

# Ligand-based targeted therapy: a novel strategy for hepatocellular carcinoma

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**Abstract:** Hepatocellular carcinoma (HCC) is the most common primary liver cancer with high morbidity and mortality worldwide. Chemotherapy is recommended to patients with intermediate or advanced stage cancer. However, the conventional chemotherapy yields low desired response rates due to multidrug resistance, fast clearance rate, nonspecific delivery, severe side effects, low drug concentration in cancer cells, and so on. Nanoparticle-mediated targeted drug delivery system can surmount the aforementioned obstacles through enhanced permeability and retention effect and active targeting as a novel approach of therapeutics for HCC in recent years. The active targeting is triggered by ligands on the delivery system, which recognize with and internalize into hepatoma cells with high specificity and efficiency. This review focuses on the latest targeted delivery systems for HCC and summarizes the ligands that can enhance the capacity of active targeting, to provide some insight into future research in nanomedicine for HCC.

**Keywords:** targeted therapy, hepatocellular carcinoma, ligand, drug delivery system, nanoparticle

## Introduction

Hepatocellular carcinoma (HCC), the most common primary liver cancer, represents the sixth most common cancer worldwide, which results in the third cause of death from cancer per year.<sup>1-3</sup> The epidemiology, risk factors, and pathogenesis were summarized in a previous highlight article.<sup>4</sup> According to the Barcelona Clinic Liver Cancer staging system, patients with intermediate or advanced stage cancer are recommended local or systemic chemotherapy.<sup>1,2,5,6</sup> However, conventional chemotherapy yields low objective response rates. Some randomized trials showed that the response rates of combined chemotherapy have been slightly increased, but those patients did not gain survival benefit.<sup>7</sup> Sorafenib (Nexavar<sup>®</sup>; Bayer Pharmaceuticals, Berlin, Germany) is a small multikinase inhibitor which blocks several tyrosine protein kinases such as vascular endothelial growth factor receptors 1, 2, and 3 and platelet-derived growth factor receptor  $\beta$ , as well as Raf kinases and intracellular serine/threonine kinases.<sup>8,9</sup> Unfortunately, the median survival of patients treated with sorafenib was just prolonged ~3 months.<sup>10</sup> It is known that the undesired outcome is associated with multidrug resistance (MDR),<sup>11</sup> fast drug elimination from bloodstream, narrow therapeutic window, nonspecific delivery, a range of side effects, low drug concentration in targeted cells, and aggregation formation due to their poor solubility, and so forth.<sup>12</sup> Thus, development of some new approach of anticancer agents is an imperative task to improve the therapeutic efficacy of HCC.

In recent years, nanoparticle (NP)-mediated targeted drug delivery system (NTDDS) has attracted researchers' great interest as a novel approach of thera-

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peutics for HCC. The NTDDS involves three essential components: a potent therapeutic such as chemotherapeutic agent, gene, or photosensitizer; a rational delivery vehicle to transport therapeutics in high concentration into tumor cells with long circulation time and excellent stability; and a surface ligand that mediates delivery vehicle to interact with and internalize into hepatoma cells with high specificity and efficiency.<sup>13–15</sup> Targeted delivery could overcome the deficiencies of conventional administration routes as mentioned earlier. As NPs for targeted delivery of therapeutics and small interfering RNAs (siRNA) in HCC have been reviewed in a recent review,<sup>16</sup> this review focuses on the latest NTDDS for HCC and summarizes the ligands that can specifically bind with the matching receptors on hepatoma cell membrane and trigger the receptor-mediated endocytosis, to provide some insight into future research in nanomedicine for HCC.

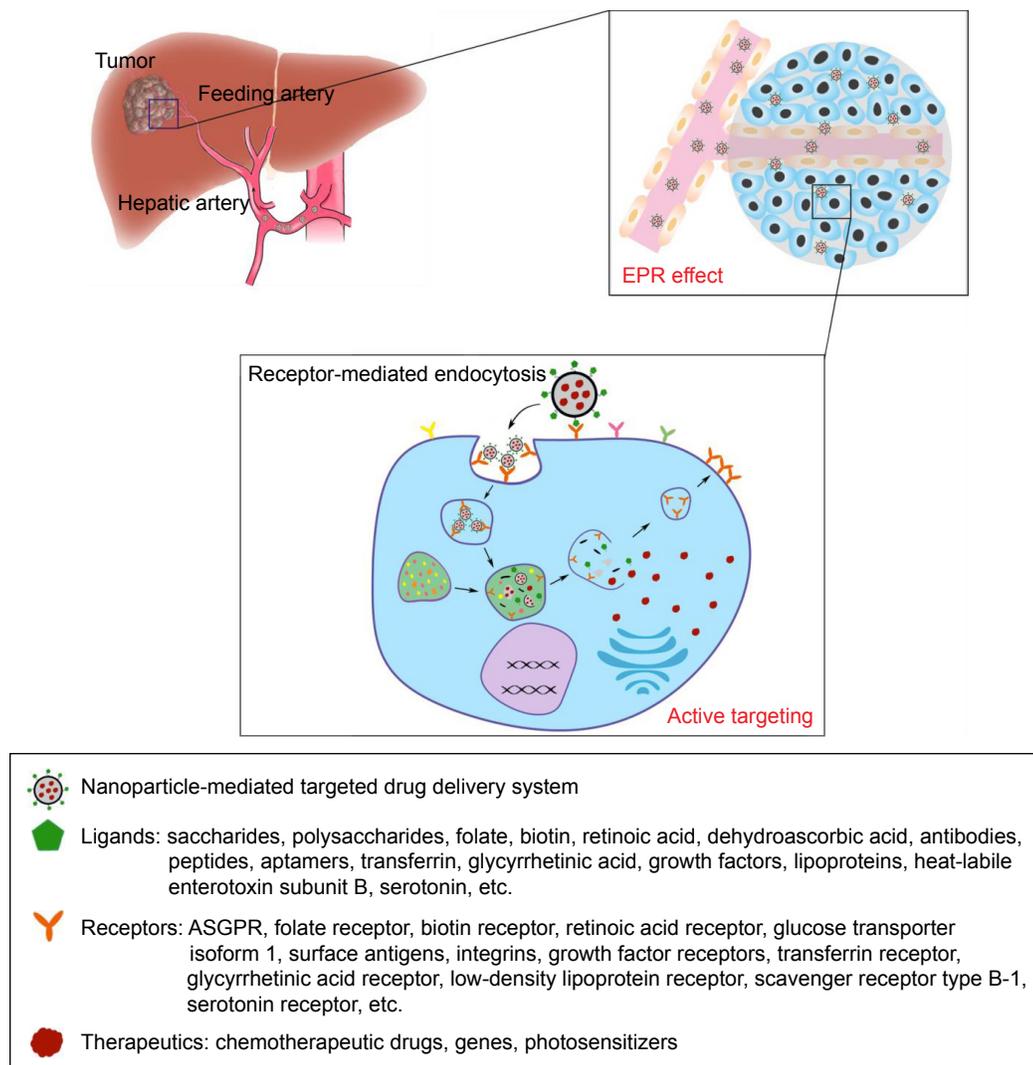
## Enhanced permeability and retention (EPR) effect in HCC

Tumor angiogenesis is a process in which new blood vessels in tumor tissue form from the existing ones. The angiogenesis is induced by various growth factors such as hypoxia inducible factor,<sup>17</sup> vascular endothelial growth factor (VEGF),<sup>18</sup> platelet-derived growth factor,<sup>19</sup> and basic fibroblast growth factor (bFGF)<sup>20</sup> because more oxygen and nutrients need to be provided to the tumor mass by dedicated blood supply to sustain fast growth beyond a size of 2 mm<sup>3</sup>.<sup>21</sup> However, the tumor vasculature is dramatically different from normal tissue blood vessels. The abnormal vascular architecture is the pathophysiological basis of passive targeting in that drug delivery systems accumulate in tumor mass much more than they do in normal tissues.<sup>22</sup> For example, Kaminskas et al<sup>23</sup> investigated the pharmacokinetics and targeting capability of PEGylated dendrimer with 12 nm in diameter and PEGylated liposome with 89 nm in hepatoma cell-bearing mice model, demonstrating that both the systems could promote increased tumor targeting of doxorubicin (DOX) and enhance tumor regression through EPR effect. In 1986, Gerlowski and Jain<sup>24</sup> and Matsumura and Maeda<sup>25</sup> first defined the phenomenon as EPR effect. The abnormal tumor vasculatures include extensive angiogenesis, inefficient lymphatic drainage, slow venous return, as well as defective vascular architectures such as discontinuous endothelial lining, lack of smooth muscle cells and pericytes, fenestrations, and so on.<sup>26,27</sup> The abnormal vasculature in HCC tissue was observed using scanning electron microscopy in Dufour's laboratory.<sup>28</sup>

The EPR effect mainly depends on the size of drug delivery system. The drug delivery system is eliminated by liver, kidney, and reticuloendothelial system (RES), which is based on their physicochemical properties.<sup>29,30</sup> Kidneys filter various metabolites and wastes from the blood into urine through the glomerulus, the pores of which are ~10 nm.<sup>31</sup> Thus, NPs with hydrodynamic diameters <10 nm will be cleared by the kidneys.<sup>32</sup> On the contrary, NPs >100 nm are removed from blood through RES such as macrophage cells in the liver and spleen.<sup>33</sup> It is reported that the NPs with 30–200 nm in hydrodynamic diameter accumulate with high efficiency in many solid tumors by EPR effect.<sup>34</sup> Distinguishingly, the fenestrations in the liver sinusoidal endothelium facilitate the substrate transfer into space of Disse between the liver sinusoid and hepatocytes in normal liver. The diameter of the fenestrations is ~78 ± 12 nm in wild-type mice.<sup>35</sup> Therefore, the size of NTDDS for HCC should be considered in order to avoid them entering into the space of Disse. Furthermore, long circulation time of the NPs without being eliminated in bloodstream is also extremely significant for their accumulation into tumor tissues through the EPR effect.<sup>36,37</sup> In normal liver, the Kupffer cells, also known as stellate macrophages, are located inside the sinusoids, thus avoiding the capture by Kupffer cells is very important for designing NTDDS for HCC. Many studies have demonstrated that NPs with more hydrophobic surface are more liable to be cleared by liver, spleen, and lungs,<sup>38</sup> whereas hydrophilic surface could make the NPs escape macrophage capture.<sup>36</sup> To achieve prolonged blood circulation, a strategy of surface functionalization has been developed by coating hydrophilic polymer such as poly(ethylene glycol) (PEG),<sup>39,40</sup> poly(vinyl pyrrolidone),<sup>41</sup> and so on on the surface of the NPs and by designing block copolymers with hydrophilic and hydrophobic domains.<sup>42</sup>

## Ligand-based active targeting

As described in Figure 1, the NTDDS could disperse in tumor mass through feeding arteries and then could accumulate into tumor interstitial fluid through fenestration by EPR effect. More importantly, the specific NP–cell surface interactions play a critical role in facilitating internalization of NPs into targeting tumor cells.<sup>43</sup> The receptor-mediated endocytosis, an approach of active targeting, is one of the most common strategies for HCC to further improve the targeting property.<sup>44,45</sup> Fortunately, some proteins and molecules are overexpressed on the surface of hepatoma cells or intratumoural angiogenesis compared to normal cells in Figure 2, thus their ligands, including (poly)saccharides (Table 1), vitamins (Table 2), antibodies (Table 3),



**Figure 1** The schematic diagram of ligand-based targeted therapy of NTDDS for HCC through EPR effect and active targeting.

**Abbreviations:** NTDDS, nanoparticle-mediated targeted drug delivery system; HCC, hepatocellular carcinoma; EPR, enhanced permeability and retention; ASGPR, asialoglycoprotein receptor.

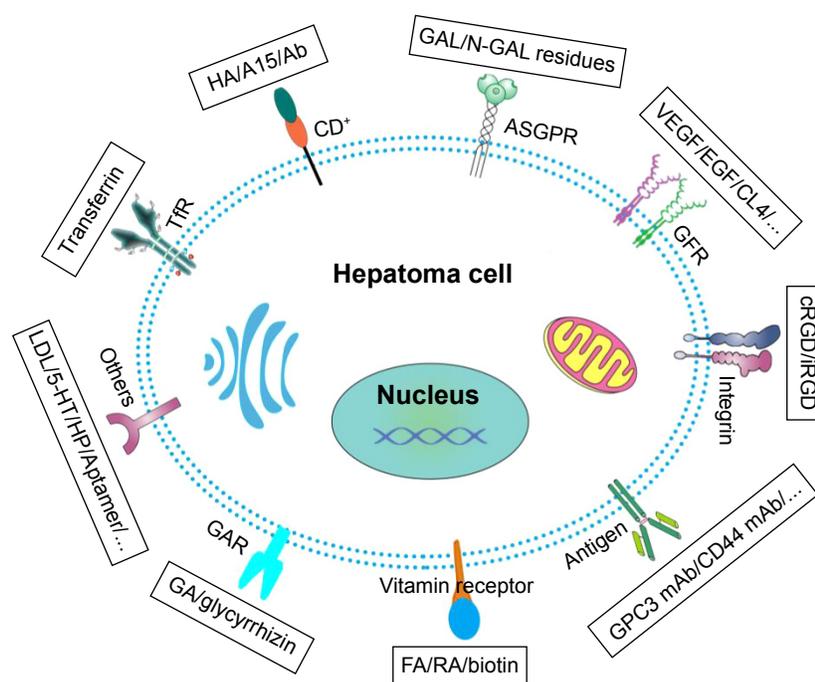
peptides (Table 4), aptamers (Tables 4 and 5), transferrin (Tf) (Table 6), other small molecules, growth factors, and so on (Table 7), were utilized to decorate drug delivery system in order to enhance the recognition between NPs and tumor cells. Subsequently, the NTDDS was internalized into cells by receptor-mediated endocytosis triggered by the ligands on the surface of NPs, and then the therapeutics in NTDDS was released into cytoplasm in order to kill the tumor cells.

## Saccharide- or polysaccharide-based active targeting

### Saccharides

Various glycans attached to protein molecules as glycoproteins and proteoglycans on the exterior surface of cancer cells are often upregulated as compared with the healthy cells,

which renders saccharides or polysaccharides as potential ligands for liver-targeting drug delivery.<sup>46,47</sup> Particularly, the asialoglycoprotein receptor (ASGPR), also known as “The Ashwell-Morell Receptor,” was first isolated and characterized by Baenziger and Maynard.<sup>48</sup> It is known that the ASGPR is the most common target site of NTDDS for HCC because of the high expression on hepatocytes and hepatoma cells but minimal expression on extrahepatic cells.<sup>49–51</sup> For example, the *in vitro* ASGPR analysis showed that the hepatoma cell HepG2 expresses 76,000 ASGPRs/cell with a high density on membrane.<sup>49</sup> These receptors can recognize and bind D-galactose (Gal) and *N*-acetylgalactosamine residues with high specificity and efficiency. The common ligand moieties to ASGPR in previous literatures were enumerated in Figure 3. The centroid consisted of six amino acids in H1



**Figure 2** The summary of receptors overexpressed on hepatoma cell and their ligands for targeted therapy of HCC.

**Abbreviations:** HCC, hepatocellular carcinoma; Tfr, transferrin receptor; FA, folic acid; RA, retinoic acid; GA, glycyrrhetic acid; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; LDL, low-density lipoprotein; HP, hematoporphyrin.

**Table 1** Summary of saccharide- or polysaccharide-based active drug/gene delivery systems for HCC to enhance the targeting effect

Ligands	Matching receptors	Therapeutic agents	Delivery vehicles	References
Tetraacetylbro- $\alpha$ -D-galactose	ASGPR	Mitoxantrone	Liposome	53
1-Thio- $\beta$ -D-galactose derivative	ASGPR	Plasmid pXL3031	PEGylated lipids	54
Lactobionic acid	ASGPR	10-Hydroxycamptothecin	Chitosan	59
		Paclitaxel	Poly(ethylene glycol)- <i>b</i> -poly( $\epsilon$ -caprolactone)	60
		Doxorubicin	Poly(ethylene glycol)-poly( $\epsilon$ -caprolactone)	61
		Doxorubicin	Poly( $\epsilon$ -caprolactone)- <i>g</i> -SS-lactobionic acid	62
		Paclitaxel	Poly(ethylene glycol)- <i>b</i> -poly(mono-2,4,6-trimethoxy benzylidene-pentaerythritol carbonate-co-acryloyl carbonate)/poly(ethylene glycol)- <i>b</i> -poly( $\epsilon$ -caprolactone)	63
		Oridonin	Chitosan- <i>g</i> -poly( <i>N</i> -isopropylacrylamide)	64
		Doxorubicin	Chitosan/polyvinyl alcohol	65
		Doxorubicin	Hollow mesoporous silica nanoreservoir	66
		Docetaxel	Solid lipid nanoparticles	67
		pEGFP-N1	Chitosan	68
		pEGFP-C1	Polyethylenimine- <i>g</i> -poly(ethylene glycol)	69
		pDNA	Poly[2-(2-aminoethoxy)ethoxy]phosphazene	70
Lactose	ASGPR	N/A	Poly(ethylene glycol)-poly(L-lactide)	71
		pcDNA3.1(+)-RASSF1A	Carboxymethyl chitosan	72
Galactosamine	ASGPR	Paclitaxel	Poly( $\gamma$ -glutamic acid)-poly(lactide)	73
		Docetaxel	D- $\alpha$ -Tocopherol polyethylene glycol 1000 succinate-poly(lactide)	74
2-Lactobionamidoethyl methacrylamide	ASGPR	Iodoazomycin	Poly[di(ethylene glycol) methyl ethyl methacrylate]- <i>st</i> -[ <i>N,N'</i> -methylenebis(acrylamide)]	76
Hyaluronic acid	CD44	Metformin	Gold nanoparticles	88
Pectin	ASGPR	pEGFP-C2 plasmid DNA	Pectin	89
		Methotrexate	Pectin	90
		5-Fluorouracil	Pectin	91
Pullulan	ASGPR	Paclitaxel/combretastatin A4	Poly( $\beta$ -amino ester)/poly(lactic-co-glycolic acid)	93

**Abbreviations:** ASGPR, asialoglycoprotein receptor; CD44, cluster determinant 44; EGFP, enhanced green fluorescent protein; HCC, hepatocellular carcinoma; N/A, not applicable.

**Table 2** Summary of vitamin-based active drug/gene delivery systems for HCC to enhance the targeting effect

Ligands	Matching receptors	Therapeutic agents	Delivery vehicles	References
Folic acid	Folate receptors	Triptolide	Pluronic F127/pH-sensitive peptide	13
		Doxorubicin	Poly(ethylene glycol)-poly(D,L-lactide)	100
		Docetaxel/iSur-pDNA	Poly(ethylene glycol)-distearoylphosphatidylethanol-amine	101
		Doxorubicin/BCL-2 siRNA	Poly(ethylene glycol)- <i>b</i> -poly(glutamic acid)	103
		pGL3-EA4D-tBid	Polyethylenimine-cyclodextrin	104
		Doxorubicin/PDTC	Chitosan	105
		Paclitaxel	Poly(ethylene glycol)- <i>b</i> -poly(lactide)	109
		Paclitaxel	Poly(ethylene glycol)/poly[N-(N',N'-diisopropylaminoethyl)aspartamide]/cholic acid	110
		Doxorubicin	Poly(ethylene oxide)-trimellitic anhydride chloride	113
		Doxorubicin	Poly(ethylene glycol)- <i>b</i> -poly[N-(N',N'-diisopropylaminoethyl)glutamine]	114
		Sorafenib	PEGylated poly(lactide-co-glycolide)	115
		Gemcitabine	Poly(acrylic acid)/chitosan	116
		psiRNA-TBLR I	Poly(ethylene glycol)- <i>g</i> -polyethylenimine	117
Biotin	Biotin receptor	Adriamycin	Pullulan acetate	121
		N/A	Silica nanoparticles	124
Retinoic acid	Retinoic acid receptor $\alpha$	Doxorubicin	Chitosan-albumin	128
Dehydroascorbic acid	Glucose transporter isoform I	Doxorubicin	Poly(ethylene glycol)-pLys-pPhe nanomicelle	135

**Abbreviations:** HCC, hepatocellular carcinoma; PDTC, pyrrolidinedithiocarbamate; N/A, not applicable.

subunit of ASGPR which forms an active ligand-binding site.<sup>49</sup> The ASGPR is a calcium-dependent receptor, that is to say, calcium is essential for recognition and interaction between ASGPR and ligand moieties.<sup>52</sup>

Galactosylated polymers were developed as a drug/gene carrier system for targeted delivery in HCC therapy.<sup>53,54</sup> Zhang et al<sup>53</sup> coupled Pluronic P123 with tetraacetyl bromo- $\alpha$ -D-galactose (Gal-P123) through Koenigs–Knorr reaction and then fabricated mitoxantrone (MX)-loaded

Gal-P123-modified liposomes (MX-LPG) for targeting cancer cells and reverse of MDR in HCC. Compared with free MX, MX-LPG had 2.3-fold higher cytotoxicity in hepatoma Huh-7 cells and a 14.9-fold increased intracellular MX accumulation in breast cancer resistance protein-overexpressing MDCKII cells in vitro. Moreover, MX-LPG strengthened the capability of antitumor and tumor targeting in mice bearing orthotopic xenograft HCC tumors in vivo. All the results indicated that Gal moiety is a potential active ligand

**Table 3** Summary of antibody-based active drug/gene delivery systems for HCC to enhance the targeting effect

Ligands	Matching receptors	Therapeutic agents	Delivery vehicles	References
HAb18 mAb F(ab') <sub>2</sub>	HAb18G/CD147	Doxorubicin	Poly(D,L-lactic-co-glycolic acid)-poly(ethylene glycol)	140
CD147 mAb	CD147	$\alpha$ -Hederin	Chitosan	141
SM5-I scFv	SM5-I binding protein	Paclitaxel	Poly(lactic-co-glycolic acid)	146
SM5-I mAb F(ab')	SM5-I binding protein	PE38KDEL-I	Poly(lactic-co-glycolic acid)	147
EGFR mAb	EGFR	N/A	Poly(lactic acid-co-L-lysine)	150
9B9 mAb	EGFR	ACHe gene	Polyethylenimine- <i>g</i> - $\alpha$ , $\beta$ -poly(N-3-hydroxypropyl)-D,L-aspartamide	151
Anti-VEGF mAb	VEGF	<sup>131</sup> Iodine	Dextran magnetic nanoparticles	152
CD44 Ab	CD44	Doxorubicin or triple fusion plasmid	Liposomal nanoparticles	154
GRP78 mAb	GRP78	5-Fluorouracil	Bovine serum albumin	157
AF-20 mAb	AF-20 antigen	N/A	Liposome	159
Anti-GPC3 heavy chain A (HN3)	Glypican-3	N/A	N/A	165, 166
GPC3 mAb	Glypican-3	siRNA	Chitosan-poly(ethylene glycol)-polyethylenimine	167

**Abbreviations:** mAb, monoclonal antibody; CD147, cluster determinant 147; EGFR, epidermal growth factor receptor; AChE, acetylcholinesterase; VEGF, vascular endothelial growth factor; CD44, cluster determinant 44; GRP78, glucose-regulated protein 78; siRNA, small interfering RNA; HCC, hepatocellular carcinoma; N/A, not applicable.

**Table 4** Summary of peptide or aptamer-based active drug/gene delivery systems for HCC to enhance the targeting effect

Ligands	Matching receptors	Therapeutic agents	Delivery vehicles	References
RGD peptide	Integrin $\alpha\beta3$	Paclitaxel	Liposome	171
c(RGDfK) peptide	Integrin $\alpha\beta3$	Doxorubicin/Verapamil	Poly(lactic acid-co-glycolic acid)-magnetic nanoparticles	174
iRGD peptide	Integrins $\alpha\gamma$	Paclitaxel	Poly( $\epsilon$ -caprolactone)-poly( <i>N</i> -vinylpyrrolidone)	176
		Salinomycin	1,2-Distearoyl- <i>sn</i> -glycero-3-phosphoethanol-amine- <i>N</i> -[methoxy(polyethylene glycol)-2000]	177
		Doxorubicin/Sorafenib	1,2-Distearoyl- <i>sn</i> -glycero-3-phosphoethanol-amine- <i>N</i> -[methoxy(polyethylene glycol)-2000]/egg lecithin	178
GE11 peptide	EGFR	Doxorubicin	1,2-Distearoyl- <i>sn</i> -glycero-3-phosphoethanol-amine- <i>N</i> -[methoxy(polyethylene glycol)-2000]	181
		<sup>131</sup> Iodide	Polyethylenimine-poly(ethylene glycol)	182
YC21 peptide	EGFR	AChE gene	Polyethylenimine- $\beta$ -cyclodextrin	183
CY11 peptide	FGFR	pDNA	Polyethylenimine- $\beta$ -cyclodextrin	186
SP94 peptide	N/A	Doxorubicin	PEGylated liposome	187
		N/A	Heat shock protein cage	188
		Aldoxorubicin	Protein cage encapsulin	189
T7 peptide	Transferrin receptor	Doxorubicin/pORF-hTRAIL	Poly(ethylene glycol)-modified polyamidoamine dendrimer	191, 194
A15 and CL4 aptamers	CD133 and EGFR	Salinomycin	Poly(D,L-lactic-co-glycolic acid)	198
EpCAM aptamer	EpCAM	Doxorubicin	Carboxymethyl cellulose	200

**Abbreviations:** EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; AChE, acetylcholinesterase; CD133, cluster determinant 133; EpCAM, epithelial cell adhesion molecule; HCC, hepatocellular carcinoma; N/A, not applicable.

**Table 5** The sequence of aptamers reported in previous publications for HCC

Aptamers	Sequence
A15	5'-CCCUCUACAUAGGG-3'
CL4	5'-GCCUUAGUACGUGCUUUGAUGUCGAUUCGACAGGAGGC-3'
TLS11a	5'-ACAGCATCCCCATGTGAACAATCGCATTGTGATTGTTACGGTTTCCGCCTCATGGACGTGCTG-3'
AS1411	5'-GGTGGTGGTGGTGGTGGTGGTGGTGG-3'
LY1	5'-TTGGGTGTTAGGCTGGTCTTAATCGGGTCGGGTTGCTG-3'

**Abbreviation:** HCC, hepatocellular carcinoma.

**Table 6** Summary of transferrin-based active drug/gene delivery systems for HCC to enhance the targeting effect

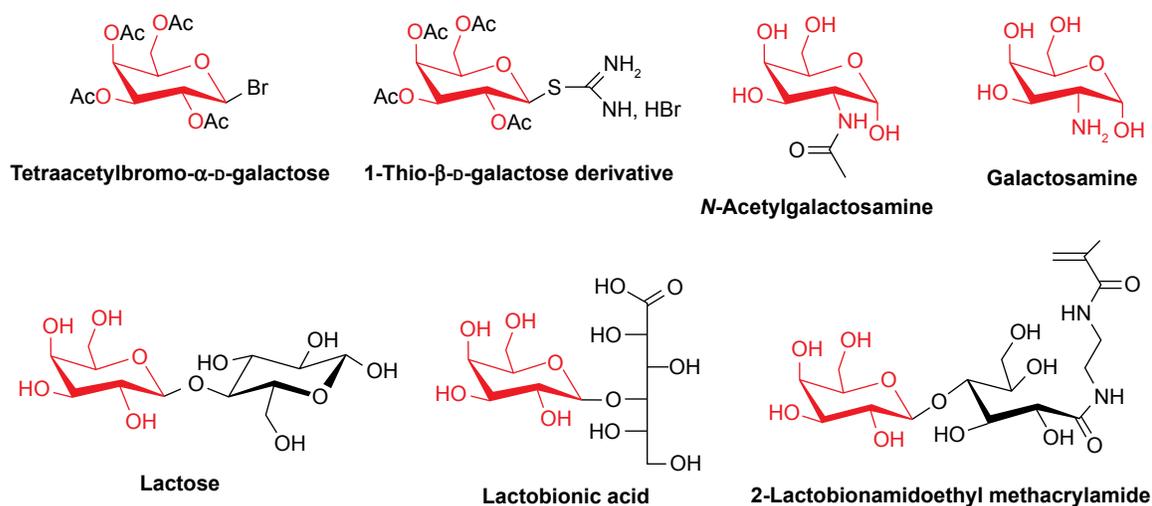
Ligands	Matching receptors	Therapeutic agents	Delivery vehicles	References
Transferrin	Transferrin receptor	Doxorubicin/Sorafenib	Poly(vinyl alcohol)/albumin	210
		Anti-miR-221	Liposome	211
Apotransferrin	Transferrin receptor	Doxorubicin	Apotransferrin	213
Apotransferrin/lactoferrin	Transferrin/lactoferrin receptor	Doxorubicin	Apotransferrin/lactoferrin	215, 216

**Abbreviation:** HCC, hepatocellular carcinoma.

**Table 7** Summary of other ligands-based active drug/gene delivery systems for HCC to enhance the targeting effect

Ligands	Matching receptors	Therapeutic agents	Delivery vehicles	References
Glycyrrhetic acid	Glycyrrhetic acid receptor	Doxorubicin/shAkt1	Polyethylenimine	219
		Curcumin	Dextran	220
Epidermal growth factor	EGFR	Plasmid pCMV/luc <sup>27</sup>	Polyethylenimine	223
LDL	LDL-R	Doxorubicin/siRNA	<i>N</i> -Succinyl chitosan	227
		Osthole	<i>N</i> -Succinyl chitosan	228
		Docosahexaenoic acid	Low-density lipoprotein	229
Hematoporphyrin	LDL-R	Doxorubicin	Bovine serum albumin	231, 232
Reconstituted high-density lipoprotein	Scavenger receptor type B-I	Chol-siRNA	Lipoplexes	235
Heat-labile enterotoxin subunit B	Ganglioside GM1	5-fluorouracil	Bovine serum albumin	238
Epigallocatechin gallate	67 kDa laminin receptor	[Ru(bpy) <sub>2</sub> (4-B)](ClO <sub>4</sub> ) <sub>2</sub> ·2H <sub>2</sub> O	Ruthenium nanoparticles	242
5-HT	5-HT receptor	Plasmid DNA	Liposome	246
Octreotide	G-protein coupled receptors	Hydroxycamptothecine	Liposome	250

**Abbreviations:** EGFR, epidermal growth factor receptor; LDL-R, low-density lipoprotein receptor; 5-HT, serotonin; HCC, hepatocellular carcinoma.



**Figure 3** Chemical structures of saccharides for targeted therapy of HCC.  
**Abbreviation:** HCC, hepatocellular carcinoma.

for targeted therapy in HCC. Gal-decorated gene carrier was also developed to investigate the transfection efficiency, demonstrating that galactosyl ligand could enhance the validity of targeted gene transfer for HCC.<sup>54</sup>

Lactobionic acid (LA), comprising gluconic acid and Gal moiety, is the common ligand for hepatoma-targeted delivery.<sup>55–57</sup> The Gal residues on drug delivery system can recognize and bind specifically to the ASGPR on the hepatoma cells,<sup>58,59</sup> thus they facilitate drug delivery into the cells, which are inhibited and killed by therapeutic agents in delivery vehicles. Zhong's group developed a series of Gal-directed hepatoma-targeting delivery system to enhance the accumulation of anticancer drugs into and antitumor activity toward hepatoma cells.<sup>60–63</sup> The anticancer drugs were transported into and released in hepatoma cells through receptor-mediated endocytosis between Gal residues and ASGPR on the cells. Consequently, these Gal-directed hepatoma-targeting delivery systems inhibited the proliferation of the human hepatoma cells *in vitro* and tumor growth *in vivo* with enhanced efficiency compared with nontargeting delivery systems or free drugs. Galactosylated chitosan (GC) was conjugated with other polymers to enhance the drug-loading capacity and uptake of drug into hepatoma cells.<sup>64,65</sup> By coincidence, the targeting agent LA was immobilized on the surface of hollow mesoporous silica nanoreservoirs through linking with  $\beta$ -cyclodextrin.<sup>66</sup> Besides, a large number of research studies reported that LA-modified delivery system could enhance the uptake of drugs or genes into hepatoma cells as a novel therapeutic strategy for HCC.<sup>67–70</sup>

Lactose is a disaccharide sugar composed of Gal and glucose groups. In aqueous solution, the glucose group

becomes the chain structure, which can react with amino group.<sup>51,71,72</sup> The Gal group yet maintains a stable ring structure. In addition, some research studies indicated that only a small amount of NPs without galactosamine was internalized into hepatoma cells, whereas the galactosamine-modified NPs lead to high targeting to hepatic tumor and facilitate their cellular uptake.<sup>73,74</sup> Hence, lactose and galactosamine are also potential ligands to ASGPR. To develop galactosylated polymer for drug and gene delivery to hepatoma cells and hepatocytes, Narain's group synthesized 2-lactobionamidoethyl methacrylamide including Gal residues,<sup>75,76</sup> which could be used in composing copolymer through reversible addition-fragmentation chain transfer polymerization technique.

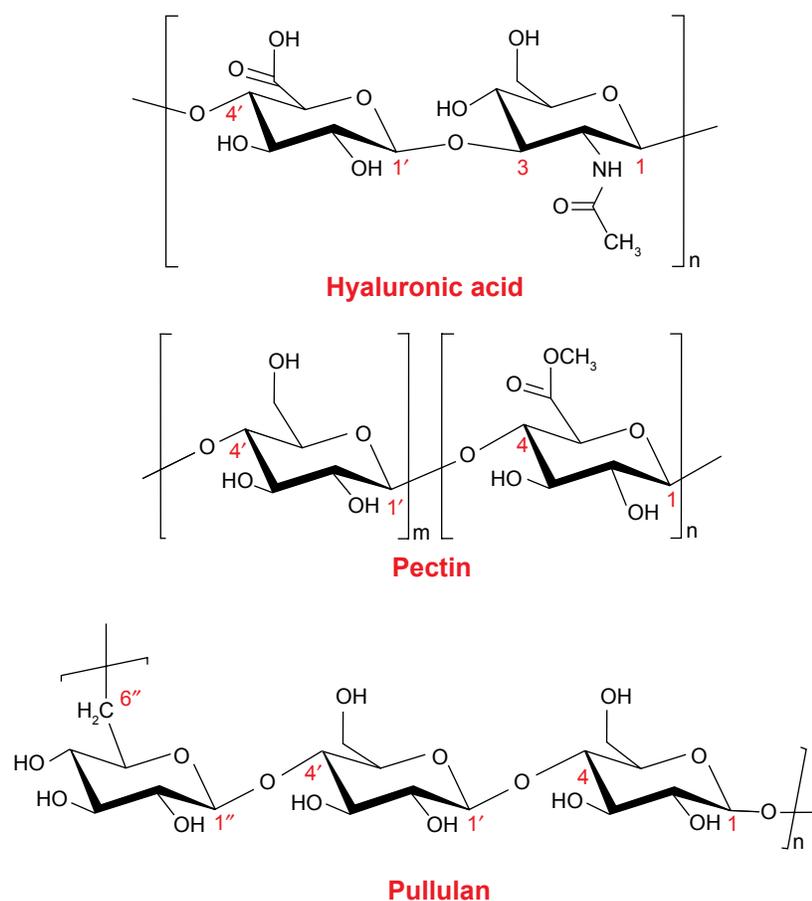
The endocytotic pathway involved in the ASGPR-mediated endocytosis of NPs was investigated through pretreating hepatoma cells with inhibitors of clathrin-mediated endocytosis (CIME), caveolae-mediated endocytosis (CaME), and micropinocytosis. The experiments demonstrated that galactosylated NPs stimulate intracellular uptake by CIME and CaME.<sup>59,67</sup> On the other hand, the "cluster effect" has impact on the receptor–ligand interaction.<sup>77</sup> It is confirmed that the Gal density on drug delivery system is important for effective recognition by ASGPR and internalization into hepatoma cells.<sup>78,79</sup> Definitely, the uptake capacity is enhanced with increase in the ratio of Gal moieties. The space between Gal moieties and vehicle surface is also another parameter of targeting specificity. The adequate PEG spacer could further increase the targeting capacity of ASGPR-overexpressing hepatoma cells and thus improve the antitumor efficacy.<sup>80</sup> The route of administration is another concern. A recent study demonstrated that galactosylated

polymer carrier still retained the capacity to enhance the accumulation of chemotherapeutic agent sorafenib into liver through oral administration compared with the non-targeting polymer carrier.<sup>81</sup>

### Polysaccharides

In addition to saccharides, hyaluronic acid (HA), a natural biodegradable and biocompatible linear polysaccharide, is a multifunctional glycosaminoglycan composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, linked through alternating  $\beta$ -1,4 and  $\beta$ -1,3 glycosidic bonds in Figure 4,<sup>82,83</sup> and has been utilized in arthritis treatment, tissue augmentation, ocular surgery, and so forth.<sup>82</sup> Various HA receptors including cluster determinant 44 (CD44), receptor for hyaluronate-mediated motility, HA receptor for endocytosis (HARE), and lymphatic vessel endothelial hyaluronan receptor-1 were found in different tissues for different biological functions.<sup>82</sup> In recent years, HA has also been investigated as a targeting moiety of NTDDS for cancer therapy because of the overexpression of CD44 in

various cancer cells.<sup>84-86</sup> A recent study demonstrated that HA could actively mediate NPs into hepatoma cells. For instance, the surface modification of superparamagnetic iron oxide (SPIO) NPs was performed by conjugating HA, and the in vitro magnetic resonance (MR) imaging of CD44<sup>+</sup> HepG2 cells and in vivo MR imaging of mice HepG2 cell-bearing tumor xenografts confirmed the high efficiency and targeting of liver carcinoma.<sup>87</sup> Furthermore, a HA-capping gold NPs (AuNPs) loaded with metformin (MET) were developed through the amide bond formation between carboxyl groups of HA on AuNPs and amine group of MET,<sup>88</sup> in which the HA serves as a targeting agents for liver cancer cells. The cell apoptosis assay indicated that the drug system pronounced higher apoptotic behavior in HepG2 cells than CD44<sup>-</sup> NIH 3T3 cells due to the higher cellular uptake in HepG2 cells through HA-CD44-mediated endocytosis. All the studies suggest that the HA could be a promising targeting agents for drug delivery in HCC therapy. On the other hand, the interaction of HA and HARE receptors on liver sinusoidal endothelial cells occurs, thus



**Figure 4** Chemical structures of polysaccharides for targeted therapy of HCC.

**Abbreviation:** HCC, hepatocellular carcinoma.

HA could hypothetically be beneficial for liver targeting.<sup>82</sup> Consequently, it is a new problem that how to escape the endocytosis of liver sinusoidal endothelial cells when the HA-functioned NTDDS targeted to HCC are injected into blood stream. As a polyanionic polymer, HA can be used as the constituent of pH-sensitive NTDDS owing to its degradability by hyaluronidase (HAase) widely distributed in the acidic tumor extracellular matrix, which is discussed further in the following subsection of dual-ligand-mediated active targeting.

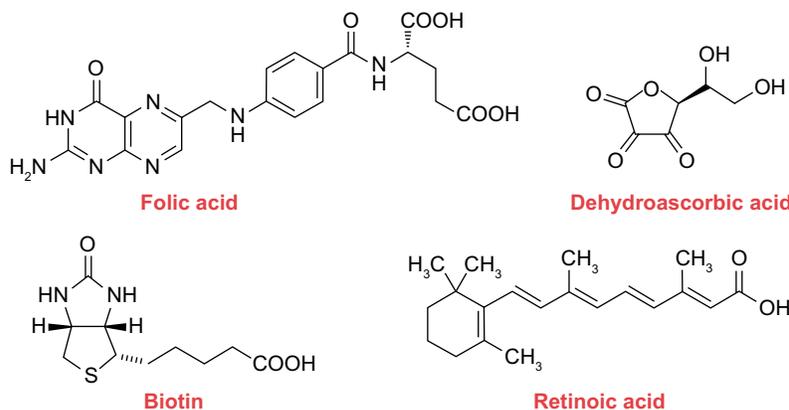
Pectin, another polysaccharide with anion, water solubility, and non-toxicity, has attracted attention for targeted therapy in HCC.<sup>89-91</sup> It is a natural linear polymer mainly consisting of  $\alpha$ -(1-4)-linked D-polygalacturonic acid residues in Figure 4 and extracted from citrus peels or apple pomaces. The D-polygalacturonic acid is an oxidized form of Gal, thus the pectin displays active targeting ability to hepatoma cells contributing to the specific interaction between galacturonic acid residues and ASGPR on the cells. The pEGFP-C2 plasmid DNA (pDNA), encoding enhanced green fluorescent protein (EGFP), was loaded into pectinate micro/nanoparticles through ionotropic gelation by Opanasopit et al.<sup>89</sup> The transfection efficiency of pEGFP-C2 pectinate NPs in hepatoma Huh7 cells was examined, displaying that the NPs were capable of mediating the transfection into Huh7 cells compared with free pDNA, and the extent of transfection depended on the ratio of pectin to DNA and the amount of pectinate NPs. The conjugation and release of chemotherapeutic agent methotrexate (MTX) in pectinate NPs were investigated in a subsequent study.<sup>90</sup> The results verified that pectin can deliver MTX to HepG2 cells with high efficiency and specificity and exhibited sustained drug release. Recently, *in vitro* and *in vivo* evaluation of pectinate

NPs loaded with anticancer drug 5-fluorouracil (5-FU) for HCC was carried out, and the pectin served as drug delivery vector of chemotherapy agent and natural targeting ligand to ASGPR in this NTDDS.<sup>91</sup> The content of 5-FU in HepG2 cells incubated with the nano-system was significantly higher than that in cells treated with free 5-FU due to the high efficiency of NTDDS. The ASGPR-mediated recognition and subsequent endocytosis of pectinate NPs was proved by blocking the ASGPR on HepG2 cells by free Gal, indicating that it is a promising platform for targeted therapy of HCC through specific binding of galacturonic acid residues of pectin and ASGPR on hepatoma cells.

Another polysaccharide pullulan, consisting of maltotriose units connected by  $\alpha$ -1,4 and  $\alpha$ -1,6 glycosidic bonds, is also a natural ligand for ASGPR, thus it conduces to HCC-targeted drug delivery.<sup>92</sup> Mediating through interaction between polysaccharide backbone of pullulan and ASGPR, the pullulan-coated drug carrier significantly inhibited hepatoma cell proliferation and migration, as well as tumor growth and angiogenesis.<sup>93</sup>

### Vitamin-based active targeting

Vitamins are a series of organic compounds and vital nutrients that all living cells require for their survival. Rapid proliferation of tumor cells, in particular, requires certain vitamins in excess such as folate, biotin, retinoic acid (RA), and dehydroascorbic acid (DHAA) to sustain their rapid growth. The chemical structures of these vitamins are presented in Figure 5. Compared to the normal cells, the receptors involved in the uptake of the vitamins are thus upregulated on tumor cell surface. Consequently, these vitamin receptors serve as beneficial target substrates for tumor-targeted drug delivery.



**Figure 5** Chemical structures of vitamins for targeted therapy of HCC.  
**Abbreviation:** HCC, hepatocellular carcinoma.

## Folate

Folate or folic acid (FA), referred to as water-soluble vitamin B<sub>9</sub>, vitamin M, and vitamin B<sub>c</sub>, is required by eukaryotic cells for facilitating the transfer of one-carbon units from donor molecules into vital biosynthetic pathways such as methionine, purine, and pyrimidine biosynthesis as various coenzymes.<sup>94,95</sup> Besides, it plays an important role in the interconversion of serine and glycine, as well as histidine catabolism.<sup>95</sup> Unfortunately, animal cells need to capture exogenous folates to sustain life because of lacking key enzymes of the biosynthetic pathway themselves.<sup>94</sup> The receptor-mediated endocytosis is the main mechanism of the cellular internalization through folate receptors (FRs) with a high affinity ( $K_D \sim 10^{-10}$  M).<sup>96</sup>

Natural product triptolide has been proved to be highly effective against many tumor cells including cholangiocarcinoma,<sup>97</sup> pancreatic cancer,<sup>98</sup> HCC,<sup>99</sup> and so on. However, the clinical applications have been limited by poor solubility and extreme side effects. Hence, Ling et al<sup>13</sup> synthesized smart pH-sensitive nanoformulated triptolide (Nf-Trip) coated with folate as a targeted therapeutic strategy for HCC. The hepatoma cells BEL-7402 with positive FRs and normal hepatocyte MIHA with negative FRs were selected to investigate the in vitro cellular uptake. When incubated with Nf-Trip, the MIHA cells showed very little cellular uptake, whereas the BEL-7404 cells were highly efficient. However, the FRs on BEL-7404 cells were competitively blocked by an excess amount of free FA, the cellular uptake of Nf-Trip dramatically decreased, suggesting that folate is an excellent hepatoma cell-specific ligand that effectively facilitate the endocytosis of Nf-Trip. In HCC orthotopic xenograft model, the biodistribution in vivo showed that a large amount of specific accumulation of the Nf-Trip was detected in the liver tumor tissue. Moreover, the Nf-Trip reduced tumor burden and improved survival without systemic toxicity.

Although FA-functionalized drug delivery loaded with small molecular drugs such as DOX and docetaxel could induce tumor cell apoptosis,<sup>100,101</sup> hepatoma cells may advance several mechanisms to resist apoptosis. Downregulation of antiapoptotic genes enhanced the sensitivity of hepatoma cells to chemotherapeutic agents by RNA interference technology.<sup>101–104</sup> Therefore, Cao et al<sup>103</sup> developed a multifunctional drug carrier that co-delivered DOX and siRNA against the antiapoptotic *BCL-2* gene. The DOX and *BCL-2* siRNA was much more efficiently transferred into BEL-7402 cells by the FA-modified nanocomplex than the nontargeting one. Moreover, the

DOX-induced cell death was enhanced by the co-delivery of *BCL-2* siRNA, indicating that co-loading siRNA and chemotherapeutic agent in a targeted drug delivery enables simultaneous delivery of specific siRNA and drug into hepatoma cell with synergistic effect in antitumor activity, which was also proved by another study.<sup>101</sup> Co-delivering two chemotherapeutic agents in a drug delivery is alternative strategy to overcome MDR of HCC. For example, multifunctional DOX loaded-folate-chitosan self-assembly micellar NPs co-delivered pyrrolidinedithiocarbamate, a NF- $\kappa$ B inhibitor, to achieve specific targeting and to surmount the DOX MDR.<sup>105</sup>

Tumor metastasis remains the major obstacle for the improvement in the long-term survival after HCC resection.<sup>106,107</sup> Intrahepatic metastasis is the most frequent, followed by extrahepatic pulmonary metastasis.<sup>108</sup> NP-mediated targeted therapy is a promising antimetastatic strategy for pulmonary metastasis. FA and paclitaxel (PTX) containing composite micelles (FA-M[PTX]) were fabricated by co-assembling the ligand FA polymer conjugate and chemotherapeutic agent PTX polymer conjugate.<sup>109</sup> Compared with M[PTX] and free MTX with equivalent dose of PTX, the pulmonary metastasis in intravenously injected murine hepatoma 22 bearing BALB/c mice models was efficiently inhibited and the survival time was significantly prolonged by FA-M[PTX].

Multifunctional delivery systems that carry therapeutic and diagnostic imaging agents with FA are emerging concept for effective targeted therapy. The diagnostic imaging agents contain fluorophores, quantum dots (QDs),<sup>110</sup> small-molecule paramagnetic agents,<sup>111,112</sup> SPIO,<sup>113–117</sup> and so forth. Wang et al<sup>110</sup> designed a smart multifunctional polymeric micelle for targeted therapy of HCC. The pH-sensitive polymeric vehicle turns PTX release “off” at neutral environment but “on” inside acidic lysosomes, and the QD encapsulation tracks pH-tunable drug release behavior and monitors the therapeutic effect. Moreover, the targeting capacity of the micelle was enhanced through the specific interaction and internalization with high affinity between ligand FA on micelle and FRs on human hepatoma cells, which was proved by cellular uptake in vitro and tumor growth inhibition in vivo. Multifunctional NPs encapsulated with SPIO is another approach to monitor the tumor progression using magnetic resonance imaging (MRI). A large quantity of FA-functioned polymeric NPs loaded with SPIO was developed,<sup>113–116</sup> which mediates by FA-FRs interaction; hence, these nanosystems could specifically accumulate in the tumor tissue and inhibit the growth of tumor.

## Biotin

Biotin, also known as vitamin H, vitamin B<sub>7</sub>, or coenzyme R, is one of the water-soluble B complex vitamin families and is a growth promotor at the cellular level.<sup>118</sup> It is reported that the biotin receptors are overexpressed more than the FRs in various cancer cells such as leukemia, colon, mastocytoma, lung, renal, and breast cancer cells.<sup>118</sup> A study demonstrated that modification of drug delivery with biotin is an effective pattern to enhance cell specificity against the cancer cells overexpressed with biotin receptors on the cell surfaces and to accelerate the internalization of the drug delivery into the targeted cancer cells through receptor-mediated endocytosis.<sup>119</sup> To verify the potential value of biotin for targeted liver neoplasms, Mishra and Jain<sup>120</sup> designed biotin-modified erythrocytes loaded with MTX by combining with *N*-hydroxysuccinimide ester of biotin. In vivo study showed that the MTX level of liver administrated with biotinylated erythrocytes was increased ~3-fold compared with free MTX and 1.8-fold compared with nonbiotinylated erythrocytes at 1 h after injection into rats, indicating that this drug system could be used to place hepatic arterial catheters for locoregional treatment of liver neoplasms.

In order to improve the cancer-targeting activity and internalization of pullulan acetate (PA) NPs, Na et al<sup>121</sup> synthesized the biotinylated PA through coupling biotin with PA between carboxyl groups of biotin and hydroxyl groups of PA. The conjugated polymers self-assemble to form the biotinylated PA NPs through a diafiltration method. The cellular uptake investigation exhibited that the biotinylated PA NPs were strongly internalized into HepG2 cells, while only slight PA NPs were absorbed in to cells. Moreover, the efficiency of internalization was enhanced with increasing biotin content.

In our previous work, the biotin-decorated fluorescent silica NPs loaded with aggregation-induced emission fluorophores (FSNP-biotin), a series of molecules emitting strong fluorescence in aggregated structure but non-fluorescence or weak fluorescence in disperse state,<sup>122,123</sup> were fabricated to verify the targeting efficiency and mechanism of endocytosis into hepatoma cells.<sup>124</sup> After 3 h of staining, strong fluorescence was emitted from the hepatoma cells BEL-7402 with overexpression of biotin receptors, whereas dim fluorescence was observed from normal liver cells LO2 with low expression of biotin receptors. To prove the mechanism of ligand–receptor interactions or the occurrence of receptor-mediated endocytosis, the biotin receptors on BEL-7402 cell surface were blocked through the incubation of free biotin prior to staining. The fluorescence in these cells was markedly

decreased. All results also indicate that biotin can enhance the cellular uptake by hepatoma cells as a ligand.

## RA

RA is a metabolite of vitamin A that underlies the functions of vitamin A with a vital role in the regulation of cell proliferation and development.<sup>125</sup> Additionally, RA serves as an anti-HCC agent by regulating apoptosis and differentiation, as well as modulating the sensitivity of tumor cells to the innate immune response.<sup>126</sup> Among the RA receptor subtypes  $\alpha$ ,  $\beta$ , and  $\gamma$ , the level of mRNA and protein of RA receptor  $\alpha$  in HCC tissue was dramatically higher than those in normal liver tissue, and the expression of RA receptor  $\alpha$  mRNA and protein was higher than that of RA receptor  $\beta$  and  $\gamma$  in HCC tissue, suggesting that the RA receptor  $\alpha$  is the dominant receptor in HCC.<sup>127</sup> The RA-decorated chitosan-albumin NPs were developed using an ionic coacervation method by Varshosaz et al.<sup>128</sup> The HepG2 cells incubated with RA-decorated chitosan-albumin NPs exhibited rapid internalization and accumulation by cells, while just inefficient uptake of nontargeted chitosan-albumin NPs was observed. The targeting efficiency was investigated by cell proliferation assay. The higher growth inhibitory effect was observed in cells treated with RA-decorated chitosan-albumin NPs loaded with DOX compared with chitosan-albumin NPs loaded with DOX. All the results indicate that the RA could mediate the nanocarrier into HCC cells as a potential ligand.

## DHAA

Transporter-mediated pathway is more rapid and efficient in the transportation of small molecules compared to receptor-mediated transport. Recently, several transporters have been proved to be upregulated on the tumor cell surface to sustain the excess nutritional requirement of tumors.<sup>129,130</sup> Tumor cells avidly consume glucose for proliferation in hypoxic environment, termed the Warburg effect.<sup>131</sup> Glucose transporter isoform 1 (GLUT1), a representative member of the GLUT family, transports D-glucose into cells across the membrane and maintains an appropriate D-glucose concentration in cells.<sup>132</sup> It has been reported that GLUT1 is overexpressed on hepatoma cells. DHAA can be specifically recognized by GLUT1 contributing to its structural similarity to D-glucose<sup>133</sup> and rapidly reduced to ascorbic acid (AA) in cells.<sup>134</sup> Therefore, Guo et al<sup>135</sup> developed DHAA-modified PEG-pLys-pPhe nanomicelle for efficient therapy of HCC. The cellular uptake in vitro showed that the internalization of DHAA-modified PEG-pLys-pPhe nanomicelle was significantly enhanced compared with that of PEG-pLys-pPhe

nanomicelle and also demonstrated that the DHAA was mainly responsible for actively recognizing and binding to hepatoma cells BEL-7402 through GLUT1. Moreover, the *in vivo* imaging study proved that the micellar system possessed high tumor-targeting capacity, because of facilitating gradual accumulation in tumor cells. In addition, they also found that the transport capacity of GLUT1 would get to saturation state when the ratio of DHAA on micelles exceeded the number of GLUT1 on cell surface.

## Antibody-based active targeting

The conjugation of NPs with antibodies, which combines the properties of the NPs with the specific and selective recognition ability of the antibodies to the antigens on the surface of tumor cells, is a successful targeting strategy for hepatic malignancy.<sup>136,137</sup> For instance, iodine (<sup>131</sup>I) metuximab injection (Licartin<sup>®</sup>), a <sup>131</sup>I-labeled HAB18G/CD147-specific monoclonal antibody (mAb) F(ab')<sub>2</sub> fragment, has been approved for the treatment of primary HCC by the China State Food and Drug Administration.<sup>138</sup> The HAB18G/CD147, an antigen for being homologous to CD147, is highly expressed on HCC cells and tissues and can bind to the bivalent fragment HAB18 F(ab')<sub>2</sub> of HAB18 mAb with high affinity.<sup>139</sup> Jin et al<sup>140</sup> fabricated the DOX-poly(D,L-lactic-co-glycolic acid)-poly(ethylene glycol) (DOX-PLGA-PEG) micelle and further decorated with bivalent fragment HAB18 F(ab')<sub>2</sub> to improve the therapeutic effect for HCC, demonstrating that the cellular uptake was enhanced through antigen-antibody recognition between the micelle and tumor cells, and the therapeutic action was improved due to higher accumulation of DOX in tumor cells. Recent research also demonstrated that CD147 could mediate cellular internalization of NPs through the caveolae-dependent pathway and lysosomal escape with high specificity.<sup>141</sup>

SM5-1 is a mouse mAb, which binds to a SM5-1 binding membrane protein of ~230 kDa.<sup>142</sup> The membrane protein is specifically expressed on HCC, melanoma, and breast cancer cells, indicating that it is a promising binding site of NPs for diagnosis and therapy of HCC.<sup>143-145</sup> Kou et al<sup>146</sup> fabricated negatively charged PTX-loaded poly(lactide-co-glycolide) (PLGA) NPs and then successfully coated them with cationic polypeptide polylysine fused to SM5-1 single-chain antibody (SM5-1 scFv), which was derived from SM5-1 mAb. The results demonstrated that the nanosystem retained high specific affinity to SM5-1 binding protein and could induce specific and efficient death of SM5-1 binding protein-positive Ch-hep-3 cells. In addition, the F(ab') fragments of SM5-1 mAb, also known as SM5-1 mAb F(ab'), could bind to the

SM5-1 binding protein. Gao et al also developed PE38KDEL type I mutant-loaded PLGA NPs conjugated with SM5-1 mAb F(ab').<sup>147</sup> The results showed that the NPs were specifically internalized into Ch-hep-3 cells with high efficiency and exhibited potent cytotoxicity of hepatoma cells.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase overexpressed in some solid tumors such as HCC,<sup>148</sup> and some studies proved that the upregulation of EGFR was positively correlated with the tumor progression.<sup>149</sup> The human hepatoma cells can be effectively inhibited through blocking the signal transduction of EGFR. On the other hand, the EGFR can also be used as a mediator for the targeted delivery system. The EGFR mAb-modified poly(lactic acid-co-L-lysine) (PLA-PLL) NPs were fabricated by Liu et al,<sup>150</sup> which were internalized into hepatoma cells with more efficiency and specificity mediated by ligand-receptor recognition. 9B9 mAb is another anti-EGFR/EGFRv III mAb, which can specifically react with EGFR/EGFRv III antigen on hepatoma cells. Wang et al<sup>151</sup> designed biodegradable cationic therapeutic gene AChE-loaded-polyethylenimine-grafted- $\alpha,\beta$ -poly(*N*-3-hydroxypropyl)-DL-aspartamide (PHPA-PEI) NPs and then conjugated with 9B9 mAb to enhance the HCC-targeting abilities and therapeutic efficiency. The *in vivo* SMMC-7721 tumor-bearing mice administrated with intraperitoneal injection demonstrated that the tumor inhibition rate of PHPA-PEI/AChE NPs with 9B9 mAb was threefold that of PHPA-PEI/AChE NPs without 9B9 mAb contributing to high interaction and internalization between 9B9 mAb on the surface of NPs and EGFR on hepatoma cells, which indicates that 9B9 mAb is a potential delivery ligand for liver cancer gene therapy.

Dextran magnetic NPs labeled with <sup>131</sup>I and anti-VEGF mAb (Sc7269) were prepared for radioimmunotherapy of liver cancer.<sup>152</sup> The nanocomplex could accumulate in tumor tissues through both intratumoral and intravenous injections, whereas very low radioactivity in normal tissues was detected, indicating that targeting to VEGF such as VEGF165, VEGF189, and VEGF121 secreting from liver cancer cells is feasible for HCC therapy.

CD44 is a cell surface glycoprotein involved in immune recognition, in cell-cell and cell-matrix interactions, in cell migration, and so on, is associated with the tendency to vascular invasion, and may have implications for metastasis and poor prognosis in patients with HCC.<sup>153</sup> Wang et al<sup>154</sup> developed anti-CD44 antibody-mediated liposomal NPs loaded with chemotherapy drug DOX or triple fusion suicide genes, including the herpes simplex virus-truncated

thymidine kinase, renilla luciferase, and red fluorescent protein, for the treatment of HCC by targeting cancer stem cells (CSCs) overexpressing CD44. The results demonstrated that the nanocarrier could target liver tumor cells and monitor the response to therapy *in vivo*.

Glucose-regulated protein 78 (GRP78), also known as binding immunoglobulin protein (BiP) or heat shock 70 kDa protein 5 (HSPA5), is overexpressed on the cell surface in HCC.<sup>155,156</sup> GRP78 mAb-conjugated bovine serum albumin (BSA) loaded with 5-FU was developed to promote 5-FU into HCC cells and inhibit the adhesion, invasion, and metastasis of HCC,<sup>157</sup> which was verified by cell administrations.

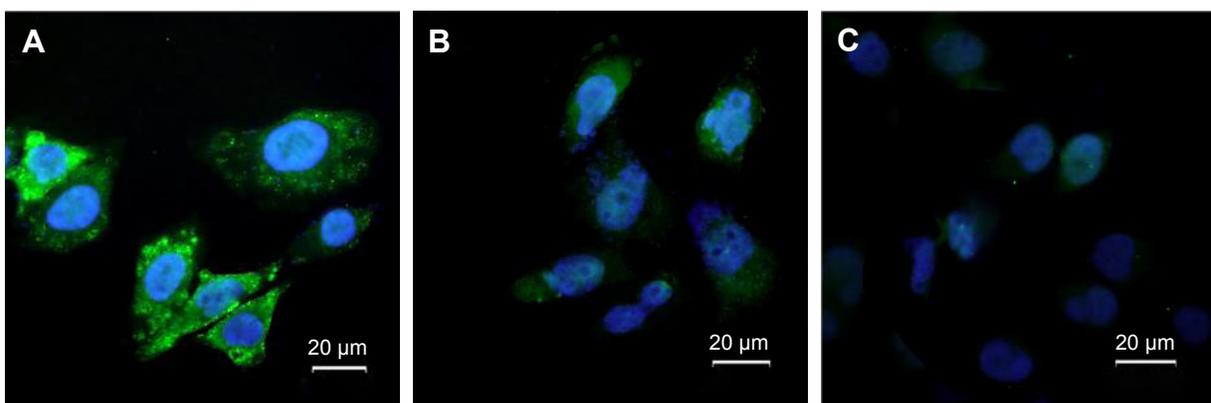
The AF-20 mAb has a high affinity for AF-20 antigen, which was found to distribute uniformly on 15/15 HCC tissues tested but was not evident on adjacent normal liver or in most normal human tissues.<sup>158</sup> Moradpour et al<sup>159</sup> synthesized immunoliposomes by coupling AF-20 with liposomes containing carboxyfluorescein. AF-20-conjugated targeted liposomes (AF20TL) were found to bind to all human cancer cell lines examined, including FOCUS, HuH-7, HepG2, Hep3B, SK-HEP-1 human HCC cell lines, and other human cancer cell lines expressing the AF-20 antigen, whereas control liposomes conjugated with C7-57 showed virtually no binding toward these cell lines. Competitive inhibition experiments conducted on FOCUS, HuH-7, and Hep3B cells using AF-20 mAb proved the specificity of the interaction of AF20TL with target cells.

It is known that glypican-3 (GPC3) is a member of the glypican family of heparin sulfate proteoglycans on some tumor cells.<sup>160</sup> A study showed that GPC3 was not detectable in hepatocytes from normal liver tissue and benign liver lesions but expressive in 72% of HCCs.<sup>161</sup> Therefore, several anti-GPC3 mAbs have been produced for immunotherapy of HCC, but none of them can inhibit cell proliferation or induce

apoptosis.<sup>161–164</sup> Feng et al<sup>165</sup> identified a human heavy chain variable domain antibody (HN3) targeting GPC3 by phage display technology and proved that HN3 could recognize a unique conformational epitope in the GPC3 core protein with high affinity. The HN3 inhibits cell growth in several hepatoma cell models *in vitro* and significantly inhibits the growth of HCC tumor-bearing nude mice *in vivo*. Additionally, Hanaoka et al<sup>166</sup> compared the HN3 and anti-GPC3 YP7, a whole IgG antibody, with regard to their relative therapeutic effects, demonstrating that the HN3 possesses parallel high tumor accumulation but superior homogeneity within the tumor tissue compared with YP7. Due to the high affinity and specificity of GPC3 mAb and the high expression of GPC3 on HCC cells, a GPC3 mAb-functionalized theranostic nanovector loaded with siRNA against luciferase was developed for targeted delivery of siRNA to HCC,<sup>167</sup> indicating that the GPC3 is a target substrate for cancer nanomedicines in HCC.

### Peptide-based active targeting

Arg-Gly-Asp peptide (RGD), a tripeptide with selectivity and affinity for integrins, regulates cell–cell and cell–extracellular matrix interactions and can be utilized as an effective targeting motif in cancer therapy since it can achieve dual targeting for both angiogenic endothelial cells and several tumor cells through the integrin  $\alpha\beta3$ .<sup>168,169</sup> Moreover, RGD peptides enjoy the advantage of minimal risk of immune reactivity, simple and inexpensive synthesis, and tight control over ligand presentation.<sup>170</sup> To confirm the expression level of the integrin  $\alpha\beta3$  on hepatoma cells, this study detected them by immunofluorescence staining. As shown in Figure 6, the fluorescent intensity of HepG2 cells is much higher than that of normal LO2 cells and HK-2 cells, indicating that the integrin  $\alpha\beta3$  is overexpressed on the hepatoma cells. Chen et al<sup>171</sup>



**Figure 6** The expression level of the integrin  $\alpha\beta3$  on HepG2 cells (A), LO2 cells (B), and HK-2 cells (C).

developed PTX-containing liposomes (LP) modified by RGD to enhance the targeting specificity and therapeutic effect, finding higher cellular uptake efficacy and anti-proliferative activity in HepG2 cells and greater anti-tumor effect with enhanced tumor penetration in nude mouse HCC xenograft models compared with LP-PTX without RGD. To improve the biological properties and pharmacokinetics of RGD, it undergoes cyclization, *N*-methylation, variation of stereochemical configuration or other kinds of modification.<sup>172</sup> Compared with linear RGD, cyclic RGD manifests enhanced binding selectivity and affinity for the integrin  $\alpha v \beta 3$  and is less susceptible to biodegradation.<sup>173</sup> Cyclo(Arg-Gly-Asp-D-Phe-Lys) (c(RGDfK)) peptide was employed by Shen et al to synthesize cRGD-DOX/VER-MNP-PLGA NPs for targeted cancer therapy.<sup>174</sup> The *in vitro* cytotoxicity investigation showed that cRGD-targeted DOX/VER-MNP-PLGA NPs led to lower cell viability in HepG2 cells than cRGD-unconjugated NPs, indicating that cRGD enhanced the targeting efficiency of the NPs. Moreover, the result of biodistribution studies confirmed the integrin  $\alpha v \beta 3$  specificity of the cRGD-conjugated DOX/VER-MNP-PLGA NPs *in vivo* while suggesting that MNP improved the targeting by cRGD.

Internalizing Arg-Gly-Asp peptide (iRGD) combines the tumor-homing capability of RGD and the tissue penetrating property of C-end Rule, enabling the targeting of extravascular tumor parenchyma.<sup>175</sup> The mechanism of the iRGD includes three steps: the RGD motif targets  $\alpha v$  integrins on tumor endothelium, then iRGD undergoes a proteolytic cleavage acquiring the ability to bind to neuropilin-1 to achieve tissue penetration.<sup>175</sup> Linear iRGD (CRGDRCPDC) was conjugated to poly( $\epsilon$ -caprolactone)-poly(*N*-vinylpyrrolidone) (PCL-PVP) NPs by Zhu et al.<sup>176</sup> The result of cytotoxicity and uptake investigation indicated that the iRGD-conjugated PCL-PVP NPs possessed higher cytotoxicity than the unconjugated NPs against murine hepatic H22 cell at 12 and 24 h after incubation. Through *in vivo* studies on H22-transplanted solid tumor model, they further demonstrated the superior antitumor efficacy of iRGD-conjugated NPs over the unconjugated counterparts. Moreover, immunofluorescence staining and near-infrared fluorescence imaging confirmed the capability of iRGD to facilitate the accumulation and penetration of NPs in tumor. The *in vitro* and *in vivo* studies on iRGD (CRGDKGPDC)-conjugated DSPE-PEG2000 nanomicelles (M-SAL-iRGD) were developed by Mao et al,<sup>177</sup> which corroborated the functions of iRGD. Selective toxicity toward liver CSCs within the HepG2 cell population was found

using M-SAL-iRGD compared with using M-SAL or free salinomycin (SAL). The result of *in vivo* anti-CSC assay further proved this. The targeting capacity of iRGD was also corroborated by a recent report.<sup>178</sup>

GE11, one of the 11 antigens in the Gebrich blood group system with relatively high prevalence,<sup>179</sup> binds specifically and efficiently to EGFR leading to elevated internalization level while being safe for its much lower mitogenic activity compared with EGF.<sup>180</sup> GE11 was incorporated on DOX-loaded liposomes as a targeting moiety by Tang et al.<sup>181</sup> Compared with the untargeted liposomes, GE11-modified liposomes exhibited improved cellular uptake by human hepatoma cell SMMC-7721 and enhanced antitumor efficacy in SMMC-7721 HCC xenograft models though their blood circulation time decreased. LPEI-PEG-GE11/NIS polyplexes were developed by Klutz et al<sup>182</sup> using linear polyethylenimine (LPEI), PEG, the synthetic EGFR-specific peptide GE11, and a sodium iodide symporter (NIS) expressing plasmid. LPEI-PEG-GE11/NIS polyplexes led to a 22-fold increase in iodide uptake in HCC HuH7 cells as well as high tumor-specific iodide accumulation, which inhibited tumor growth and increased survival in HCC xenograft bearing nude mice. YC21 (GEL) peptide, another EGFR targeting oligopeptide composed of 21 amino acid units, is GE11 peptide with a linker sequence (GGGGS)<sub>3</sub> at the carboxyl terminal.<sup>180</sup> Liu et al<sup>183</sup> formulated the EGFR-targeted gene vectors (YPCs) by coupling YC21 with the PEI600-CD (PC) vectors composed of  $\beta$ -CD and low molecular weight polyethylenimine (PEI). Highly efficient gene delivery ability of EGFR-positive cells was proved in their *in vitro* study on SMMC-7721 cells. The *in vivo* investigation of gene transfection and antitumor activity also suggested its prominent gene transfection ability and therapeutic effects in the inhibition of tumor growth.

Fibroblast growth factors (FGFs) are another family of growth factors, and their receptors (FGFRs) are transmembrane proteins which are overexpressed in various human solid cancers and relative to angiogenesis.<sup>184</sup> A study demonstrated that a heptapeptide sequence (MQLPLAT) is binding to FGFR with high affinity,<sup>185</sup> suggesting that peptide is also a ligand to FGFR. Hu et al<sup>186</sup> developed CY11 (CGMQLPLATWY)-coupled gene delivery to specifically transfect plasmid DNA into FGFR<sup>+</sup> HepG2 cells. The results indicated that the CY11 could enhance the gene delivery efficiency due to the specific binding between the FGFR on the surface of hepatoma cells and the peptide on vectors.

SP94 (SFSIIHTPILPL) is a peptide that can selectively target HCC cells both *in vitro* and *in vivo* through an unknown

target molecule.<sup>187</sup> Lo et al<sup>187</sup> synthesized a targeted drug delivery system by coupling SP94 with PEGylated liposomal DOX (SP94-LD). Studies that they implemented on mice bearing HCC xenografts confirmed the targeting efficiency of SP94 and demonstrated the superior targeting ability of SP94 over the phage clone PC94 and the enhanced therapeutic effect of SP94-LD compared with control peptide conjugated LD (CP-LD) and LD. Toita et al<sup>188</sup> formulated HspG41C-SM(PEG)*n*-SP94 peptide conjugates by modifying a naturally occurred heat shock protein cage (HspG41C cage) with SP94 through the heterobifunctional linker (SM(PEG)*n*). The cellular binding investigation demonstrated the binding selectivity of this conjugate to human HCC-derived cell lines, while suggesting that the binding efficiency could be influenced by the amount of SP94 peptides on Hsp cages, conjugation site of SP94 peptide, and linker length between a Hsp cage and a SP94 peptide. They further found that the optimized strategy was conjugating the Hsp cage with high levels of SP94 at the N-terminus of SP94 through a longer linker, which might as well guide the usage of other peptide ligands. SP94 was also utilized by Moon et al<sup>189</sup> to modify a protein cage, encapsulin, for HCC cells targeting, and SP94 was displayed on the surface of encapsulin through conjugation or genetic insertion. The studies they conducted proved the effective and specific targeting capability of SP94.

T7 (HAIYPRH), a Tf receptor-specific peptide, binds to a different site of Tf receptor compared with Tf and can exhibit enhanced uptake due to the help of endogenous Tf.<sup>190</sup> Han et al conjugated PEG-modified polyamidoamine dendrimer (PAMAM) with T7 to deliver DOX to Tf receptor-overexpressed tumors.<sup>191</sup> Enhanced cellular uptake and *in vitro* antitumor effect were detected in BEL-7402 cells. In the investigation of *in vivo* antitumor effect, PAMAM-PEG-T7/DOX NPs showed higher accumulation in tumor tissues and led to more remarkable inhibition of tumor growth than PAMAM-PEG/DOX NPs and saline. It has been reported that the complex of DOX and DNA do not leak DOX in the bloodstream contributing to intercalation of DOX within the DNA strand.<sup>192,193</sup> Accordingly, the PAMAM-PEG-T7 NPs loaded with therapeutic gene encoding human tumor necrosis factor-related apoptosis-inducing ligand (pORF-hTRAIL) and DOX were developed to improve the antitumor activity.<sup>194</sup> This system yet exhibited improved cellular uptake and gene expression and consequently higher level of apoptosis in BEL-7402 cells compared with the unmodified system. Both the studies demonstrated the promising potential of T7 as a targeting ligand for liver cancer.

## Aptamer-mediated active targeting

Aptamers are single-stranded DNA, RNA, or unnatural oligonucleotides with a molecular weight of 10–15 kDa and bind with high specificity and affinity for a wide range of target molecules including other nucleic acids, proteins, peptides, and small molecules.<sup>195</sup> Although functionally similar to protein antibodies, aptamers also called as “chemical antibodies” offer several advantages over antibodies in terms of biomedical applications.<sup>196</sup> Aptamer-conjugated NPs are thus empowered by these attributes to selective delivery vehicles for therapeutic applications to the target.<sup>197</sup>

CESN, SAL-loaded NPs conjugated with CD133 aptamers A15 and EGFR aptamers CL4, was developed using an emulsion/solvent evaporation method by Jiang et al to enhance the delivery of SAL and target not only HCC CSCs but also a large percent of non-CSCs, thus improving the therapeutic effect.<sup>198</sup> The cytotoxicity investigation showed that A15-conjugated SAL-loaded NPs (CSN) and CL4-conjugated SAL-loaded NPs (ESN) could be 1.38- or 2.01-fold effective than SN against Huh7 cells, respectively, while the CESN was the most effective. Similar experiment using Hep3B cells corroborated this trend. Moreover, the *in vivo* antitumor assay further confirmed the superiority of CSN and ESN over nontargeting SAL-loaded NPs.

Epithelial cell adhesion molecule (EpcAM) is upregulated in HCC and could be selectively bound by DNA aptamers.<sup>199</sup> For instance, a DNA-based EpcAM aptamer was utilized by Pilapong et al as a targeting moiety to develop a theranostic nanoprobe (called as EpcAM-MNPs) for HCC treatment and MRI.<sup>200</sup> *In vitro* study indicated that the DOX-loaded EpcAM-MNPs had higher specificity in drug delivery for HBV-associated HCC cells and better intracellular uptake compared with free DOX. Moreover, they were located mainly in endosomes/lysosomes, where the relatively low pH enabled the ready release of DOX.

The aptamers in NTDDS for HCC were limited in previous literatures. However, current studies have established some targeted systems using aptamers to target molecules overexpressed on HCC cells, attaining higher cellular specificity and better therapeutic effect. The thiolated TLS11a aptamers were attached to AuNP-modified glassy carbon electrode surface by Sun et al,<sup>201</sup> to enhance the specificity of cancer cell detection based on the hybrid nanoelectrocatalysts and enzyme for signal amplification. The specificity study using various types of cells revealed the excellent selectivity of the cytosensor for HepG2 cells. This aptamer was also used for ultrasensitive and selective electrochemical cytosensing for HCC cells.<sup>202</sup> There are studies using the conjugates or

adducts of aptamers and diverse kinds of therapeutic materials for treatment of HCC, as AS1411 (AGRO100)-DOX adduct,<sup>203</sup> LY-1-conjugated QDs and magnetic particles,<sup>204</sup> TLS11a-GC-guided DOX delivery,<sup>205</sup> and so on. These aptamers in Table 5 are potential ligands for targeted therapy for HCC, and the specificity and efficiency would be investigated in further works.

## Tf-based active targeting

Tf, an iron-transporting glycoprotein, has two homologous domains for ferric iron and can internalize them efficiently into cells with expression of Tf receptors (TfRs) by receptor-mediated endocytosis.<sup>206</sup> When iron is removed from holo-Tf (iron-binding Tf), Tf becomes apotransferrin (apoTf).<sup>206</sup> TfR1 and TfR2 are two chief TfRs responsible for cellular iron uptake, while TfR1 is more ubiquitously expressed and shows significantly higher affinity for Tf compared with TfR2.<sup>207</sup> It is reported that the mRNA level of TfR1 is upregulated in human HCC but that of TfR2 is downregulated.<sup>208</sup> Consistent conclusion has been drawn in a similar study on a rat model of HCC.<sup>209</sup>

The Tf-decorated core-shell NPs loading DOX in poly(vinyl alcohol) nanocore and sorafenib in albumin nanoshell were developed using sequential freeze-thaw/coacervation method by Malarvizhi et al.<sup>210</sup> Upon conjugation of Tfs, the cellular uptake of the nanomedicine was significantly increased in HepG2 cells. Besides, notably improved synergistic cytotoxicity and cell death have been observed upon incubation of HepG2 and PLC/PRF/5 cells with the Tf-targeted NPs. In order to mimic in vivo system, studies of cellular uptake and cytotoxicity were performed in three dimensional HCC spheroids. The results suggested remarkable penetration and internalization of NPs into the HCC spheroids and promising synergistic cytotoxicity inflicted on HCC cells.

To enhance the transfection efficiency of negatively charged liposomes encapsulating miRNA-221 antisense oligonucleotide (anti-miR-221), Zhang et al<sup>211</sup> synthesized anti-miR-221-encapsulated Tf-targeted liposomes (Tf-RL) by conjugating Tfs to the liposomes. In vitro study indicated that the Tf-RL delivered miRNA more efficiently to the hepatoma cells than nontargeted liposome containing anti-miR-221 (RL), showing higher silencing efficiency and expression of targeted downstream genes. Concordant results have been found in HepG2 tumor-bearing xenografted mice after intravenous injection of Tf-RL. Moreover, the biodistribution in tumor-bearing mice suggested that the Tf-RL could result in higher intensity of miRNA in tumors compared to RL and, as a consequence, induce more significant apoptosis in HepG2 cells through active targeting.

ApoTf, a form of iron-removed Tf, has a weaker binding affinity for TfR than diferric Tf<sup>212</sup> but can also be used as targeting ligand and carrier for NTDDS. The conjugated and non-conjugated forms of Dox to apoTf NPs have been prepared by Krishna et al<sup>213</sup> and the outcome suggested that the non-conjugated form (direct-nano) is superior to the conjugated form (conj-nano) in various aspects, including drug localization in nucleus, sustainability of half-life, efficiency of delivery and so on. Besides, lactoferrin (Lf), also known as lactotransferrin, is an 80 KDa glycoprotein in the Tf family and has notably high affinity for iron.<sup>214</sup> Research has also been also conducted using apoTf or Lf as the targeting moiety and drug carriers for chemotherapy of HCC. Golla et al<sup>215</sup> synthesized DOX-loaded apoTf NPs (Apodoxonano) and Lf NPs (Lactodoxonano), and administered the drug intravenously in rats, minimizing the cardiotoxicity caused by DOX while attaining relatively high drug accumulation in liver and extended bioavailability. To sustain optimal concentration levels of drug and simplify the modality of administration, oral formulation of DOX-protein NPs are developed and administered in rats though doubts remain about whether the oral adsorption would also be effective in humans and how to determine the dosage in human studies.<sup>216</sup>

## Other ligands for HCC

Glycyrrhetic acid (GA) is the hydrolysis product of glycyrrhizin which was extracted from the root of the traditional Chinese medicine *Glycyrrhiza glabra* (licorice).<sup>217</sup> It is demonstrated that the GA and glycyrrhizin could mediate the active hepatic-targeting drug delivery system through GA receptor and glycyrrhizin receptor, respectively.<sup>218–220</sup> In 1991, Negishi et al<sup>221</sup> confirmed that the quantity of GA binding sites was higher than that of glycyrrhizin binding sites, that is to say, the GA could be more effective than glycyrrhizin to HCC. Thus, the GA has been developed in targeted therapy for HCC as a ligand, which was summarized in a review.<sup>218</sup> In a recent work, the capacity of the GA to HCC was also proved through a drug and gene codelivery carrier modified with GA.<sup>219</sup> The drug concentration and gene transfection efficiency of GA-modified carrier was remarkably higher than that of GA-modified carrier adding free GA. Moreover, the GA-modified carrier possessed much better antitumor efficacy on xenograft liver tumor. All results indicate that GA as a ligand plays an important role in enhancing HCC-targeting efficacy through active targeting.

In the aforementioned section, the EGFR was described as a targeting site to match antibodies for HCC. Epidermal growth factor (EGF), derived from the cleavage of prepro-EGF and generated primarily in kidney in humans, can bind

with EGFR as well, which promotes embryonic development and stem cell regeneration and regulates ion transport.<sup>222</sup> To resolve the problems of *in vivo* gene delivery, Wolschek et al<sup>223</sup> synthesized PEGylated EGF-containing DNA/PEI complexes for HCC targeting. The study on 2 xenotransplantation models for human HCC showed that expression levels within the tumors were up to 97%–99% of the total transgene expression *in vivo*, which is two logs higher than levels in the major expressing organ, liver. However, the transfection levels were significantly lower upon systemic application of EGF-free complexes, indicating that specific delivery is mediated by the interaction between EGF and EGFR, which corroborated the conclusion of the *in vitro* competition study using free mEGF.

Low-density lipoprotein (LDL) particle, with an average diameter of ~22 nm (ranging from 18 to 25 nm), has an apolar core consisting of cholesteryl esters, triglycerides and free unesterified cholesterol, and a cholesterol-containing phospholipid monolayer shell wrapped by a single protein of apoB100.<sup>224</sup> LDL is the main carrier of cholesterol to peripheral tissues and is internalized through interaction between its ApoB ligand and the LDL receptor (LDL-R).<sup>225</sup> Increased expression of LDL-R was found in HCC compared with adjacent liver tissue.<sup>226</sup> *N*-succinyl chitosan NPs were coupled with LDL for target co-delivery of cholesterol-conjugated siRNA and DOX by Zhu et al.<sup>227</sup> The LDL-decorated delivery system exhibited superior cytotoxicity against HepG2 cells over non-targeted system and also manifested enhanced liver tumor-targeting effects and relatively lower systemic toxicity in mice bearing hepatoma cell tumor, suggesting their potential for HCC therapy. LDL was also utilized to modify osthole-loading *N*-succinyl chitosan NPs, attaining high targeting efficacy indicated by investigations *in vitro* and *in vivo*.<sup>228</sup> Hepatic arterial infusion of LDL-based NPs was employed for docosahexaenoic acid (DHA) transporting.<sup>229</sup> LDL-DHA showed selective cytotoxicity against rat (H4IIE), mouse (Hepa1C7, TIB-75) and human (SK-Hep1) HCC cells, and its hepatic artery injection generated improved therapeutic and biologic effects compared with LDL NPs loaded with triolein or sham surgery controls.

Hematoporphyrin (HP) can also bind to LDL-R on tumor cell membrane as a ligand.<sup>230</sup> HP was conjugated to NPs by Chang et al for specific targeting of DOX to HCC cells.<sup>231</sup> *In vitro* investigation on HepG2 cells showed that HP-modified DOX-loaded NPs (HP-NPs) led to enhanced cellular uptake and strongest cytotoxicity compared with untargeted DOX-loaded NPs and free DOX. HP-NPs also resulted in increased AUC ratio of DOX in the liver (target organ) to DOX in the heart (an organ of major side effects),

improving targeting efficiency and minimizing side effects. To enhance anticancer efficacy of photodynamic therapy for HCC, this team also employed HP to modify NPs but HP here was not only a targeting ligand for LDL-R on hepatoma cells but also a photosensitizer.<sup>232</sup> Their study found increased cellular phototoxicity *in vitro* and remarkably lower tumor growth *in vivo* caused by HP-NPs compared with free HP, which may result from the HCC specificity of HP-NPs and/or the cytotoxicity of DOX itself.

Recombinant high-density lipoprotein (rHDL) particle is composed of phosphatidylcholine, apolipoprotein A-1, cholesterol, and cholesteryl esters and can efficiently deliver drugs since cancer cells can acquire HDL core components through scavenger receptor type B-1 (SR-B1).<sup>233</sup> The SR-B1 is broadly overexpressed among various cancer cells including HCC and SR-B1 receptor-mediated HDL uptake makes HDL a promising target delivery vehicle for therapeutics.<sup>234</sup> The rHDL was utilized as a biomimetic nanovector by Ding et al,<sup>235</sup> mediating HCC-targeted cholesterol-conjugated siRNA (Chol-siRNA) delivery. rHDL could enhance cellular uptake and *in vitro* cytotoxicity of the siRNA and specifically target it into cytoplasm. Besides, rHDL-targeted complexes could effectively accumulate in tumor with prolonged retention time compared with the non-targeted lipoplexes, leading to significant tumor growth suppression *in vivo*.

Heat-labile enterotoxin subunit B (LTB) is a subunit of heat-labile enterotoxin (LT) which is a heat-sensitive enterotoxigenic factor<sup>236</sup> and is composed of a single A subunit (LTA) and a ring of five B subunits (LTB).<sup>237</sup> LTB binds specifically to the monosialoganglioside GM1 which is the host receptor of LT.<sup>236</sup> A novel NP was developed utilizing a mixture of LTB and BSA for HCC-targeted delivery of 5-FU.<sup>238</sup> Investigation on SMMC-7721 cells showed enhanced cytotoxicity and cellular uptake of BSA-LTB NPs compared with BSA NPs, suggesting the promising targeting efficiency of LTB.

Epigallocatechin Gallate (EGCG) is the most abundant catechin in green tea infusions<sup>239</sup> and has efficient antioxidant activity possibly due to a low reduction potential and its ability to chelate metal ions.<sup>240</sup> EGCG binds with great affinity to 67 kDa laminin receptor (67LR) which is upregulated in HCC.<sup>241</sup> EGCG-functionalized ruthenium NPs (RuNPs) loaded with luminescent ruthenium complexes were developed to improve the treatment of liver cancer.<sup>242</sup> Significant internalization of EGCG-RuNPs was found and could be suppressed upon treatment with 67LR-blocking antibody or laminin before the addition of NPs, indicating that the uptake of RuBB-loaded EGCG-RuNPs was mediated

by 67LR. Moreover, high antitumor efficacy was found on tumor-bearing nude mice.

Serotonin (5-HT), a famous neurotransmitter and vasoactive substance which also mediates many gastrointestinal functions,<sup>243</sup> is involved in tumor growth of HCC<sup>244</sup> and binds to 5-HT receptors. There are 7 receptor classes including 14 subtypes of 5-HT receptors, among which 5-HT receptors 1B and 2B were significantly increased in HCC compared with nontumoral tissue.<sup>245</sup> Gopal et al<sup>246</sup> utilized 5-HT as the targeting moiety for liposomes delivering DNA and observed enhanced targeted transfection in HepG2 cells compared with a non-targeted control liposome. Since there are several classes of 5-HT receptors located in places other than liver,<sup>245</sup> the feasibility of employing 5-HT as a HCC-targeting ligand in vivo remains to be further investigated.

Somatostatin (SST) is a regulatory peptide produced by neuroendocrine, inflammatory, and immune cells and acts as an inhibitory regulator of diverse functions, including inhibiting hormone and growth factor hypersecretion from tumors which facilitates tumor growth and producing antiproliferative effect giving rise to variable tumor shrinkage.<sup>247</sup> The functions of SST are achieved through high-affinity G-protein-coupled receptors which comprise five subtypes (SSTR1-5)<sup>247</sup> and are expressed in cultured hepatoma cells and HCC.<sup>248</sup> Octreotide, a somatostatin analog which binds primarily to SSTR2,<sup>249</sup> was used to develop octreotide-polyethylene glycol(100) monostearate (OPMS) as a targeting moiety for the delivery of hydroxycamptothecin (HCPT) loaded in nanostructured lipid carrier (NLC).<sup>250</sup> Enhanced cellular uptake by SMMC-7721 cells was observed and the results indicated that OPMS-modified NLC had remarkable targeting efficiency both in vitro and in vivo. Somatostatin was also attached to AuNPs to establish a targeted delivery system for antitumor agents.<sup>251</sup> Investigation on HCC-1806 cell lines revealed that cellular uptake of AuNPs-SST was higher than citrate-AuNPs and can be suppressed upon addition of antagonist, suggesting the modification of AuNPs using SST could enhance drug internalization by HCC-1806 cells through the specific interaction between SST and SSTR.

## Dual-ligand modification to further enhance active targeting

The efficiency and specificity of active targeting depends on direct point-to-point recognitions and interactions of the ligands on the surface of NPs and the matching receptors on tumor cell membrane.<sup>252,253</sup> However, this receptor-mediated endocytosis is a saturated pathway due to the

limited number of receptors and the recycling of receptors during endocytosis.<sup>254-257</sup> On the other hand, most receptors such as ASGPR and FR are not only expressed on hepatoma cells but also on some normal cells, resulting in unfavorable uptake in these non-targeted cells.<sup>258</sup> Recently, some studies have focused on further enhancement of targeting effect and reduction of non-specific uptake. As has been noted in this review, hepatoma cells overexpress multiple surface receptors. To overcome this obstacle, dual-ligand based NPs is a promising strategy.<sup>198,255-260</sup> In our previous report, dual-ligand Gal moieties and GA-modified chitosan NPs were prepared as a drug or gene carrier for HCC.<sup>261</sup> The cellular uptake in vitro and biodistribution in vivo demonstrates that this dual-ligand decorated nanosystem can enhance the specific recognition and internalization by hepatoma cells, compared with single-ligand decorated nanosystem. Besides, it seems to be another good alternative to modify HA with a small-molecular ligand to further enhance the targeting of HA vehicle. For example, GA-HA conjugate was developed by chemical reactions and loaded with chemotherapeutic agents.<sup>262,263</sup> All results indicate that GA-HA NPs seem to be a potential drug carrier with “double target sites” for HCC intracellular delivery. Due to degradation of HA by HAase, HA has been applicable to compose smart delivery system as HAase-responded shell and active ligand. At the HAase-rich tumor microenvironment, HA shells are disintegrated and other ligand-decorated carriers are exposed to the interstitium in tumor tissue, then the active targeting is triggered.<sup>259</sup>

## Conclusion

This study reviewed the ligands in NTDDS which recognize the membrane receptors and proteins on the surface of hepatoma cells. The further investigation should focus on the detection of targeting properties of these ligands for preclinical trials and explore new target sites on membrane of hepatoma cells. Apart from ligands mentioned earlier, there are ligands whose receptors were found to have increased expression on HCC cells or play a role in HCC development, such as adenosine A2b receptor,<sup>264</sup> gamma-aminobutyric acid A receptor theta subunit,<sup>265</sup> androgen receptor,<sup>266</sup> E prostanoind receptor,<sup>267</sup> mitogenic insulin receptor A,<sup>268</sup> receptor of insulin-like growth factor 2,<sup>269</sup> and so on. On the other hand, the spatial properties of ligands can affect the targeting performance.<sup>78,80</sup> It is known that the targeting performance is enhanced as increasing the density of ligands on the NPs until the saturation of ligand-receptor interaction is occurred, whereas the space between ligand and vehicle is not definite. A recent study demonstrated

that the space plays a key role in transfection efficiency,<sup>80</sup> but the effect should be further investigated.

A great deal of studies have shown that immunotherapy is a potent therapeutic option for HCC.<sup>270–272</sup> However, it is uncertain whether that combination with immunotherapy and NTDDS can induce synergistic effect of anti-HCC. Furthermore, the high expression of MDR results in the poor response of HCC to chemotherapy.<sup>273</sup> Thus it is a potential approach to treat HCC cells by composite delivery system co-loaded with siRNA for MDR genes and chemotherapeutic agent or multiple complementary chemotherapeutic agents to improve the prognosis of HCC.<sup>274</sup>

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## Disclosure

The authors report no conflicts of interest in this work.

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