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NARRATIVE REVIEW

Targeting Wnt-β-Catenin Signaling Pathway for Hepatocellular Carcinoma Nanomedicine

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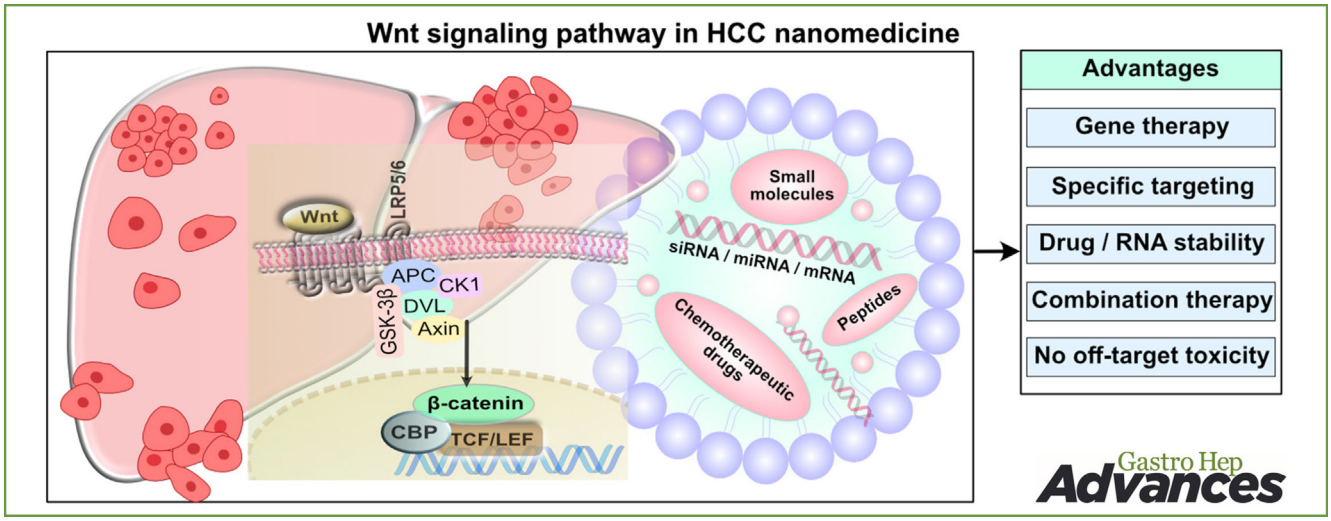

Hepatocellular carcinoma (HCC) represents a high-fatality cancer with a 5-year survival of 22%. The Wnt/β-catenin signaling pathway presents as one of the most upregulated pathways in HCC. However, it has so far not been targetable in the clinical setting. Therefore, studying new targets of this signaling cascade from a therapeutic aspect could enable reversal, delay, or prevention of hepatocarcinogenesis. Although enormous advancement has been achieved in HCC research and its therapeutic management, since HCC often occurs in the context of other liver diseases such as cirrhosis leading to liver dysfunction and/or impaired drug metabolism, the current therapies face the challenge of safely and effectively delivering drugs to the HCC tumor site. In this review, we discuss how a targeted nano drug delivery system could help minimize the off-target toxicities of conventional HCC therapies as well as enhance treatment efficacy. We also put forward the current challenges in HCC nanomedicine along with some potential therapeutic targets from the Wnt/β-catenin signaling pathway that could be used for HCC therapy. Overall, this review will provide an insight to the current advances, limitations and how HCC nanomedicine could change the landscape of some of the undruggable targets in the Wnt/β-catenin pathway.

Keywords: Hepatocellular Carcinoma; Liver Cancer; Nanoparticle; Wnt Signaling; HCC Targeting

Introduction

Hepatocellular carcinoma (HCC) is a major global challenge and the predominant form of primary liver cancer constituting almost 90% of all liver cancers and the third leading cause of cancer-related deaths after lung and colon cancers, respectively.[1]

Hepatocarcinogenesis is a complex multistep process involving several cellular phenomena such as hypoxia,

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Abbreviations used in this paper: APC, adenomatosis polyposis coli; API, active pharmaceutical ingredient; ApoE, apolipoprotein E; ASGPR, asialoglycoprotein receptor; CAF, cancer-associated fibroblast; CBP, CREB-binding protein; ECM, extracellular matrix; GSK3β, glycogen synthase kinase 3β; HCC, Hepatocellular carcinoma; HCV, hepatitis C virus; HDL, high-density lipoprotein; HSC, hepatic stellate cell; NASH, nonalcoholic steatohepatitis; NP, nanoparticle; siRNA, small interfering RNA; TCF, T-cell factor; TfR, transferrin receptor; TME, tumor microenvironment; tMNV, therapeutic milk-derived nanovesicles; VEGF, vascular endothelial growth factor; WGS, whole genome sequencing.

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inflammation, oxidative stress, and changes in the tumor microenvironment (TME).[2] HCC development often involves a long process of continuous inflammatory damage, necrosis, and fibrotic deposition.[3] Additionally, HCC mostly prevails in the setting of chronic liver diseases such as nonalcoholic steatohepatitis (NASH), alcoholic liver disease, hepatitis C and hepatitis B, accounting for an annual HCC incidence of 1%–6%.[4] Furthermore, the etiology of HCC burden shows geographical variation such that infection by hepatitis B virus accounts for 60% cases in Asia and Africa whereas most cases in Europe and North America have been dominated by chronic hepatitis C virus (HCV) infections.[5] This is expected to shift towards NASH and alcoholic liver disease, given the advent of highly effective antivirals against HCV that are able to achieve cure in 99.7% of cases.[6,7]

The current therapeutic options for HCC include hepatic resection and transplantation, transarterial chemoembolization and systemic treatments depending on the disease stage advancement. In the past few years, HCC management has substantially advanced with 6 systemic therapies currently approved for the treatment of advanced-stage HCC. As the first-line treatment, the combination of atezolizumab (anti-programmed death-ligand 1 antibody) and bevacizumab (antivascular endothelial growth factor antibody) has shown promising results in the overall improvement in progression-free survival outcomes as compared to tyrosine kinase inhibitors sorafenib and lenvatinib that are used as single-drug therapies in HCC patients.[8–10] Other systemic drugs acting as tyrosine kinase inhibitors such as regorafenib and cabozantinib, and anti-vascular endothelial growth factor receptor 2 antibody ramucirumab are also being used as second-line therapy for HCC management.[11]

Although the recent years have shown enormous advancement in HCC therapy with exciting opportunities that lie ahead, the current challenges in HCC therapy cannot be overlooked. HCC treatment is complex and multifaceted since the expected outcomes often depend on the time of diagnosis and any pre-existing comorbidities. Considering that HCC mostly prevails in the setting of other liver diseases, understanding the underlying liver TME through its signaling pathways and safely and effectively selecting a target for the tumor cells are still the major objectives in this field of research. Owing to these challenges, the field of nanotechnology could serve as the shepherd required to deliver therapies from the bloodstream to its target cells and subcellular compartments by virtue of their excellent qualities of controlling physiological surface properties and architecture for optimal therapeutic targeting devoid of any off-target toxicities. Over recent years, nanotechnology has visibly grown into a fast-advancing field of therapeutics owing to its unprecedented advantages over traditional therapeutics. Some of these advantages include specific targeted delivery avoiding systemic toxicities, enhancement of pharmaceutical properties increasing drug penetration and absorption, drug stability during circulation, visualization of treatment through image-guidance and codelivery of multiple drug moieties.[1] Also, nano carriers are invariably nontoxic, biocompatible, biodegradable and well-tolerated permitting limited to no adverse effects. In 1995, the first-in-human cancer nano therapy, Doxil® (doxorubicin), was clinically approved owing to its enhanced drug circulation time and increased drug stability. Ever since, several other cancer nanomedicines have been FDA approved including DaunoXome®, Myocet®, Abraxane®, Oncasper®, Lipusu®, Genexol-PM®, DepoCyt®, Mepact®, Marqibo®, Onivyde®, Vyxeos®, and Apealea®, and numerous nanotherapeutics lie within the clinical phase for different cancers.[12,13]

Although nanotherapeutics offers an unmatched improvement in drug delivery, due to the limited impact and treatment-resistance demonstrated by the current systemic therapies, there is currently no approved nanomedicine for the treatment of HCC. Therefore, it is of utmost importance to dig deeper into understanding the signaling pathways that govern hepatocarcinogenesis and identify novel targets that can be used to develop more specific and targeted nano therapies. Several molecular mechanisms and cellular signaling pathways have been reported in HCC. Amongst these, the most commonly affected pathways include; Wnt/ β-catenin pathway, Ras/Raf/MEK/ERK pathway, insulin-like growth factor pathway, PI3K/AKT/mTOR pathway, hepatocyte growth factor/c-MET pathway and growth factor-regulated angiogenic pathway.[14,15] Amongst these, evidence suggests that the Wnt/ β-catenin pathway is a pivotal signaling pathway orchestrating HCC and its recurrence and could, therefore, be the key to novel therapeutic targets in the field of HCC research.[16–18]

Is nanomedicine the key to a safe and effective HCC therapy?

Although most of the drugs achieve high hepatic concentrations, it is of utmost importance to safely and effectively deliver these drugs to the tumor cells devoid of any toxicity. The field of cancer nanomedicine has achieved immense advances in overcoming the numerous shortcomings of conventional cancer therapies and nanotechnology; therefore, presents a promising solution to the therapeutic challenges encountered due to the complexity and heterogeneity of HCC tumors.[19,20]

Briefly, nanoparticles (NPs) can be defined as particles within the size range of 10–100 nm that are primarily composed of lipids and/or polymers to deliver active therapeutic moieties such as drugs, proteins, peptides, and nucleic acids.[21] NPs can be broadly classified as organic, inorganic and hybrid NPs that further include various subtypes such as polymeric NPs, dendrimers, quantum dots, solid lipid NPs, nanoemulsions, liposomes, metallic NPs, magnetic NPs, and porphyrin-based NPs (porphysomes).

The type of nano carrier used for drug delivery ultimately determines the fate of the nanoparticle in the body. Different NPs have different properties and behave differently when taken up by the liver to target HCC cells. With regard to this,

inorganic NPs such as iron oxide and zinc oxide NPs have shown higher surface-to-volume ratio resulting in increased surface charges that promotes cellular uptake; however, these are more prone to oxidation, and therefore require either a short circulation time or surface modifications to avoid oxidative degradation. At the same time, inorganic NPs often require active targeting and offer limited delivery of chemotherapeutic drugs and peptides, and it is not feasible to encapsulate nucleic acids using this type of NPs. On the other hand, lipid NPs have shown superiority in HCC targeted delivery and are preferentially being used for various reasons. First, lipid NPs have a natural affinity for apolipoprotein E (ApoE) due to their structural resemblance to lipoproteins. ApoE is a protein that is involved in fat metabolism in the liver and lipid NPs exploit this characteristic to specifically target liver cells enhancing cellular uptake and distribution to hepatocytes. When lipid nanoparticles (LNPs) enter the bloodstream, they come in contact with ApoE-rich lipoproteins such as high-density lipoprotein (HDL) and low-density lipoprotein, which possess ApoE on their surface. Hydrophobic interactions and electrostatic forces facilitate the binding of LNPs to ApoE that leads to the formation of complexes recognized by low density lipoprotein receptors on the liver cell surface. In response, this triggers receptor-mediated endocytosis, causing internalization of ApoE-LNP complexes into HCC cells through clathrin-coated pits. Within the cell, these complexes are subsequently transported from early to late endosomes where the acidic environment of the later prompts the dissociation of ApoE from the LNPs. At this stage, LNPs need to undergo endosomal escape bypassing lysosomal degradation and to release its cargo. Endosomal escape occurs in various ways such as: 1) proton sponge effect, 2) membrane fusion, 3) disruption of endosomal membrane integrity, and 4) escape through exocytosis.[22] In the proton sponge effect, the LNP surface becomes more protonated due to the decrease in endosomal pH leading to osmotic swelling of the endosome, an increase in water and ion influx and rupture of endosome releasing LNPs into the cytoplasm.[23,24] On the other hand, membrane fusion only occurs when LNPs are specifically designed with fusogenic lipids or peptides that fuse with the endosomal membrane causing the release of the encapsulated drugs into the cytosol.[25,26] Additionally, designing LNPs with cationic lipids or peptides with aminoadipic acid residues and histone to alanine substitutions can lead to membrane destabilization by interacting with the negatively charged endosomal membrane and releasing the LNP payload into the cytoplasm.[27] Apart from these mechanisms, endosomal escape could also occur via exocytosis. In this, the LNPs can be released into the extracellular space during the transport of LNPs from late endosomes to the plasma membrane. As a result of endosomal escape, the LNPs are released into the cytoplasm, where they either undergo drug release or further uptake into the nucleus. Henceforth, the interaction between ApoE and LNPs, followed by low density lipoprotein receptors binding and endosomal escape aids efficient uptake into the liver cells making the liver an attractive target organ for drug delivery systems and a promising strategy for HCC therapeutics. Overall, nanomedicine offers a plethora of advantages over conventional HCC therapies such as:

Improved pharmacokinetics

The key properties of NPs such as particle size and surface charge are critical determinants of the fate of the NPs in the body.[21] The ideal nanoparticle size ranges between 10 and 100 nm and it is important to maintain the particle size greater than 10 nm since this size has been estimated as the threshold for first-pass metabolism by the kidneys whose glomerular capillary wall measures approximately 10 nm in diameter.[28] Furthermore, HCC tumors often have a leaky vasculature favoring enhanced permeability and retention of NPs with research showing that NPs in the 50–100 nm size range are capable of penetrating large liver tumors post intravenous drug administration and any particle greater than 2 nm will be restricted from exiting the normal systemic circulation and will be routed to the liver.[29] Similarly, the nanoparticle surface charge also plays a crucial role in the delivery of the NPs to the liver. Sterically stabilized NPs with slightly negative or positive surface charges would have least interactions and better stability. Additionally, blood vessels and cell surfaces are negatively charged, hence a highly negative charged nanoparticle would be more prone to repulsion within the body. Also, increased nanoparticle surface charges attract macrophage scavenging and are more easily cleared by the reticuloendothelial system rendering the nanoparticle system less stable and efficient to liver targeting.[30]

Subsequently, the controlled release of the drug, long nanoparticle time, efficient tumor perfusion, proper tumor tissue penetration and high tumor vascular permeability play a vital role in effective treatment as well as decreasing the volume of distribution that further helps attenuate drug localization in HCC tumor cells. Neither should the NPs be eliminated from the systemic circulation too rapidly nor too late. It is of utmost importance that NPs should be designed such that they exhibit a controlled rate of elimination from the blood.[31] Also, the NPs should comprise of lipids and polymers such that they prevent any drug release at nontarget sites as these could contribute to off-target toxicities during HCC treatment. Eventually, the rate of drug delivery to the HCC tumor site should be optimized to enable adequate drug concentration and produce a therapeutic effect.[32]

Selectivity and specificity (targeted delivery)

Drug encapsulation in NPs is a conventional approach termed as passive targeting that is adapted to increase drug efficacy and limit off-target toxicities. However, passive targeting is not always sufficient to achieve effective drug delivery, and therefore active targeting using targeting moieties coupled onto NPs is essential. NPs offer the advantage of specific targeted delivery to HCC tumor cells through the addition of targeting ligands to its surface (Figure 1).

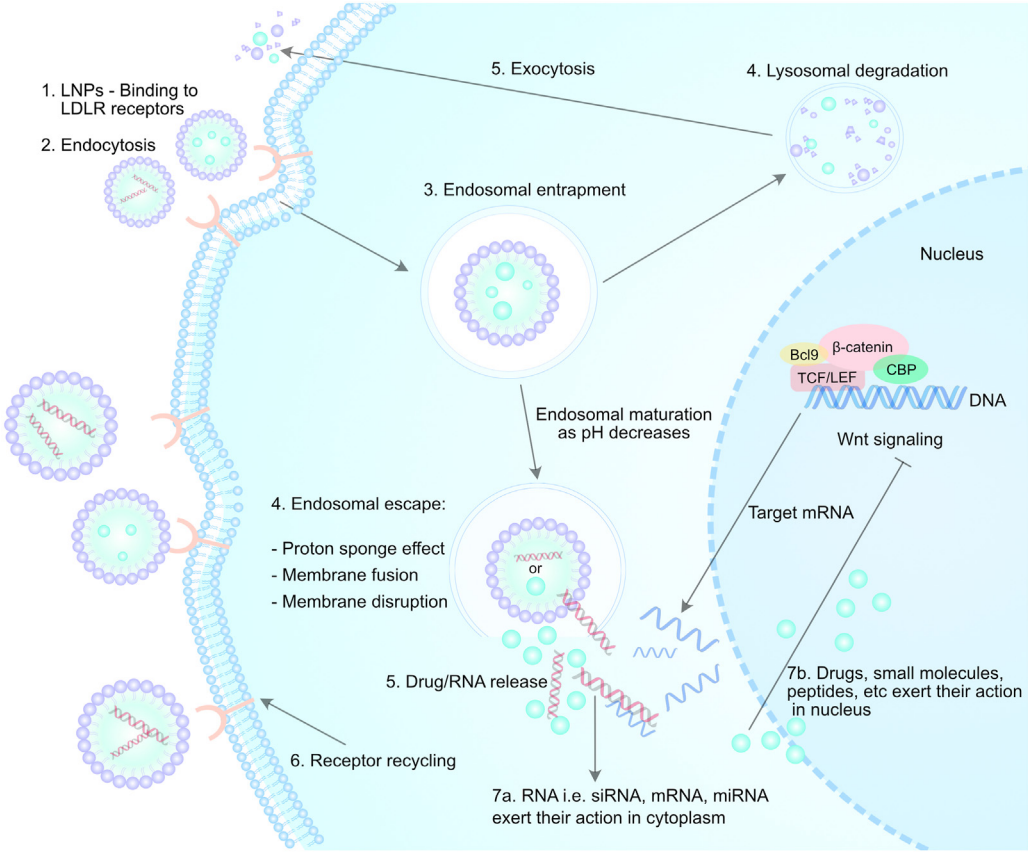

Figure 1. Fate of nanoparticles in the HCC tumor cell.

Moreover, the large surface areas of NPs provide an opportunity to attach ligands such as small molecules, antibodies, peptides, and other targeting moieties that could act as vehicles to direct the NPs to the liver or have additional therapeutic effects in the body. It is also crucial to carefully select targeting ligands since certain ligands could act as binding site barriers and subsequently lead to less efficient tumor penetration. Also, active targeting is extremely necessary when delivering small interfering RNA (siRNA)–based agents since these need to be delivered into the HCC tumor cells as opposed to chemotherapeutic drug-based NPs.[33]

Specific liver targeting can be achieved using the surface biomarkers such as galactose, galactosamine, lactoferrin, and lactose that target the asialoglycoprotein receptors (ASGPRs) which are abundantly present on hepatocytes (500,000 ASGPR/hepatocyte) and are specifically expressed on the sinusoidal and basolateral hepatocellular membranes. However, ASGPR has also been reported to be overexpressed in viral hepatitis; therefore, sole ASGPR targeting in hepatitis induced HCC could likely result in toxicity to the surrounding liver tissue.[34] Apart from that, glypican-3 is another core protein that is overexpressed in the cell membrane and cytoplasm of HCC cells as compared to normal or cirrhotic liver cells, and therefore glypican-3 targeting antibodies such as GC33, YP7, HN3 and MDX-1414 could be incorporated onto the surface of NPs for specific HCC targeting. Another specific receptor widely used for actively targeting HCC cells is a transferrin receptor (TfR). The TfR takes part in the uptake of iron and the liver being the major organ linked to iron metabolism, TFR1 is highly expressed in the liver, and therefore surface transferrin, LT7, apotransferrin and TfR-specific peptides are often being utilized to specifically deliver the active moieties to the liver through nanomedicine. Furthermore, folic acid is another attractive target for drug delivery to the liver due to the overexpression of folic acid receptors in HCC. Folic acid is an ideal surface target due to its small size, stability, inexpensive and nonimmunogenic characteristics. In addition to these, scavenger receptor class B type I is a multiligand membrane receptor protein that is highly expressed on hepatocytes and is the central protein responsible for HDL uptake; thus, making HDL mimics such as apolipoprotein A-1 an efficacious target for HCC tumor cell delivery[1,35] (Figure 2).

Protection of active ingredient

Drug encapsulation in a nanosized matrix enables active pharmaceutical ingredient (API) stabilization and protection throughout its journey from ingestion/injection to reaching the target HCC tumor cells. For orally ingested nanodrugs, the drug is protected from both chemical and enzymatic degradation that could inactivate the drug in the gastrointestinal tract before it reaches the target site for a therapeutic effect. Studies have shown that lipid NPs remain stable when

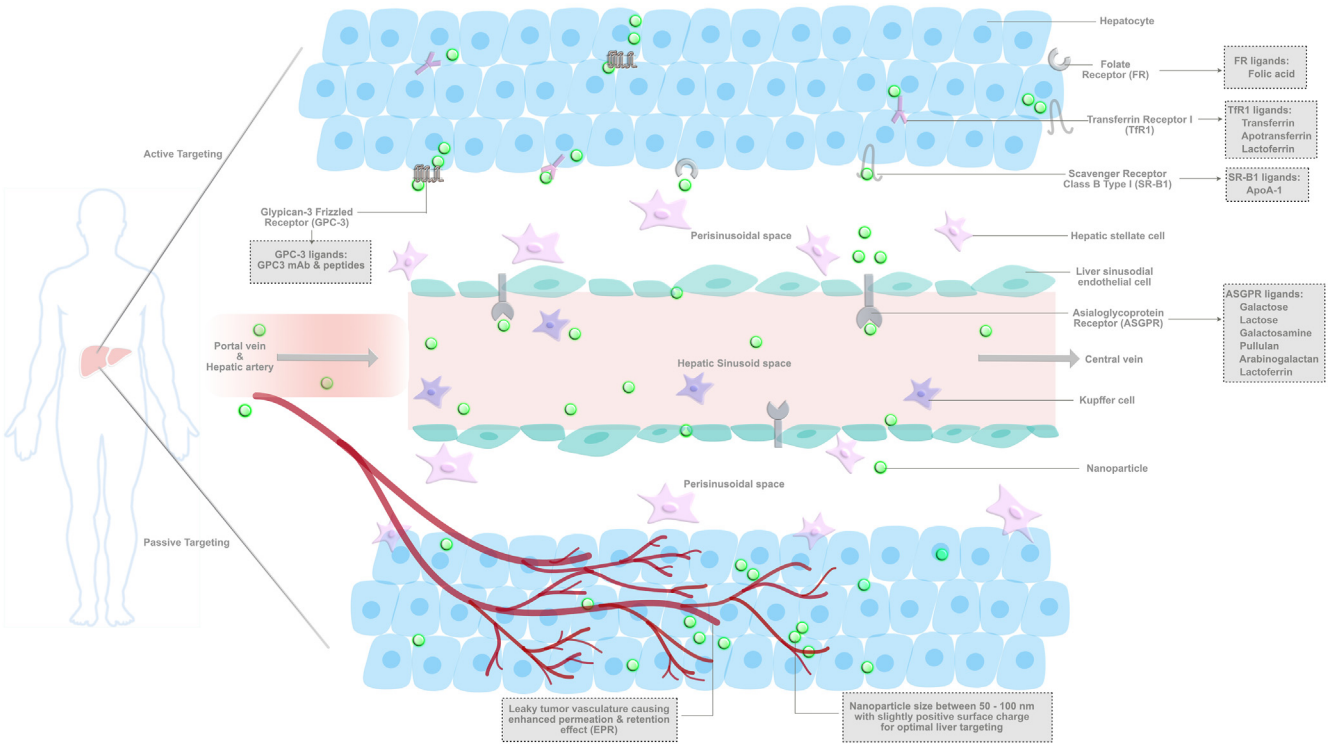

Figure 2. Targeting strategies to HCC tumor cells.

exposed to pH values as low as 1.2 and it has also been observed that slightly increasing the levels of polyethylene glycol in the lipid NPs enhances its potency.[36] Similarly, nucleic acid gene therapies are extremely unstable and prone to degradation in the systemic circulation and at the tumor site by rapid clearance by mononuclear phagocytic cells.[37,38] Studies have shown that naked siRNA is rapidly cleared by the kidneys with a circulatory half-life of <5 minutes that could be increased to >30 minutes in case of cholesterol conjugated siRNAs. To overcome these drawbacks and to increase siRNA stability and circulation time in the body for the most effective therapy, different types of NPs are used to encapsulate siRNA and deliver it to the HCC tumor cells. Interestingly, lipid NPs have successfully entered the clinic for the delivery of siRNA and messenger ribonucleic acid (mRNA) gene therapies such as the first FDA-approved siRNA drug Onpattro and the 2 authorized mRNA COVID-19 vaccines.[39–46] These clinical approvals prove the stability and efficacy of delivering small molecules through nanotechnology and provide hope for the development of small molecule nanoformulations for HCC therapy.

Reduced toxicity

A major drawback of the current conventional HCC therapies is the associated toxicities due to nonspecificity, whereby the drugs are biodistributed to various organs post systemic circulation and exert a therapeutic effect. Subsequently, NPs offer the advantage of directing the drug specifically to the liver avoiding any off-target toxicities as well as increasing the drug concentrations at the HCC target site. In the clinic, reduced toxicities would have a direct effect with an increased probability of extended survival that is merely 6–9 months with the current therapies. Additionally, materials used to design NPs are biologically inert, nontoxic and biocompatible offering an added advantage of eliminating carrier-based toxicities such as hypersensitivity reactions that occurred in patients receiving the cremophor-EL based paclitaxel formulation and is now overcome by Abraxane that consists of a nontoxic albumin nano carrier to deliver paclitaxel.[47]

Combination therapies

Another explicit advantage of nanotechnology is that it offers the ability to coformulate multiple drugs in one nanoparticle enabling the delivery of 2 or more active moieties for optimal therapeutic outcome and reduction of undesired cytotoxic effects.[48] Moreover, combination therapies also offer the advantage of precision medicine whereby 2 or more drugs or nucleic acids can be combined depending on the subtype of HCC for a diverse patient population. In 2017, the FDA-approved liposomal formulation of daunorubicin and cytarabine (Vyxeos) was the first-in-class demonstration of the advantage of a combination drug therapy through nanomedicine. For decades, the combination of cytarabine with daunorubicin (7 + 3 regimen) had remained the standard treatment in acute myeloid leukemia with unfavorable adverse effects in intermediate and high-risk groups of patients. The clinical study results of CPX-351 (Vyxeos) showed that the liposomal encapsulation delivered a synergistic 5:1 drug ratio to

the leukemia cells with a significant improvement in the overall survival of all acute myeloid leukemia patients leveraging a combination drug nanotherapy superior to the conventional drug combination.[49–51] Currently, the combination therapy of atezolizumab and bevacizumab is the mainstay of clinical HCC care that has shown improved overall survival as compared to sorafenib and Lenvatinib.[8] Furthermore, studies have also combined C-X-C motif chemokine receptor 4-targeted p53 mRNA and anti-PD1 drugs in NPs and have successfully demonstrated the reversal of immunosuppression in preclinical HCC models using this combination therapy.[52] Also, another group of researchers showed that a dual anti-PD-1/vascular endothelial growth factor receptor 2 therapy can overcome treatment resistance to either treatment alone or enhance overall survival in HCC.[53] Therefore, designing combination therapies using chemotherapeutic agents, immunotherapeutic agents and nucleic acids within a single nanoparticle could be a game-changer in HCC management considering disease complexity and heterogeneity.[54]

Current challenges to HCC nanomedicine

Formation of protein corona

The protein corona is a layer of loosely (soft corona) or tightly (hard corona) associated proteins that are spontaneously adsorbed onto the nanoparticle surfaces, as these NPs travel through the biological systems to their target tissue.[4,55–57] Interestingly, this adsorption varies with changes in nanoparticle material, size, surface modifications and biological environment through which the nanoparticle travels.[58–62] This phenomenon may occur due to several encounters including hydrophobic interactions involving nonpolar amino acids, electrostatic attraction or repulsion, protein-protein interactions, ligand exchange, hydrogen bonding, cation bridging, chelation and displacement of proteins by other biomolecules and often leads to biological incompatibility and altered drug release profile of NPs.[63] Studies have confirmed that equilibrium in the protein corona is achieved within a few minutes post exposure of NPs to blood plasma and the most abundant corona proteins include apolipoprotein B 100, complement factor H, fibronectin, complement C3, gelsolin, complement C4 B, apolipoprotein A, complement C1r subcomponent, thrombospondin 1, prothrombin, coagulation factor V, and myosin 9.[4,64–66] In order to overcome this challenge, researchers are designing NPs using antifouling agents and zwitterionic “stealth” polymers that would minimize the surface interactions.[67] Polyethylene glycol was earlier used as an antifouling agent; however, it tends to lose its antifouling property at elevated temperatures above 35 °C, and therefore novel polymer ligands such as cysteine-conjugated silica NPs, sulfobetaine-coated gold NPs, cysteine-coated cadmium selenide quantum dots, amphoteric natural starch-coated polymer NPs and zwitterionic gold NPs have attracted interest as protein-corona free NPs. Consequently, the neutral surface charge on zwitterionic NPs compromise tumor cell internalization, and therefore to address this issue, charged reversal micelles are being designed that possess the ability to switch surface properties from neutral to positive through pH-sensitive interactions.[68] Interestingly, protein corona due to an interaction with apolipoproteins, could be leveraged for hepatocyte targeting in HCC.[64] This has been confirmed by researchers who designed a class of lipoprotein NPs that mimic apolipoproteins to successfully deliver siRNA to hepatocytes; however, more fundamental studies are still required to determine the biological fate and percentage of apolipoprotein adsorption on different types of NPs.[69]

Understanding nanoparticle path for effective liver targeting

NPs utilize this complex 5-step circulation, accumulation, penetration, internalization and release cascade-conveying process to enter tumor cells: a) systemic circulation (C); b) accumulation within tumor tissue (A); c) penetration diffusion at the tumor site (P); d) internalization by tumor cells (I); and e) drug release within tumor cell (R).[70] For successful cellular uptake of NPs, they need to overcome several barriers including the high cell density, dense extracellular matrix (ECM), osmotic pressure in HCC tumor cells and finally the cellular membrane of tumor cells. NPs are often internalized through endocytosis pathways and need to avoid lysosomal and Kupffer cell traps and degradation before releasing the drug in a controlled manner at the cellular level.[71]

The HCC TME plays a significant role in determining the fate of NPs as they reach the liver. The TME consists of tumor stromal cells such as activated hepatic stellate cells (HSCs), myofibroblasts, cancer-associated fibroblasts (CAFs), immune cells (cytotoxic T cells, regulatory T cells, tumor-associated neutrophils and macrophages, surrounding tumor stroma (ECM proteins, chemokines, growth factors and stromal degrading enzymes).[72–74] The CAFs comprise of the major part of the tumor stroma that promotes tumor growth and metastasis by generating collagen, fibrin, and other parts of the ECM to form a thick matrix that shields the HCC tumor cells. This hinders drugs from reaching the tumor cells either physically or by altered cell signaling pathways in the HCC tumor cells. Since CAFs play a crucial role in the growth and metastasis of tumor cells by its interaction with HCC cells, targeting CAF-expressed molecules (fibroblast-specific protein-1, transforming growth factor beta, matrix metallopeptidases, vascular endothelial growth factor (VEGF), platelet-derived growth factor and splice variant of fibronectin) using specific targeting moieties could enhance nanoparticle penetration through this barrier into the tumor cells.[75] NPs possess the ability to deliver drugs to specific parts of the HCC tumor tissue and combat different TME stimuli depending on their design and presence of markers and secreted factors overexpressed in HCC tumor cells.

Furthermore, the TME is often hypoxic and acidic as a result of increased metabolic activities, increased nutritional and oxygen needs at the HCC tumor tissue.[76] This leads to the production of reactive oxygen species that in return interferes with DNA repair mechanisms and destabilizes the genome further causing treatment resistance. Owing to this, NPs can come to the rescue since they can be designed to be activated in a hypoxic physiological condition in the TME using a combination of NPs and bio-reducible prodrugs that improve the tumor hypoxic environment and enhance the therapeutic effect on HCC tumor cells. In addition, there is increased glycolysis and lactic acid accumulation in the TME to meet the increased oxygen needs that increases the risk of tumor metastasis and drug resistance in HCC. Interestingly, pH-sensitive NPs can overcome this challenge and can be designed to improve acidity and specifically target HCC tumor cells to prevent tumor progression and drug resistance.[77]

Accurate preclinical testing

Most investigational drugs do not reach the clinic due to the gap between preclinical and clinical aspects of the disease. Although most preclinical models offer the crucial insights into HCC development, several limitations still persist in order to accurately mimic and reproduce the complexity and heterogeneity of HCC. Additionally, the majority of the currently available artificial HCC tumor models in immunodeficient mice do not recapitulate the human setting of the disease that often occurs in the presence of liver diseases such as hepatitis, liver fibrosis and cirrhosis.[78] Therefore, there is dire need to develop and utilize spontaneous and humanized mouse models using patient-derived tumors, as these will be best suited to investigate the immunological changes, safety, and efficacy of the designed nanomedicine. In recent practice, Friedman et al, have developed a simple diet and chemical induced cirrhotic HCC mouse model using high-fat, high-fructose, and high-cholesterol diet combined with low dose weekly intraperitoneal carbon tetrachloride as an accelerator.[79] This mouse model exhibits the histological, immunological and transcriptomic features of human NASH-HCC and would be an extremely useful tool for preclinical testing. However, patient-derived orthotopic xenografts remain superior and the first choice as these would retain the genetic heterogeneity and multitude of mutations present in the HCC patient population.[80–82] Although these models possess a limitation in accurate tumor monitoring during the study, it cannot be overlooked that orthotopic tumors on the liver replicate the TME and would be most ideal to study the pharmacokinetics, safety and efficacy of the nanoformulation.

Targeting the Wnt/β-catenin signaling pathway in HCC

Molecular mechanisms of Wnt/β-catenin signaling

Wnt/β-catenin signaling in the liver is crucial for the regulation of diverse processes that include cell growth, cell survival, cell fate, homeostasis, repair and stem cell maintenance.[83] Aberrant activation of this pathway and mutations in genes encoding key components are characteristic to hepatocarcinogenesis and promote tumor growth and dedifferentiation. The Wnt/β-catenin signaling pathway can be activated by the binding of a Wnt ligand to membrane receptors Frizzled and low-density lipoprotein receptor-related protein 5/6[84] (Figure 3). This leads to the disassembly of the cytoplasmic destruction complex, which consists of proteins such as casein kinase I isoform delta, glycogen synthase kinase 3β (GSK3β), Axin, and adenomatosis polyposis coli (APC), resulting in the accumulation of stabilized β-catenin in the cytoplasm.[84] The Wnt signaling inhibits GSK3β activity by sequestering this kinase in multivesicular endosomes. β-catenin is then translocated to the nucleus and works as a transcriptional activator along with DNA-binding proteins T-cell factor (TCF)/lymphoid enhancer factor, regulating target genes such as cellular myc, Cyclin D1, axis inhibition protein 2 (AXIN2), VEGF, matrix metallopeptidase 7, CD44, cytochrome P450, endothelial growth factor receptor, and epithelial cellular adhesion molecule (EpCAM).[84–87] When the Wnt ligand is absent, cytoplasmic β-catenin is continuously phosphorylated by casein kinase I isoform delta and GSK3β, components of the degradation complex, and subsequently degraded by the proteasome resulting in the inhabitation of the Wnt/β-catenin signaling.[88]

Catenin beta 1 mutations in HCC

A previous study using whole genome sequencing (WGS) demonstrated that alterations in the Wnt/β-catenin signaling were observed in 66% of HCCs, with *catenin beta 1* (*CTNNB1*) mutations detected in up to 31% of these cases.[89] *AXIN2* and *APC*, which are other genes relevant to Wnt/β-catenin signaling, were found to have mutation rates of 6% and 2% in HCC cases respectively.[89] Other WGS studies have also revealed that *CTNNB1* mutations and deregulation of the Wnt/β-catenin signaling were among the major clusters of associated alterations in HCC.[90,91] WGS studies also have suggested that different mutated genes were involved to associate with the aberrant activation of Wnt/β-catenin signaling in HCC, such as *Cyclin D1*, *NCOR1* and *FGF19*, *TERT*, *MLL2*, *APOB*, *ARID2* and *NFE2L2*.[89,90] The high frequencies of *CTNNB1* mutations have been reported in populations where the primary risk factors for HCC are alcohol related HCCs, HCV infection, and hepatitis B virus infection.[92] The overall rate of *CTNNB1* mutations was higher in Europe (25.2%) and the Americas (21.8%) compared to Asia (15.9%).[91] Additionally, *CTNNB1* mutations in HCC have been observed at different frequencies in several countries. In France, the mutation frequency ranges from 19% to 40%.[93] In Germany, it is reported as 25%, while in Japan, it is as high as 41.7%. Italy has a mutation frequency of 17.5%, and in the USA, it is 19.2%.[93] Furthermore, the *CTNNB1* mutation is an important indicator for classifying the subclasses of the "non-proliferation class" in HCC molecular

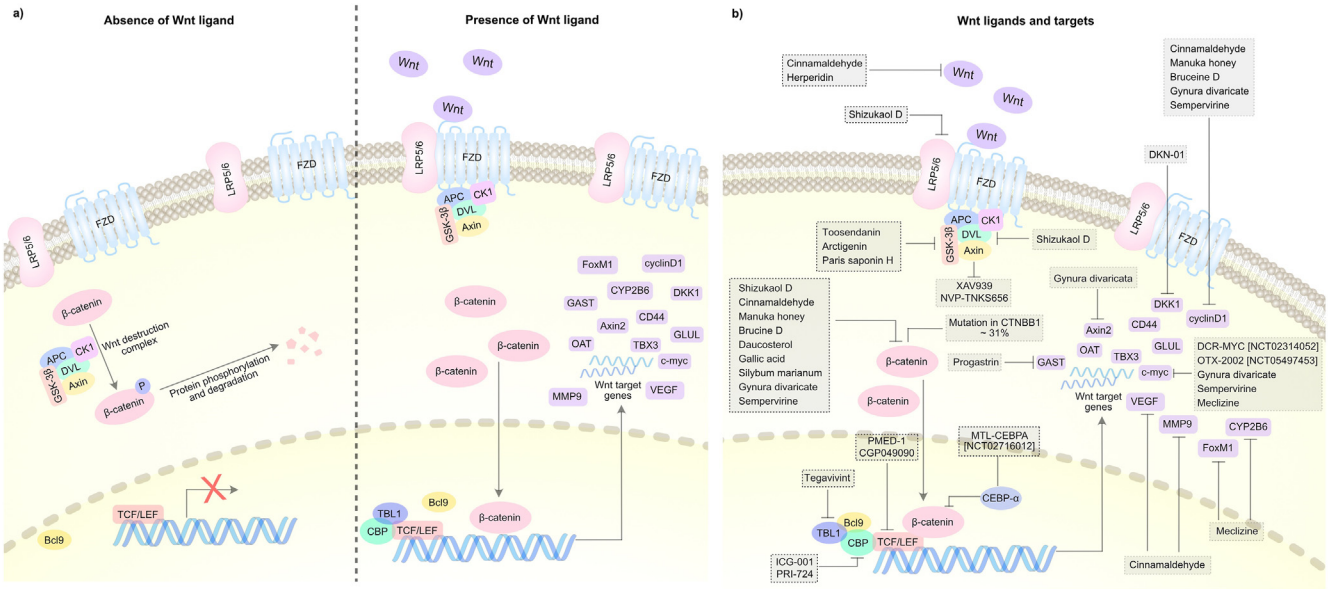

Figure 3. Wnt/β-catenin signaling in HCC. (A) Wnt signaling pathway in the presence and absence of Wnt ligands; (B) Wnt ligands, targets and inhibitors.

subtypes.[94] This subgroup with *CTNNB1* mutations exhibited immune exclusion and resistance to immune checkpoint blockers in HCC.[94] Importantly, multiple mechanistic and clinical research have also reported that the aberrant activation of Wnt/β-catenin signaling potentially mediates resistance to tyrosine kinase inhibitors and immune checkpoint inhibitors in HCC.[95,96] *CTNNB1* mutations are prevalent in HCC and result in the stabilization of cytoplasmic β-catenin and nuclear translocation that prevent phosphorylation and degradation of β-catenin due to abrogation of the phosphorylation of target residues, such as S33, T41, S45, K335 and N387.[97–101] Impairment of phosphorylation targets within the N-terminal domain of β-catenin impedes the phosphorylation of β-catenin, thereby preventing its subsequent proteasomal degradation. A study has shown that 18.1% missense point mutations were observed in exon 3 of CTNNB1 among HCC cases.[102] These mutations were found to occur more frequently in codons 32, 33, 38, and 45 with serine being the most prominently affected amino acid.[102] The *CTNNB1* mutations lead to the activation of Wnt/β-catenin signaling to enhance cell proliferation, cell migration, cancer stemness and tumor size.[97–101] Therefore, the Wnt/β-catenin signaling is considered a potential therapeutic target against HCC.

Current molecular targets identified in Wnt/β-catenin signaling in HCC

Recently, liver-targeted gene therapy has garnered a lot of interest as this enables introducing new genes to restore, increase or decrease gene expression hindering tumor progression through the Wnt/β-catenin signaling pathway. Moreover, NPs act as efficient carriers preventing nuclease damage and degradation of the genetic cargo for cancer treatment. Currently, there are 4 ongoing clinical trials using inhibitors (Table 1) and other 3 ongoing clinical trials using nanoformulations (Table 2) to target Wnt/β-catenin signaling in HCC, focusing on downstream genes or transcriptional coactivators of β-catenin. Targeting the Wnt/β-catenin signaling in HCC is an attractive therapeutic approach; however, there is lack of success due to the limited knowledge on the effective targets affecting this pathway and further defining the specific patient population that would benefit from this therapy. Therefore, some of the Wnt/β-catenin therapeutic targets are discussed below (Figure 3).

Targeting CTNNB1/β-catenin. Given the presumed cancer-causing effect of CTNNB1 mutations, targeting the Wnt/β-catenin signaling holds great promise for precision medicine approaches aimed at enhancing outcomes in HCC. β-catenin is a key component of the Wnt signaling signaling frequently mutated in HCC. However, targeting β-catenin directly is challenging due to its flat surfaces lacking the deep binding pockets targeted by small molecule inhibitors.[103] Moverover, β-catenin can interact with more than 240 proteins and be regulated by different posttranslational modifications.[103] These interactions could alter the binding pockets of β-catenin for small molecule inhibitors.[103] In ligh of that, it is valuable to investigate alternative strategies to indirectly modulate β-catenin activity by developing novel therapeutic modalities like nanoparticle-mediated siRNA therapy. NPs can be engineered to specifically deliver siRNA to cancer cells with high precision, thereby minimizing off-target effects and reducing potential damage to healthy tissues. By selectively targeting β-catenin, nanoparticle-mediated siRNA therapy can also minimize systemic toxicity compared to conventional treatments that may affect healthy cells and tissues.

Moreover, β-catenin is frequently associated with drug resistance in HCC.[104–106] Nanoparticle-mediated siRNA therapy can potentially overcome this resistance by directly inhibiting β-catenin expression and restoring sensitivity to other treatments. Importantly, recent studies have demostrated that the promising knockdown effect of mutant β-catenin in HCC by siRNA.[107–111] Therefore, nanoparticle-mediated siRNA targeting of β-catenin in HCC holds promise as a highly specific, effective, and potentially personalized therapeutic approach with the potential to revolutionize cancer treatment.

Targeting proteasomal degradation of β-catenin. The destruction complex of β-catenin is comprised of APC, Axin, casein kinase 1α and GSK3β, which together promote the phosphorylation, ubiquitination and proteasomal degradation of β-catenin.[112,113] Enhancing the activity of the destruction complex by different inhibitors or siRNA targeting the related genes regulating the complex is another feasible strategy of inhibiting the WNT/β-catenin signaling. The tankyrase inhibitor, XAV939 and NVP-TNKS656 has been demonstrated to inhibit HCC by targeting tankyrases TNKS1/2 to downregulate β-catenin thought stabilizing AXIN levels.[114–116] Other study has shown that using NVP-TNKS656 or siRNA against TNKS1/2 were able to downregulating mutant β-catenin in HCC.[116] Other inhibitors, such as pyrvinium, SSTC3 and CGK062, are reported to potentiate casein kinase 1α activity in other cancer types.[113,117] Similarly, ISG12a, methylselenic acid and Celecoxib have been shown to inhibit the degradation of ubiquitinated AXIN, enhance or activate GSK3β activity respectively[118,119,120] yet more studies of these inhibitors in HCC are needed.

Targeting transcriptional coactivators of β-catenin. The stablized β-catenin can enter into nucleus to bind with TCF family and CREB-binding protein (CBP) to trigger the expression of downstream genes during HCC carcinogenesis. In order to inhibit the downstream Wnt/β-catenin signaling, another approach is to block the interaction of the β-catenin associated transcriptional cofactors including TCF and CBP. ICG-001, a CBP inhibitor, has been reported to disrupt the interaction between CBP and CTNNB1, thereby increasing radiation-induced DNA damage in HCC and enhancing the efficacy of radiotherapy.[121] Another CBP inhibitor, PRI-724, has been elucidated to the downregulate cell proliferation and the upregulation of apoptosis-related proteins in HCC.[122] Recent studies have investigated that PMED-1 and CGP049090, which are small molecules, potentially reduced the β-catenin-TCF interactions in HCC.[123,124] Moreover, studies has shown that using dnTCF4, which is the negative form of Wnt-effector transcription factor for suppressing the binding of β-catenin-TCF, can improve survival and reduces tumors significantly in the HCC mouse modes drived by S45Y/S33Y-CTNNB1 with hMet[125] or S45Y/S33Y-CTNNB1 with G12D-Kras.[111] However, the study on inhibiting the interaction of β-catenin with TCF/CBP in HCC is still limited. To date, no anti-β-catenin-TCF/CBP complex agents have been approved for clinical use in HCC, emphasizing the ongoing requirement for drug discovery and development. In addition, targeting the interaction of β-catenin-TCF/CBP by small molecules is also challenging due to the large interaction surface.[103] Therefore, nanoparticle-mediated therapy, directly targeting the interaction between TCF/CBP with β-catenin, can serve as an alternative approach to inhibit Wnt/β-catenin signaling in HCC.

Targeting different oncogenes in HCC with mutant CTNNB1 background. Besides solely targeting the Wnt/β-catenin signaling, combination therapies that simultaneously address multiple targets are considered effective in treating HCC successfully. Recent studies have employed different mouse models, including xenografts and hydrodynamic transfection with Sleeping Beauty transposon-mediated somatic integration, to recapitulate HCC triggered by the aberrant Wnt/β-catenin signaling for therapeutic evaluation (Table A1). Importantly, studies have suggested that the activation of Wnt/β-catenin alone may not be adequate to HCC tumorigenesis.[126,127] Instead, codelivery of different oncogenes along with mutant β-catenin is required for HCC development in the hydrodynamic transfection with Sleeping Beautymouse models. The therapeutic effect of using a mTORi inhibitor (Everolimus) has been examined to decrease HCC tumor burden in T41A-CTNNB1 with G31A/T80K-NFE2L2 context.[128] Targeting c-MET by EMD1214063 has demonstrated to improve overall survival in overexpressed S45Y-CTNNB1 and c-MET-V5 induced HCC.[129] An androgen receptor degradation enhancer, ASC-J9, has been investigated to suppress ΔN90-CTNNB1 with FAK-induced HCC formation.[130] Furthermore, xenograft models can be offered as subcutaneous or orthotopic of cell lines carrying mutant CTNNB1 in mice for evaluating therapeutic efficacy. A study has used HepG2 cell line carrying exon3 deletion-CTNNB1 with overexpression of glutamine synthetase to generate HCC subcutaneous xenograft and suggested Crisantaspase and the glutamine synthetase inhibitor methionine-L-sulfoximine can inhibit tumor growth.[131] Other study has overexpressed S45P-CTNNB1 in Hepa1-6 cell line for the development of HCC orthotopic xenograft under choline-deficient high fat diets to demonstrate tumor inhibition effect by using siRNA against TNFRSF19.[132]

Natural products targeting Wnt/β-catenin signaling for HCC therapy

For the past few years, natural agents have been evaluated as potential anticancer drugs by inhibiting Wnt/β-catenin signaling, with a preference for targeting tumor cells and minimizing cytotoxic effects on normal cells.[113] Moreover, various nanoformulations have been utilized to enhance targeted drug delivery, increase bioavailability and aqueous solubility, prolong drug retention time, and mitigate potential side effects.[133] The nanoformulations with natural products are also capable of protecting against

Table 1. Clinical Studies Targeting Wnt/β-Catenin Signaling Pathway in HCC

No.	Title and clinical trial identifier	Phase and current status	Wnt target	Mechanism of action
1	DKN-01 inhibition in advanced liver cancer (NCT03645980)	1 Recruiting	DKK1	DKN-01 is a targeted anti-DKK1 mAb
2	Tegavivint for the treatment of recurrent or refractory solid HCC tumors (NCT04851119)	1/2 Recruiting	Transducin beta-like protein (TBL1)	Tegavivint binds to TBL1 inhibiting b-catenin from binding to TBL1
3	Predictive value of progastrin titer at diagnosis and of progastrin kinetics during treatment in HCC cancer patients (ONCOPRO) (NCT03787056)	Not applicable Recruiting	Gastrin (GAST)	Progastrin gene GAST is a direct target gene of the Wnt signaling pathway
4	Meclizine for hepatocellular carcinoma (OPTIM) (NCT03253289)	1 Active, not recruiting	CYP2B6, MYC and FOXM11	Meclizine is a constitutive androstane receptor (CAR) inverse agonist. CAR activation induces HCC, and therefore meclizine may decrease the expression of downstream CAR target genes – CYP2B6, MYC and FOXM1

environmental factors, such as pH variations, enzymatic degradation, and the possibility of biochemical degradation.[133] Recently, several studies have suggested that various natural products have demonstrated inhibitory effects on Wnt/β-catenin signaling in HCC (Table 3). Shizukaol D and Hesperidin were reported to downregulate β-catenin via inhibiting the Wnt upstream regulators.[134,136] Cinnamaldehyde, Manuka honey, Bruceine D, Daucosterol, Gallic Acid, Silybum marianum, Gynura divaricate, and Sempervirine exhibited suppression effect of β-catenin.[135,137–139,142–144,146] Moreover, Bruceine D, Toosendanin, Arctigenin, and Paris saponin H were suggested to promote the activity of proteasomal degradation for β-catenin.[138,140,141,145] Applying nanoformulations with natural products is a potential diection to enchence the therapeutic effect for HCC treament.

Preclinical studies using nanomedicine targeting Wnt/β-catenin signaling in HCC

Several preclinical studies investigating nanoparticle-related therapeutic agents for targeting the Wnt/β-catenin signaling in HCC have shown promising results (Table A2). The agents are able to target either β-catenin directly,[147–150] the upstream regulators of WNT,[148] or the downstream targets[148–153] of WNT β-catenin signaling. The therapeutic milk-derived nanovesicles (tMNV) that target EpCAM on HCC cells and are loaded with siRNA to β-catenin (EpCAM-tMNV) showed effective β-catenin-targeting in vitro and in vivo.[147] Moreover, administration of EnCore lipid NPs with small siRNA directly targeting *CTNNB1* has demonstrated the significant decreased tumor burden in the HCC mouse model induced by S45Y/S33Y-CTNNB1 and G12D-Kras.[111] The Niclosamide in Niclosamide-loaded pluronic NPs was an FDA-approved anthelmintic agent used for schistosomiasis treatment, and its potential in anticancer treatment has been demonstrated via targeting the Wnt/β-catenin signaling upstream regulators, β-catenin and downstream targets, supported by reduction of HCC tumour size in mouse model.[148] Similarly, sBBI&PDP and SeNPs/QCT showed promising results in targeting β-catenin and the downstream Cyclin D1 to suppress HCC.[149,150] Furthermore, the nanoparticle therapeutics, Anti-miR-17 LNP, can target MYC-regulated genes such as the MiR-17, which resulted in delayed tumor progression and reduced tumour volume via inhibiting MYC-induced transcriptional program.[151] Suppressing known WNT targets, such as VEGF,[154] by DOX/Cur-NPs (Curcumin and Doxorubicin

Table 2. Clinical Studies Targeting Wnt/β-Catenin Signaling Pathway in HCC Using Nanoformulations

No.	Title and clinical trial identifier	Phase and current status	Wnt target	Type of nanoformulation
1	DCR-MYC in patients with HCC (NCT02314052)	1b/2 Terminated	MYC	MYC siRNA LNPs
2	OTX-2002 in patients with HCC – MYCHELANGELO I (NCT05497453)	1/2 Recruiting	MYC	MYC mRNA LNPs
3	MTL-CEBPA in patients with advanced liver cancer (NCT02716012)	1a/b Active, not recruiting	C/EBP-α	First-in-class, first-in-human small activating C/EBP-α RNA (saRNA) liposomes

Table 3. Natural Product Targeting Wnt/CTNNB1 Pathway in HCC

No.	Natural product	Source	Target/mechanism
1	Shizukaol D[134]	A dimeric sesquiterpene from Chloranthus serratus	- Downregulating the expression of β-catenin and its upstream regulators LRP and Dvl2 - Inhibiting cell viability and colony formation and induces apoptosis in HCC cells
2	Cinnamaldehyde[135]	Aromatic aldehyde extracted from Cinnamomum cassia	- Downregulating the expression of Wnt-3a, β-catenin, cyclin D1, MMP-9, and VEGF
3	Hesperidin[136]	Polyphenolic flavanone glycoside of citrus fruits and vegetables	- Downregulating the expression of Wnt-3a - Inhibiting Wnt3a-induced proliferation in HCC cells
4	Manuka honey[137]	Monofloral honey obtained from the Manuka tree	- Downregulating the expression of β-catenin and cyclin D1 - Inducing apoptosis in HCC cells
5	Bruceine D[138]	Quassinoid compound extracted from the seeds of Brucea javanica	- Downregulating the expression of β-catenin and cyclin D1 - Promoting proteasomal degradation of β-catenin and the depletion of its nuclear accumulation
6	Daucosterol[139]	Phytosterol-like compound extracted from acanthopanax senticosus	- Downregulating the expression of β-catenin - Reducing the proliferation, migration, and invasion capacities of HCC cells
7	Toosendanin[140]	Triterpenoid extracted from Melia toosendan Sieb et Zucc	- Promoting proteasomal degradation of β-catenin by increasing the function of APC, AXIN1, CK1, and GSK3β complex
8	Arctigenin[141]	Bioactive lignan ingredient extracted from Arctium lappa (Niubang)	- Downregulating the expression of β-catenin by increasing GSK3b activity
9	Gallic acid[142]	Phenolic compound found in several fruits and medicinal plant	- Downregulating the expression of β-catenin
10	Silybum marianum total extract[143]	Extracted from the milk thistle seeds	- Downregulating the expression of β-catenin - Reducing the proliferation in HCC cells
11	Gynura divaricata[144]	A medicinal and edible plant in folk and the roots, stems, and leaves	- Downregulating the expression of β-catenin, c-MYC, cyclin D1, and AXIN2 - Inhibiting cancer stem cell growth
12	Paris saponin H[145]	A steroid saponin derived from Rhizoma Paridis	- Downregulating the expression of β-catenin by increasing GSK3b activity - Reducing cell viability and inducing apoptosis in HCC cells
13	Sempervirine[146]	An alkaloid compound found as a constituent of Gelsemium sempervirens	- Downregulating the expression of β-catenin, c-MYC, and cyclin D1

delivered by nanoparticle) or NP-SFB-Ab (Sorafenib-loaded polymer nanoparticle) demonstrated reduced liver nodule formation and inhibited tumour growth as well.[152,153] Overall, targeting the Wnt/β-catenin pathway with nanoparticle therapeutics represents a promising strategy for the treatment of HCC.

Conclusion

The recent years have witnessed exceptional progress in research and applications in the field of HCC management with the Wnt/β-catenin signaling pathway being a key pathway regulated during hepatocarcinogenesis. Emerging targets such as β-catenin, proteasomal degradation of β-catenin, transcriptional coactivators of β-catenin and different oncogenes in HCC with mutant CTNNB1 background could be targeted for HCC management. However, the current therapies and novel targets face a challenge in safely and effectively delivering drugs to the tumor cells due to their nonspecificity, off-target toxicities and impaired liver function. To overcome this, the revolutionary field of nanotechnology offers numerous advantages that spark hope in contributing to the progress made in HCC therapy and outcomes in recent years. The utilization of nanotechnology in HCC therapy offers the potential for targeted drug delivery, enhanced bioavailability, improved tumor penetration, and reduced systemic toxicity. By encapsulating drugs, small molecules, peptides, RNAs and natural products within nanoformulations, it is possible to achieve selective and targeted release, enabling higher drug concentrations at

the tumor site, while minimizing adverse effects on healthy adjacent tissues. Furthermore, the unique physicochemical properties of NPs can facilitate active targeting and personalized medicine strategies, allowing for selective binding to tumor cells and improving treatment efficacy. With ongoing advancements in nanotechnology, there is optimism that it will continue to play a vital role in overcoming the challenges associated with HCC management and contribute to further advancements in therapeutic outcomes for patients.

Supplementary materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2023.07.012.

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