocytosis and the release of the necessary therapeutic dose of the drug with minimal losses. In some cases, high concentration of transporters in the target organ can be achieved under the action of external factors, such as magnetic field and sonication, which are also treated as active delivery instruments.[147] This approach was practically implemented in real-time targeting of, *e.g.*, difficult-to-access organs and in theranostics. In particular, the above goals are achieved using magnetic (metallic, bimetallic and iron oxide-based) nanoparticles. The last-mentioned nanoparticles are most widely used in biological systems owing to relatively low toxicity and ease of modification. In this case, the limiting factor is the velocity of blood flow through the target organ (0.05 – 50 cm s^{-1}).[148, 149] As a result, effective targeting involving magnetic nanoparticles requires relatively high magnetic moments and magnetic field gradients.

III.2.a. Active transport a a result of interaction with specific receptors

The surface of liver cells is covered with various classes of receptors, mainly transmembrane ones, that are responsible for certain functions including cell adhesion and interaction with biomolecules and transport systems. Particular receptors can initiate and regulate endocytosis and pinocytosis. There are clathrin- and caveolin-mediated endocytosis, as well as endocytosis dependent on the size of endocytic vacuoles, properties of the transporter and on how vesicles are formed.[150] Clathrin-mediated endocytosis is the most common mechanism; it is slower than phagocytosis. The mechanisms of endocytosis have been described in more detail elsewhere.[151, 152] Table 3 lists the receptors discovered in the endoplasmic membranes of liver cells. For instance, ASGPR, GAR, Glypican-3 and GLP-1R receptors are expressed in hepatocytes while RBP, PDGFR and urokinase plasminogen activator receptors are expressed in HSC. The mannose-, galactose- and fucose-specific receptors, fibronectin receptors and SR are expressed in Kupffer cells. Sinusoidal epithelial cells express stabilin receptors, laminin receptors and HAR. Overexpression of certain receptors including ASGPR, GAR, LDLR, TfR 1, TfR 2, FR, CAIX and EGFR occurs in HCC cells. The interaction of the drug delivery systems with these receptors may be followed by endocytosis and subsequent release of drugs or diagnostic agents. The best-studied target receptors include ASGPR, LDLR and β_2-adrenalin receptors.

The main and most studied target used in the design of systems for active delivery of drugs and diagnostic agents to liver parenchymal cells is ASGPR.[153, 154, 181] Alternative targets are represented by GAR and GLR.[156] Ito cells express high level of Coll-VIR, RBP and PDGFR, whereas vimentin and M6P/IGF-IIR are also expressed in other types of cells.[155] In particular, overexpression of vimentin occurs in normal mesenchymal cells, including blood vessel endothelium and smooth muscle cells. Certain tumour cells, *e.g.*, B16 melanoma cells express M6P/IGF-IIR. In this connection, the use of Coll-VIR, RBP and PDGFR for targeting HSC is more appropriate from the standpoint of selectivity. Receptors expressed in SEC, *viz.*, SR and HAR, are targets for anti-inflammatory drug delivery systems. One of the most important HAR, CD44, is overespressed not only in HCC and SEC, but also in tumour cells including prostate cancer, bowel cancer and ovarian cancer

Table 3. Receptors in liver cell endoplasmic reticulum, favouring cellular uptake of targeted delivery systems.

Receptor	Liver cell type	Ligand	Ref.
ASGPR	Hepatocytes, HCC	Asialofetuin, Gal, GalNAc, Lactoamyl, Pu, Lac, LA, xyloglucan	153 – 155
GAR	Hepatocytes, HCC	GA	155, 156
GLR	Hepatocytes, HCC	GL	155
Glypican-3	Hepatocytes, HCC	Positively charged proteins including heparin-binding growth factors	157, 158
GLP-1R	Hepatocytes, HCC	GLP-1, exenatide-4	159
STR2/3/5	Hepatocytes, HCC	SST	160, 161
LAPTM4B	Hepatocytes, HCC	LAPTM4B-binding peptides including IHGHHIISVG and EEGIHGHHIISVG	162
EGFR	Hepatocytes, HCC	EGF	163
FR	Hepatocytes, HCC	Folic acid derivatives	164
Transferrin-specific receptors (TfR 1, 2), ferritin-specific receptors	Hepatocytes, HCC, HSC	Transferrin and ferritin	61, 165
CAIX	HCC	Monoclonal antibodies against extracellular epitopes of CAIX antigen	166, 167
Ionotropic purinergic receptors	Hepatocytes	–	168
HDLR	Hepatocytes	HDL	169
LDLR	Hepatocytes and Kupffer cells	LDL	39
Cannabinoid receptors	Kupffer cells and hepatocytes	Endocannabinoids	170
SR (BI/II, AI/II, MARCO, CD36/68, dSR-C1)	Kupffer cells, SEC	Apolipoprotein A-I, apoferritin, HDL	39, 155
Fucose-specific receptors	Kupffer cells	Fucose	39, 171
Galactose-specific receptors	Kupffer cells	Gal, GalNAc	39
Fibronectin receptors	Kupffer cells	–	172
Complement receptors (C3b and C1q), LPS-R	Kupffer cells	Antigens	169
F_c-Receptors (FcγII-B2) and IgA receptors	Kupffer cells, SEC, hepatocytes	IgE and IgG	39
MR	Kupffer cells, SEC, hepatocytes	Man	39, 155
Stabilin receptors	SEC	Hyaluronic acid, chondroitin	173
Laminin receptors	SEC	Laminin, nidogen	174
Collagen receptors	SEC	Denatured collagens	174
HAR	SEC	Hyaluronic acid and its derivatives	39, 155
CD44 (one of the most important HAR)	HCC, hepatocytes	Hyaluronic acid, osteopontin, collagen, *etc*.	175, 176
RBP	HSC	Vitamin A	155
Vimentin	HSC	*N*-Acetylglucosamine	155
Thyrosine kinase receptors	HSC	–	177
Urokinase plasminogen receptors	HSC	–	178
Antidiuretic hormone receptors	HSC	–	179

Table 3 (continued).

Receptor	Liver cell type	Ligand	Ref.
PDGFR	HSC	pPB-HSA	155, 180
M6P/IGF-IIR	HSC	M6P-HSA	155
Coll-VIR	HSC	RGD-Peptide	155
α_2-Macroglobulin receptors	HSC	Antiproteases and proteins binding to growth factors and cytokines	169
PAR1/3/4	HSC	Thrombin	169

cells; therefore, CD44 can be treated as a promising target for the development of active antitumour drug delivery systems. Overexpression of MR occurs in SEC, HSC and Kupffer cells; however, its ligands demonstrate a relatively low selectivity, which may lead to off-target effects. HSC-targeted gene therapy systems are based on RBP, with retinol as specific ligand. Owing to the limitations of space, below we will consider only liver cell-targeted active drug delivery systems based on GAR, GLR and SR; ASGPR will be discussed in a separate Section.

Triterpene saponins, glycyrrhetinic acid (GA) and glycyrrhizinic acid (GL), isolated from licorice root are ligands to GAR and GLR, respectively. The molecule of glycyrrhizinic acid (Fig. 2) is composed of two glucuronic acid residues and a glycyrrhetic acid moiety. Certain derivatives of the parent saponins can be obtained by partial hydrolysis of GA.[182] The C(1) atom bears a hydroxyl group in the structure of GA, being bound to glucuronic acid dimer in the structure of GL. Both saponins are used in traditional Chinese medicine for therapy of various diseases and conditions including metabolic syndrome, uncontrollable inflammatory processes, diabetes, infectious diseases (caused by bacteria or viruses), oxidative stress, *etc*.[183] Besides, GA prevents the emergence of multidrug resistance.[184, 185]

The GAR and GLR receptors are mainly localized on the surface of hepatocytes; it should be noted that the expression level of GAR and GLR in tumour cells can be five times higher than in normal cells.[186] Lin *et al*.[186] reported more efficient delivery of GL-modified doxorubicin-loaded chitosan nanoparticles to hepatocytes *in vitro* as compared to the GA-modified analogues; GL-modified doxorubicin-loaded chitosan-PEG nanoparticles revealed no siginficant efficacy differences.[187] Recently reported GA-modified liposomes based on Chol-PEG-GA[188] appeared to be much less efficient than the unmodified analogues in sodium phosphate buffer (drug release was $32.97 \pm 1.22\%$ and $81.31 \pm 7.99\%$, respectively). Such a difference was explained by the ability of PEG to form a hydration shell around liposomes, which presents a barrier to drug release. The pharmacokinetic parameters (AUC and MRT) of the PEGylated form were more than twice as high as those of the unmodified form. It was shown that the PEG ligand is mainly accumulated in liver cells rather than other tissues and organs. Being a component of a liver cell-targeted active drug delivery system, GA can also cross the brain – blood barrier and accumulate in brain tissues.[189]

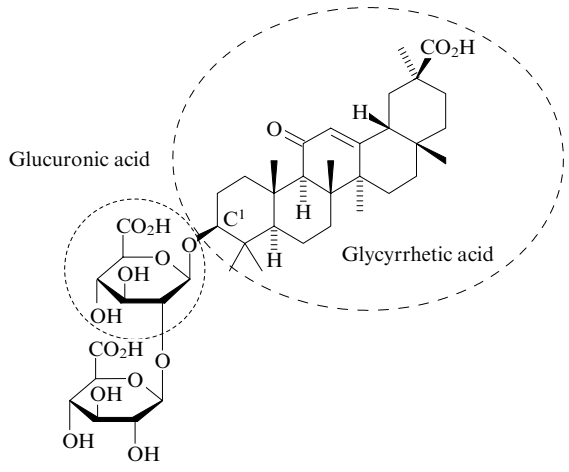


Figure 2. Structure of glycyrrhizinic acid.

Recently,[190] PEG-GA-based self-assembly micelles demonstrating high stability at low (~ 1.2) and neutral pH have been reported. The stability of micelles noticeably reduced in alkaline media (pH ~ 10, $Na_2CO_3 - NaHCO_3$) or in the presence of hydrolytic enzymes. At critical micelle concentrations in the range of $0.081 - 0.73$ mg ml^{-1}, the PEG-GA conjugates formed nano-sized micelles 120.86 ± 31.74 nm in diameter. No marked cytotoxicity against HEK293 cells (48 h, $0 - 1$ mg ml^{-1}) was revealed.

Alginic acid (alginat) is a FDA-approved nontoxic, biodegradable and biocompatible polymer.[191] Spherical doxorubicin-loaded GA-modified alginat nanoparticles ~ 274 nm in diameter were prepared in the presence of EDC and *N*-hydroxysuccinimide. The content of GA in the conjugate was in the range of 10 mass % – 14 mass %. *In vivo* experiments demonstrated that the nanoparticles preferentially accumulated in liver cells and had a more pronounced antitumour effect compared to doxorubicin. A histological study revealed tumour necrosis, which was not observed in normal liver cells.

An active liver-targeted drug delivery system based on GA-modified SCTS micelles (GA-SCTS) showed a relatively high *in vivo* efficacy.[192, 193] Using a GA-SCTS-fluorescein isothiocyanate conjugate, it was demonstrated that the micelles preferentially deposited in the mice liver cells. The cytotoxic concentration (CC_{50}, conventional MTT assay) of Dox-loaded GA-SCTS micelles against HepG2 cells was 54.7 ng ml^{-1}, whereas Dox-loaded SCTS micelles containing no GA exhibited lower activity (CC_{50} = 57.9 ng ml^{-1}). It should be noted that the concentration of the conjugate in the HepG2 cells was more than twice as high as that in the HeLa cells.

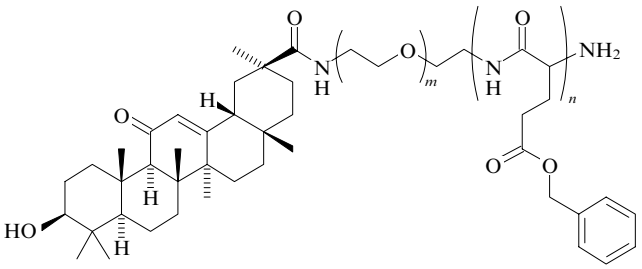


Figure 3. Structure of GA-PEG-PBLG copolymer used for the preparation of micelles for targeted delivery of doxorubicin.[194]

Huang *et al.*[194] prepared GA-PEG-PBLG-based micelles for targeted delivery of doxorubicin to liver (Fig. 3) and established a pH-dependent character of drug release obeying Fickian diffusion kinetics. The concentration of Dox in rats 2 h after intravenous administration of the drug delivery system was 44 μg g^{-1}, being almost five times higher than the concentration of free doxorubicin hydrochloride. The *in vitro* cytotoxicity of the Dox-loaded micelles against QGY-7703 cells was twice as high as in control (IC_{50} = 47 and 90 ng ml^{-1}, respectively). Based on the results obtained (see above), the GA- and GL-modified nanoformulations seem to be promising carriers for active delivery of drugs (in particular, antitumour agents) to the liver.

Scavenger receptors (SR) represent a family of surface glycoproteins recognizing modified lipoproteins (including oxidized and acetylated LDL); they bind collagen, HDL and LDL, as well as xenobiotics. In particular, SR in macrophages and SEC are responsible for the elimination and uptake of a broad range of charged substances including dextran sulfate, a product of albumin treatment with formaldehyde and negatively charged liposomes and nanoparticles. Thus, SR can be used in the design of novel active delivery systems.[195] There are two main classes of SR, *viz.*, SR-A and SR-F. The former includes alternative splicing products (SR-AI, SR-AII and SR-AIII) of a single gene. Among them, SR-AIII is not present in endoplasmic membrane and not involved in endocytosis. Note that the soluble form of SR-AIII can regulate the activity of the other two isoforms.[196] Yet another modification, SCARA5, was found in liver cells quite recently.[197,198] Low expression level of SCARA5 is associated with the growth and development of HCC. On the contrary, FAK-mediated (FAK is focal adhesion kinase) activation of gene expression causes suppression of cell invasion of HCC cells.[198] Apoferritin, apolipoprotein A-I, HDL and fetuin are natural SR ligands and can be used to design active drug delivery systems. For instance, Shin *et al.*[199] developed Paclitaxel-loaded pH-responsive HDL-like nanoparticles. Nanoparticles based on apolipoprotein A-I released Paclitaxel at low pH and were stable at moderate temperatures and physiological pH (pH 7.4). The nanoparticles 9.2 nm in diameter contained 62.2% of PTX and the apolipoprotein A-I : PTX mole ratio was 1 : 55, thus corresponding to 110 Paclitaxel molecules. *In vitro* studies revealed that the cytotoxic activity of the nanoparticles (MTT assay) depends on the expression level of SR-BI in the MCF-7 and SK-Br-3 cells. Cutrin *et al.*[200] developed an active liver cell-targeted drug delivery system based on apoferritin which is efficiently taken up from blood by SCARA 5. The system was modified with a MRI contrast agent Gd-HPDO3A [HPDO3A is 1,4,7-tris(carboxymethyl)-10-(2′-hydroxypropyl)-1,4,7,10-tetraazacyclododecane] and curcumin. Nanoparticles intraperitoneally administered in mice (63 mg kg^{-1}) demonstrated high efficacy against thioacetamide-induced liver damage (a model of hepatitis). The encapsulation of curcumin into the apoferritin cavity significantly improved its stability and bioavailability with retention of anti-inflammatory activity. Various active liver-cell targeted drug delivery systems are presented in Table 4.

Table 4. Examples of active delivery systems to liver cells.

Active molecule	Target	Delivery system	Delivery system type	Disease or activity	Model	Ref.
Dox (PK2)	ASGPR	*N*-(2-Hydroxypropyl)methacrylamide functionalized with D-galactosamine and Dox	Polymeric conjugate	HCC	Human (phase 1)	201
Dox	ASGPR	Pu-Dox, pH-responsive conjugates	Nanoparticles	HCC	HepG2 cells	202
Dox	ASGPR	LA-CS-PVA	Microbeads	HCC	HepG2 cells	203
Dox	ASGPR	Galactosylated PEG-*b*-(lactic acid-*co*-malic acid) block copolymer	Micelles	HCC	HepG2 cells	204
Dox	ASGPR	Lactoferrin-modified PEG	Liposomes	HCC	HepG2, BEL7402, and SMMC7721 cells, mice	205
Dox	ASGPR	Copolymer containing Gal and Chol	Micelles	HCC	HepG2 cells	206
Dox + PTX	ASGPR	LA-BSA	Nanoparticles	HCC	HepG2 cells, mice	207

Table 4 (continued).

Active molecule	Target	Delivery system	Delivery system type	Disease or activity	Model	Ref.
Docetaxel	ASGPR	LA-DOPE	Lipid nanoparticles	HCC	BEL7402 cells	208
PTX	ASGPR	PVLA-PLGA	Nanoparticles	HCC	HepG2 cells	209
5-FU	ASGPR	LA-Octadecylamine (stearylamine)	Solid lipid nanoparticles	HCC	HepG2 cells	210
Norcantharidin	ASGPR	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-PEG-Gal	Liposomes	HCC	HepG2 cells, rats	211
Taspine	ASGPR	Gal-modified lipids	Solid lipid nanoparticles	HCC	Mice	212
10-Hydroxy-camptothecin	ASGPR	LA-CS	Nanocrystals	HCC	HepG2 cells	213
Oridonin	ASGPR	LA-CS-Poly(*N*-isopropyl-acrylamide)	pH-responsive nanogel	HCC	HepG2 cells	214
Mitoxanthrone	ASGPR	Gal-conjugated pluronic P123	Dual-functional liposomes	HCC	Huh-7 cells, rats and mice	215
siRNA (siE2F1)	ASGPR	α,β-Poly(*N*-2-hydroxyethyl)-D,L-aspartamide derivatized with diethylenetriamine and with a galactosylated PEG derivative	Polymeric complex	HCC	HepG2, HuH7 and JHH6 cells	216
Apoptin gene	ASGPR	Asialoorosomucoid-PLL	Polymeric complex	HCC	HepG2 cells, mice	217
Antisense oligo-nucleotide anti-HBV	ASGPR	sito-G and nonionogenic surfactant (Brij 35)	Cationic liposomes	HBV	Primary rat hepatocytes and HepG2.2.15 cells	218
NIK-specific siRNA	ASGPR	sito-G	Cationic liposomes	HBV	HepG2 and HepG2.2.15 cells	219
siRNA (siFVII)	ASGPR	GalNAc-conjugated PEG lipids	Lipid nanoparticles	Thrombosis	Mice	220
Short hairpin RNAs (pGenesil-siHBV4, pGenesil-siSurvivin)	ASGPR	Gal-PEI	Polymeric conjugate	HBV	HepG2.2.15 cells, HepG2.2.15, mice	221
Ribavirin	ASGPR	Lac-functionalized copolymer	Nanoparticles	HCV	HepG2 cells	222
Acyclovir	ASGPR	Lac-*N*-succinyl-CS	Nanoparticles	Hepatitis	Mice	223
Prednisone	ASGPR	Galactosylated and fluorescein isothiocyanate labelled PCL-*g*-dextran	Micelles	Anti-in-flammatory	HepG2 cells	224
Primaquine phosphate	ASGPR	Pu – dextran sulfate	Nanoparticles	Malaria	Rats	225
siTTR	ASGPR	LA-α-CD-PAMAM	Complex	Familial amyloidotic polyneuropathy	HepG2 cells BALB/c mice	164
–	ASGPR	Galactose-terminal amino-functionalized superparamagnetic iron oxide nanoparticles	Nanoparticles	Diagnostic agent	HepG2 cells	226
Oxaliplatin	GAR	3-Succinyl-30-stearyl-GA	Liposomes	HCC	Mice, rats	227
Wogonin	GAR	3-Succinyl-30-stearyl-GA	Liposomes	HCC	HepG2 cells, mice	228
PTX	GAR	GA-HA	Nanoparticles	HCC	HepG2 cells and xenograft BALB/c mice	229

Table 4 (continued).

Active molecule	Target	Delivery system	Delivery system type	Disease or activity	Model	Ref.
PTX	GAR	GA-PEG-GA	Micelles	HCC	HepG2 cells, rats	230
5-FU	GAR	GA-CS	Nanoparticles	HCC	SMMC-7721 cells, mice	231
Curcumin	GAR	GA-Pu	Nanoparticles	HCC	HepG2 cells	232
10-Hydroxy-camptothecin	GLR	GL-BSA	Nanoparticles	HCC	SMMC7721 cells	233
Dox	GAR, HAR	GA-cycteine-HA conjugate	Nanoparticles	HCC	HepG2 cells, mice	234
Dox	GAR	GA-SCTS	Micelles	HCC	HepG2 cells	192
Brucine	GAR	Chol-PEG-GA	Liposomes	HCC	Mice and rats	188
Andrographolide	GAR	GA-PEG-poly(L-histidine)-PLGA	Micelles	HCC	Hep3B cells, mice	235
Dox	CD44	HA-PLGA	Micelles	HCC	HepG2 cells	175
PTX	CD44	HA-Peptides based on arginine and histidine	Liposomes	HCC	HepG2 cells, mice	236
pDNA	CD44	HA-PEG, PCL-poly(*N*,*N*-dimethylaminoethyl methacrylate)	Polymeric complexes	HCC	HepG2 cells, mice	237
–	M6P/IGF-IIR	M6P-HAS	Conjugates	Liver fibrosis	HSC (*ex vivo*)	238
Triplex-forming oligonucleotide (TFO)	M6P/ IGF-IIR	M6P-GFLG-HPMA-GFLG-^{32}P-TFO (GFLG = Gly-Phe-Leu-Gly, HPMA is *N*-(2-hydroxypropyl) methacrylamide]	Conjugates	Liver fibrosis	HSC-T6 cells, rats	239
Dox	M6P/ IGF-IIR	M6P-HSA-Dox	Conjugates	Liver fibrosis	HSC, rats	240
Vismodegib (GDC-0449)	M6P/ IGF-IIR	M6P-PEG-Poly(2-methyl-2-carbonylpropylene carbonate)-*graft*-dodecanol	Micelles	Liver fibrosis	Mice	241
15-Deoxy-$\Delta^{12,14}$-prostaglan J_2 (15d-PGJ2)	SR, M6P/IGF-IIR and PDGFR-β	M6P-HSA-15d-PGJ2 and pPB-HSA-15d-PGJ2	Conjugates	Liver fibrosis	HSC, rats	242
IFN-γ	PDGFR-β	pPB-HSA-PEG-IFN-γ	Conjugates	Liver fibrosis	LX2 cells, HSC, mice	243
IFN-γ	PDGFR-β	pPB-IFN-γ, pPB-PEG-IFN-γ	Conjugates	Liver fibrosis	LX2 cells, HSC, mice	244
IFN-γ mimetic (mimγ)	PDGFR-β	Bicyclic pPB-PEG-mimγ	Conjugates	Liver fibrosis	HSC, mice	245
IFN-αlb	Coll-VIR	RGD	Liposomes	Liver fibrosis	HSC, rats	246
Hepatocyte growth factor	Coll-VIR	RGD-PEG-DOPE	Liposomes	Liver cirrhosis	Rats	247
Oxymatrine	Coll-VIR	RGD-PEG-PCL	Polymeric vesicles	Liver fibrosis	HSC, rats	248
siRNA against HSP47	RBP	Vitamin A	Liposomes	Liver fibrosis	HSC	249
DsRed2-HGF vector (HGF is hepatocyte growth factor)	RBP	Vitamin A	Liposomes	Liver fibrosis	HSC	250

Table 4 (continued).

Active molecule	Target	Delivery system	Delivery system type	Disease or activity	Model	Ref.
Tyr3-Octreotide, an analogue of SST	FAR	*N*-Palmitoyl cycteine	Lipid nanoparticles	HCC	Mice	251
Coumarin-6	FAR	Ursodeoxycholic acid-BSA-lipid (phospholipids, triglycerides and octadecylamine)	Lipid nanoparticles	HCC	HepG2 and Bel 7402 cells	252
SOD	MR	Man-SOD	Conjugate with fluoresecnt dye	Antioxidant activity	Rats	253
Curcumin and Gd-HPDO3A (MRI contrast agent)	SR	Apoferritin	Encapsulation of curcumin and Gd-HPDO3A into apoferritin	Anti-inflammatory action	Mice	200
Rhodamine B	Lactose receptor	Lac-PEG-DHP	Micelles	HCC	SMMC7221 cells and mice	254
–	p38 receptor	*h*C1-3 monoclonal antibody	Conjugate	Liver fibrosis	HSC	255
Dox	LDLR (presumably)	Hematoporphyrin-modified BSA	Nanoparticles	HCC	HepG2 cells, mice, rats	256
Dox	FR	PEG-trimellitic anhydride-folic acid	Superparamagnetic Fe_3O_4 nanoparticles	HCC	Rats and rabbits	257
Diacid metabolite of norcantharidin	FR	Folic acid – PEG	Liposomes	HCC	H22 cells, mice	164
Dox	TfR	Transferrin-modified liposomes	Liposomes	HCC	HepG2 cells, mice, rats	61
Dox	TfR	T7-PAMAM-PEG, (T7 = HAIYPRH peptide)	Nanoparticles	HCC	Bel-7402 cells, mice	258

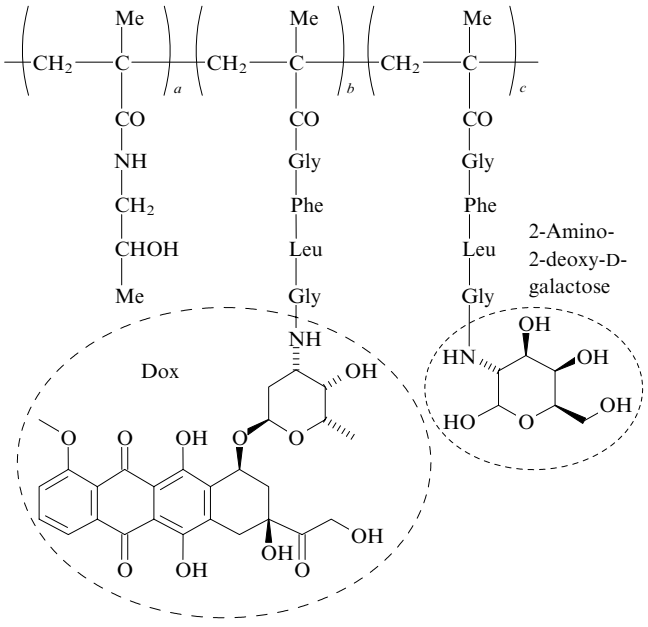


Figure 4. Structure of polymeric conjugate PK2.[201]
a = 96.1, b = 2.4, c = 1.5 mol.%.

The system PK2 was designed for active delivery of doxorubicin (Fig. 4). The drug is linked through a lysosomally degradable tetrapeptide, while the role of selective ligand is played by *N*-(2-hydroxypropyl)methacrylamide copolymers modified with D-galactosamine (2-amino-2-deoxy-D-galactose). The toxicity, selectivity and efficacy of the conjugate against liver cancer was evaluated in a Phase 1 clinical trial on 31 patients with primary (n = 25) or metastatic (n = 6) liver cancer. It was found that 24 h after intravenous administration, PK2 accumulates in tumour cells and liver cells (3.3% and 16.9% of the administered dose, respectively) , thus providing high therapeutic concentration of intratumoural Dox, which was 12- to 50-fold higher than that of the unmodified Dox.[201] The PK2 dose was increased from 20 to 160 mg m^{-2} (Dox equivalents); however, the maximum tolerated dose was associated with adverse effects including fatigue, neutropenia and mucositis. In this connection, a dose of 120 mg m^{-2} was recommended for further clinical trials.

III.2.b. The asialoglycoprotein receptor

The asialoglycoprotein receptor (Ashwell receptor,[259, 260] a lectin of the C-type lectin family) selectively binds plasma glycoproteins having no neuraminic acid residues. Consequently, ligand – receptor binding also depends on the activity of neuraminidase.[261] Specific recognition and cla-

thrin-mediated endocytosis requires modification of the proteins with galactose residues or *N*-acetylgalactosamine.[262] The affinity of *N*-acetylgalactosamine to the receptor at optimum pH > 6 is 10- to 50-fold higher than the affinity of the unmodified terminal galactose residues.[260, 263, 264] For instance, endocytosis of lactosylated HSA by hepatocytes occurs rapidly and involves the formation of vesicles 100–120 nm in size at a first-order rate constant of 3.4×10^{-8} mol litre^{-1} and $t_{1/2} \approx 3$ min.[265] Galactosylated ligands are ordered as follows with respect to 'binding valency': tetra > tri ≫ di ≫ mono, the affinity of the anomeric form $\beta_{1\to4}$ being higher than that of the $\beta_{1\to3}$ form.[266, 267] The affinity of galactosylated ligands can increase 100- to 1000-fold on going from monovalent derivatives [dissociation constant (K_d) 10^{-4} mol litre^{-1}] to trivalent ones ($K_d = 10^{-6}$, 5×10^{-9} mol litre^{-1}); further going to tetravalent derivatives shows no significant differences ($K_d = 9 \times 10^{-9}$ mol litre^{-1}).[154] Usually, the higher content of Gal in the ligand (in particular, asialoorosomucoid), the higher the ligand affinity.[268] Unlike trifluoroacetamide and propanamide substituents, bulky benzamide and phthalimide moieties at position 6 reduce the affinity of monovalent ligands due to steric hindrances, while shielding of hydroxyl groups at positions 3 and 4 leads to a significant decrease in activity.[269] Increasing the density of galactose residues favours an increase in the hepatic uptake clearance of glycosylated proteins from 4 to 400 ml h^{-1} and, as a consequence, a decrease in the rate constant for excretion of conjugates through bile ducts from 0.057 to 0.007 h^{-1}.[270, 271] Various ions except Ca^{2+} can bind the cytoplasmic domain of the receptor and thus influence the ASGPR-mediated signalling cascade.[272] ATP depletion and an increase in endosomal pH under the action of certain reactants or Na^+/H^+ ionophores (monensin, phorbol esters) causes the receptor activity to decrease.[273] Relatively high galactose concentrations (~10–27 mmol litre^{-1}) also block the ASGPR.[274] Steric factors, specific features of linker sequences, as well as hydrophilic–hydrophobic balance also influence the affinity of the galactosylated ligands and have been discussed in the literature.[275–277] High expression and high density of ASGPR (~500,000 per cell) were observed in the basolateral domain of hepatocytes and in the sinusoidal domain. The receptor contains three Ca^{2+}-dependent binding sites for multivalent ligands (tri- and tetravalent N-linked glycans). In this case, ligand–receptor binding is due to the 'cluster effect', which in turn promotes high specificity. However, if the density of galactose residues in the structure of glycoproteins is high (> 300 Gal/LDL),[278] endocytosis mainly occurs in Kupffer cells,[277, 279] which express not only ASGPR, but also other galactose receptors.[280] The asialoglycoprotein receptor is also present in other types of cells including peritoneal macrophages and monocytes, testes, sperm cells, intestinal cells, and in colorectal carcinoma cells (a result of liver cancer metastasis).[281]

Examples of ASGPR-specific ligands are provided by asialofetuin, fibronectin, asialotransferrin, asialoceruloplasmin, asialolactoferrin, hepatoglobulin, serum immunoglobulin A, xyloglucan, soybean glycosides, sito-G, arabinogalactan, carrageenan and pullulan. Ligands can contain different types of galactose residues, *viz*., Gal,[215] Lac,[282] and LA.[207] In particular, BSA modified with oligosaccharides terminated with GalNAc and Sia(α2 → 6)GalNAc(β1 → 4)GlcNAc(β1 → 2)Man is preferentially recognized by the H1 subunit of ASGPR.[283] The results of *in vivo* studies with ASGPR-deficient mice showed that glycoproteins terminated with Sia(α2 → 6)GalNAc and Sia(α2 → 6)Gal are endogenous ASGPR ligands.[284] Recent studies suggest that ASGPR plays a significant role in controlling the homeostasis of the protein components that regulate thrombi formation and blood coagulation processes.[285] Gal(GalNAc)-modified glycoconjugates promote cell adhesion.[285] Lactosylated HDL ~10 nm in diameter are highly hepatocyte-specific.[279] The expression levels of two isoforms, ASGPR-1 (1035.9) and RBP-4 (26913), in liver are respectively 43.3- and 66.8-fold higher than in other tissues and organs. The expression level of the receptor in the HepG2 and HepAD38 cells is as high as 76000 and 17000, respectively (*cf*. 3000 for the HuH-6/7, BNLCL2 and HuH-5-2 cells).[286] An immunochromatographic assay revealed expression of the receptor on 80% of rapidly proliferating HCC cells and on at most 20% of low-mitosis HCC cells.[287] It was demonstrated that the expression level of ASGPR on rapidly dividing HCC cells is much lower than on normal hepatocytes, whereas no significant differences from the baseline were observed under milder conditions.[288] Note that tetrachloromethane, ethanol, liposaccharides and anti-Fas-antibodies (apoptosis antibodies) inhibit the expression of ASGPR. A similar effect was observed in the case of diabetes and hepatectomy.[289]

The asialoglycoprotein receptor is composed of two homologous (58%) subunits, namely, ASGPR1 (HHL-1, H1) and ASGPR2 (HHL-2, H2),[281] each containing four domains, *viz*., an N-terminal cytoplasmic domain (40 amino acids), a monotopic transmembrane domain (~20 amino acids), an extracellular domain (~80 amino acids) and a binding site for galactose and its analogues (~140 amino acids). The cytoplasmic domain of ASGPR2 differs from that of ASGPR1 in an insertion of 18 amino acids. Note that mutations of the amino acid sequence of the galactose binding site, especially, in the binding sites for 3-OH and 4-OH groups, can lead to the loss of galactose specificity.[290] The binding site is formed by the amino acids Asp241,265, Asn264, Glu252, Gln239 and Trp243 from the subunit H1.[290] For instance, binding arabinogalactan and pullulan (both are endogenous ASGPR ligands) occurs through the formation of more than 10 hydrogen bonds.[291] In normal hepatocytes and HepG2 cells the ASGPR2 subunit exists as three different isoforms (2a–c), whereas the ASGPR1 subunit mainly exists as a single isoform. For instance, the H2a isoform is not involved in the structure formation of the transmembrane receptor; however, it is the largest modification composed of a part of the cytoplasmic domain and contains five amino acids homologous to the binding site of the transmembrane domain to the ectodomain.[292] This product of alternative splicing is soluble and was found in blood plasma, whereas the minor amino acid sequence plays the role of a signal sequence.[293, 294] The isoforms H2b and H2c contain no minor amino acid sequence and form a complex with the H1 subunit in hepatocyte membranes and do not undergo proteolysis. It was established that binding to hepatocyte membranes involves a total of 117 amino acids of the H1 subunit, which act as an anchor.[295, 296] On the contrary, the isoform containing no anchor amino acid sequence can be secreted into the systemic circulation. Note that these versions of both isoforms still can form functional hetero-oligomeric soluble complexes (s-ASGPR).[297]

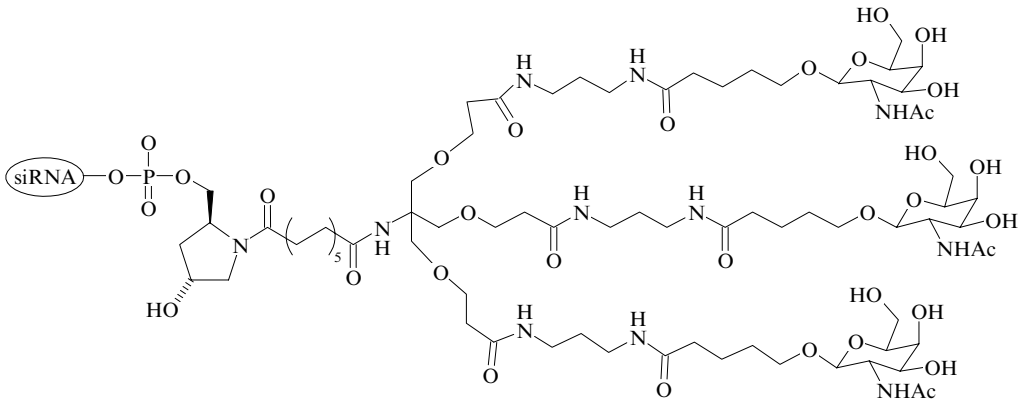


Figure 5. Covalent conjugate of ASGPR ligand with three GalNAc residues for targeted delivery of siRNA to hepatocytes.[304]

For instance, s-ASGPR can inhibit asialoorosomucoid binding to the transmembrane ASGPR. The expression level of ASGPR1 varies insignificantly compared to that of ASGPR2 (3 : 1 mole ratio), which rapidly degrades in endoplasmic reticulum in the absence of ASGPR1. Oligomerization of the subunits underlies high affinity ligand binding.[298] In the absence of ASGPR2 the receptor can bind asialo-α_1-glycoprotein and asialofetuin in COS-7 cells with $K_d < 40$ nmol litre^{-1} (*cf.* $K_d < 10$ nmol litre^{-1} for the normal receptor).[297] The number of binding sites for the ASGPR ligands on the hepatocyte surface is in the range of 10^5 to 5×10^5,[299] of which ~90% are on the sinusoidal surface, 10% on the lateral surface and ~1% on the surface of bile duct cells.[300, 301] For some diseases, the expression level of ASGPR can vary significantly. In patients with liver cirrhosis, the expression level of the receptor is 28% of that in healthy liver.[302]

The ligands mentioned above as well as their galactosylated derivatives can be incorporated into the structure of protein and polymer molecules or directly into liposomal

Table 5. Liver-targeted active drug delivery systems for therapy of various diseases developed at Alnylam Pharmaceuticals.

Medicine	System	Trial phase	Disease	Ref.
Revusiran (ALN-TRSC)	GalNAc-siRNA against TTR	Phase 2 (completed)	TTR-mediated amyloidosis	305
		Phase 2 (completed)	TTR-mediated amyloidosis	306
		Phase 2 (completed)	TTR-mediated amyloidosis	307
		Phase 1 (completed)	TTR-mediated amyloidosis	308
		Phase 3 (completed)	TTR-mediated familial amyloidotic cardiomyopathy	309
Revusiran analogue (ALN-TTRSC 02)	GalNAc-siRNA against TTR	Phase 1 (announced)	TTR-Mediated amyloidosis	310
Fitusiran (ALN-AT3SC)	GalNAc-siRNA against Serpin-C1	Phase 1/2 (recruiting participants)	Hemophilia A/B (antithrombolytic)	311
		Phase 1 (announced)	Hemophilia A/B (antithrombolytic)	312
ALN-PCSSC	ESC-GalNAc-siRNA against proprotein convertase subtilisin-kexin type 9 (MC3-lipid nanoparticles)	Phase 1 (completed)	Hypercholesterolemia	313
		Phase 1 (completed)	LDL-Chol	314
ALN-HBV	ESC-GalNAc-siRNA against X-ORF (genotypes A – J)	Phase 1/2 (recruiting participants)	HBV	315
ALN-GO1	ESC-GalNAc-siRNA against glycolate oxidase	Phase 1/2 (recruiting participants)	Primary hyperoxaluria Type 1	316
ALN-AS1	GalNAc-siRNA against 5-aminolevulinate synthase	Phase 1 (recruiting participants)	Acute intermittent porphyria	317
ALN-AAT	GalNAc-siRNA against α1-antitrypsin	Phase 1/2 (announced)	Antitrypsin deficiency	318
ALN-CC5	GalNac-siRNA against components of complement C5 (CC5)	Phase 1/2 (announced)	Paroxysmal night hemoglobinuria	319

membranes. These active delivery systems can serve carriers for some molecules including peptides, antiviral and antitumour agents (doxorubicin, Paclitaxel, norcantharidin) and oligonucleotides, *e.g.*, IFN-α2b, siRNA. The structure of the ASGPR-modified synthetic delivery systems may contain PLL, PEI, CS, PAMAM and block copolymers. In particular, cationic PLL can bind DNA molecules and form self-assembly complexes that undergo relatively fast elimination after intravenous administration and demonstrate low efficiency of *in vitro* gene transfer due to non-specific interactions with negatively charged components of the test system, including various negatively charged proteins. To improve the stability and plasma half-life of the complex, its structure is modified with PEG or *N*-(2-hydroxypropyl)methacrylamide. However, the *in vivo* selectivity of the modified complex still remains insufficient for targeted gene transfer to the focus of disease.[303] Hybrid galactose-based active delivery systems, *e.g.*, a Gal-PEG-PLL conjugate, can target Bel-7402 and HepG2 cells and transfer pDNA to them. In this case, PEG helps to reduce the toxicity of PLL through shielding of positively charged groups. Consideration of other polymeric systems including PEI, CS and copolymers modified with ASGPR-specific ligands is beyond the scope of the review. For that aspect the reader is addressed to Refs 153–155.

The greatest success in the development of conjugates for ASGPR-mediated active delivery of siRNA (Fig. 5) was achieved by Alnylam Pharmaceuticals (Table 5). The action mechanism of ALN-HBV involves inhibition of key steps of the HBV life cycle including virus replication, assembly, secretion of virus and its antigens, as was demonstrated in a number of proof-of-concept preclinical studies. In particular, it was shown that a single subcutaneous administration of ALN-HBV conjugate in mice causes the expression level of HBsAg (HBV marker) 15 days post dosing to be much lower compared to controls (a maximum decrease of log = 3.6, a mean decrease of log = 1.6). Multiple administration in rats (3 mg kg^{-1}) during the first three weeks resulted in a stable effect observed in the subsequent three months. No valuable off-target effects were observed. To assess the clinical efficacy, pharmacokinetic parameters, toxicity and tolerability of the conjugate, a Phase 1/2 clinical trial on patients with HBV infection and healthy adult volunteers was announced quite recently.

IV. Conclusion. An expert evaluation

In some cases, the use of modified liver cell-targeted drug delivery systems including micelles, liposomes, conjugates, niosomes and various nanocomplexes facilitates reduction of off-target effects, optimization of pharmacokinetic parameters and enhancement of their efficacy compared to the original drugs. However, a number of limiting factors, such as low drug loading and selectivity, instability and chronic toxicity create significant obstacles to the development and wide use of effective targeted delivery systems. Not all galactosylated systems ensure effective delivery of drugs and diagnostic agents to the liver and other target organs.[320, 321] As mentioned above, the expression level of ASGPR in HCC cells depends on the stage of tumour development. In the case of autoimmune hepatitis, the ASGPR antibodies prevent targeted delivery. Galactosylated emulsions are better systems for drug delivery to liver cells compared to galactosylated liposomes[322] that exhibit high affinity to the Gal-specific receptors in Kupffer cells in contrast to ASGPR.[323] The affinity of the galactosylated particles to the Gal-specific Kupffer cell receptors is higher compared to GalNAc. Low content of lactose in lipoproteins (< 60 Lac/LDL) promotes targeting of hepatocytes, whereas high content of the ligand (> 300 Lac/LDL) favours the uptake of the galactosylated particles by Kupffer cells. Negative charge of Gal-modified chitosan nanoparticles facilitates their preferential accumulation in endothelial cells.[324] Lactosylated HSA/adenosine monophosphate-containing conjugates are deposited in SEC. Although the efficacy of nanoparticles is to a great extent due to the EPR effect, intravenous administration of nanoparticles causes more than 90% of the dose to accumulate in various organs including liver, spleen, lungs and bronchi. Taking account of this fact, the term 'passive targeted delivery' does not reflect the essence of the observed phenomena. In this case it is rather a question of systemic circulation and local neovascularization parameters.

Disappointing results of clinical trials suggest that present-day understanding of specific features of targeted delivery is far from being sufficient for the design of effective drugs. From this point of view, considerable progress in the title field, reflected in a large number of *in vitro* and *in vivo* trials, is often non-scalable and does not lead to the desired results in the course of clinical trials. This was discussed in an accessible form by Professor Florence.[325, 326] In this connection, mention should be made of a number of clinical trials that were terminated for various reasons. In particular, a Phase 2/3 clinical trial of the antitumour drug MagneTarg™ conducted by FeRx, Inc. (USA) was terminated because the clinical endpoints were not met with statistical significance. This also holds for NS-9 liposomes (Nippon Shinyaku, Japan), although *in vitro* trials and preclinical studies demonstrated high efficacy against liver cancer, as well as for NUC-B-1000 and PEV2A/PEV2B. Despite the fact that MAXY-α (Roche R-7025) demonstrated a higher efficacy against hepatitis B and C compared to Pegasys® in preclinical studies, Maxygen, Inc. (USA) and Roche terminated a Phase 1 clinical trial because of significant deterioration of the pharmacodynamic and pharmacokinetic parameters of the substance in most patients. As a result, the alliance initiated a new programme on the design of an improved analogue. A Phase 2 clinical trial at Flamel Technologies SA (France) revealed no significant efficacy difference between IFN-α2b XL and PEGylated IFN-α2b combined with ribavirin. An increase in the therapeutic dose was accompanied by common off-target effects including flu-like syndrome, erythema and mood swings. The clinical efficacy of HCV-IET combined with EMZ702 (hydroxycobalamine), PEGylated IFN-α and ribavirin was two or three times higher than that of conventional methods for treatment of hepatitis C. However, dose-escalation therapy led to common off-target effects, mainly exanthema. As a result, the trial was terminated to determine safer therapeutic doses. Low IC_{50} values obtained in the course of *in vitro* trials do not correlate with the clinical effect.[327] Often, it is impossible to induce complete tumour regression in xenograft models; therefore, one deals with tumour regrowth and proliferation after discontinuation of treatment.[328, 329] Drug delivery to target cells should be treated exclusively as a small step on the road to the development of a novel effective targeted delivery system. From this point of view the greatest

progress was made using the siRNA delivery systems within the framework of gene therapy approaches. Here, studies on the mechanisms of intracellular transport and drug release are at least as important as systemic targeting.[330, 331]

Emphasize that in the case of active transport the *in vivo* expression level of the target receptor can vary with time.[332] In other words, the cell lines (cultures) used in the *in vitro* trials do not correspond to specific features of the primary tumour cells at a higher level of organization and in other microenvironments, especially, in patient's body. The lack of information what fraction of tumour cells expresses the target receptor and how its expression (activity) depends on time and on the stage of *in vivo* tumour development in each particular case creates even more obstacles to the development of effective active drug delivery systems. As a consequence, many targeted delivery systems show no desired efficacy in the course of preclinical studies, as was demonstrated for the folate-modified liposomes.[333] That is why many researchers cast some doubt on the efficiency of active targeting.[334]

In vivo biological barriers also preclude the development of efficient targeted delivery systems. In order to maintain a therapeutically significant concentration in systemic circulation, the nanoparticle clearance by macrophages should be relatively low. The most widely used method to improve the pharmacokinetic properties of nanotransporters is to modify their surface *via* PEGylation;[335] this to a greater extent concerns liposomes.[166] However, in some cases such a modification may lead to inhibition of transport across the tumour cell membranes and, as a consequence, to less pronounced antitumour effect.[336] The problem can be partially solved using a labile, dynamic PEG shell responsive to specific conditions in the target organ (pH, enzymes) or under the action of external factors that stimulate the elimination of PEG and drug release.[337] Having migrated through vascular endothelial walls on the way to the focus of disease, nanoparticles have to overcome other types of biological barriers including components of extracellular matrix, smooth muscle cells, pericytes, as well as pH, hypoxia conditions and increased IFP. Usually, drug molecules penetrate to at most three to five cell diameters from the blood vessel; as a result, hard-to-reach tumour cells remain unaffected.

One should also take into account the fact that target receptors are also expressed in other types of normal cells and pronounced off-target effects are thus possible at certain drug doses.[338, 339] In this connection, the specificity of expression and location of receptors play a key role.[340] For instance, ASGPR is expressed in normal hepatocytes and in HCC cells (see above) and thus researchers have to improve the selectivity. In particular, Terada and co-workers[341] reported a HCC-specific delivery system based on matrix metalloproteinase-2 cleavable DOPE/PEG-containing liposomes. The mechanisms of drug release also require more detailed consideration.[342]

Recall that, according to P.Ehrlich's original concept, the term 'magic bullet' implies a drug molecule that selectively targets a single therapeutically significant entity which is expressed in specific manner exclusively at the focus of disease.[343] If the drug molecule binds a number of clinically validated targets responsible for the therapeutic effect, it can be treated as 'magic shotgun'.[344] Actually, no DM or delivery systems acting solely at the focus of disease under systemic administration are available. Drug delivery occurs through systemic circulation, penetration through blood vessel walls and distribution within the target cells, *e.g.*, tumour cells. Taking account of this fact it is not surprising that DM can be deposited in other organs and tissues, thus inducing off-target effects and wearing-off from the effective dose. In this connection, modern interpretation of the 'magic bullet' concept implies preferential deposition of DM in the target cells with allowance for the width of therapeutic window and effective dose,[1] still being far from the original. Clinical facts suggest that at most 5% of the administered dose can be delivered to the target in the case of tumour diseases. If no breakthroughs will be achieved in the foreseeable future, prospects in the design of novel efficient delivery systems seem to be disappointing. Irrespective of progress in nanotechnologies, targeted drug delivery is essentially limited to systemic circulation. 'Overexpression' of the target receptors in human body does not ensure high selectivity of the targeted delivery system. However, this does not mean that the use of nanoparticles as drug targeting agents is unreasonable. Main research efforts should be aimed at studying the regularities that will allow one to scale promising results of preclinical studies up to clinical trial conditions.

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