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Sequential drug delivery for liver diseases

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ABSTRACT

The liver performs critical physiological functions such as metabolism/detoxification and blood homeostasis/biliary excretion. A high degree of blood access means that a drug's resident time in any cell is relatively short. This short drug exposure to cells requires local sequential delivery of multiple drugs for optimal efficacy, potency, and safety. The high metabolism and excretion of drugs also impose both technical challenges and opportunities to sequential drug delivery. This review provides an overview of the sequential events in liver regeneration and the related liver diseases. Using selected examples of liver cancer, hepatitis B viral infection, fatty liver diseases, and drug-induced liver injury, we highlight efforts made for the sequential delivery of small and macromolecular drugs through different biomaterials, cells, and microdevice-based delivery platforms that allow fast delivery kinetics and rapid drug switching. As this is a nascent area of development, we extrapolate and compare the results with other sequential drug delivery studies to suggest possible application in liver diseases, wherever appropriate.

Contents

Abbreviations: 5-Fu, 5-fluorouracil; AFLD, alcohol-induced fatty liver disease; AIHPAs, anti-inflammatory and hepatoprotective agents; ALF, acute liver failure; ASK1, apoptosis signaling kinase 1; cccDNA, covalently closed circular DNA; CCR2/5, cysteine-cysteine motif chemokine receptor-2/5; CDDP, cisplatin; CHB, chronic hepatitis B infection; DDI, drug–drug interaction; DEBs, drug-eluting beads; DILI, drug-induced liver injury; ECM, extracellular matrix; EPS, efficacy potency and safety; FXR, farnesoid X receptor; GSH, glutathione; HAIC, hepatic arterial infusion chemotherapy; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCPT, 10-hydroxycamptothecin; HGF, hepatocyte growth factor; HSCs, hepatic stellate cells; iDILI, idiosyncratic drug-induced liver injury; IFN-α, Interferon-α; IMPs, immunomodulating peptides; inDILI, intrinsic drug-induced liver injury; LOXL2, lysyl oxidase-like 2; LT, liver transplantation; MEMS, micro-electro-mechanical systems; MSCs, mesenchymal stem cells; MTX, methotrexate; NAC, *N*-acetyl cysteine; NAFLD, nonalcoholic fatty liver disease; NAs, nucleotide analogs; NASH, nonalcoholic steatohepatitis; NIR, near-infrared; PEG-IFN, pegylated interferon; PEG-L-PA, poly(ethylene glycol)-b-poly(L-alanine); PHx, partial hepatectomy; PLGA, poly(lactic-co-glycolic acid); PLLA, poly(lactic acid); PPAR, peroxisome proliferator-activated receptor; PTX, paclitaxel; PVA, polyvinyl alcohol; RGD, arginylglycylaspartic acid; SOD, superoxide dismutase; TACE, transarterial chemoembolization; TGFβ1, transforming growth factor β1; TGFβR1, transforming growth factor β1 receptor; TNF, tumor necrosis factor; TP, tea polyphenols; VEGF, vascular endothelial growth factor.

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1. Introduction

Liver diseases account for more than 800 million cases and lead to approximately 2 million deaths every year worldwide [1,2]. The high morbidity of liver diseases is due to not only the high prevalence of liver-specific insults such as viral infection and misuse of drugs but also increasing population with metabolic syndromes such as nonalcoholic fatty liver disease (NAFLD). The mortality of liver diseases is mainly attributed to end-stage diseases such as cirrhosis and hepatocellular carcinoma (HCC), both of which are chronically developed [3].

Liver anatomy and physiology with complex cell-scale repeating lobules and blood homeostasis [4], exhibiting synthetic [5], metabolic [6,7], and excretory functions [8], imply that delivering therapeutic agents to the liver is a daunting task with many challenges [9,10]. When blood travels to almost every cell in the liver, anything that the cells are exposed to lasts for only a short duration (sec to minutes) [11]. Foreign agents present in the liver cells are quickly converted or metabolized to a nontoxic form and lose their original properties.

To overcome the challenges of delivering therapeutic agents such as small molecules and peptide drugs, advances in novel drug delivery platforms have improved the pharmacodynamics/pharmacokinetics of the drugs [12], resulting in increased efficacy and reduced hepatotoxicity. For instance, lipidization of a somatostatin analog enabled better association with liver cell membrane, thus enriching drug accumulation in the liver by 3.8-fold [13]. Use of cell type-specific ligands could further distinguish the population (i.e., hepatocytes and Kupffer cells) and even the sub-population of cells (i.e., quiescent and activated hepatic stellate cells (HSCs) [14]). When encapsulated in pegylated liposomes, the antitumor agent doxorubicin exhibited a unique concentration profile characterized by maintained concentration with a reduced peak value compared to conventional liposome. It confers better tumor inhibition and reduced liver damage [15]. As for combination therapy, multiple drugs with diverse modes of action can fully cover the spectrum of disease pathology so that each drug at reduced concentration (and thus hepatotoxicity) is administered to synergize with each other for improved efficacy (E), potency (P), and safety (S) [16]. Delivery of drugs for combination therapy of liver diseases can be through simultaneous or sequential delivery. The simplicity of simultaneous delivery of multiple drugs has unwanted side effects of undesirable drug–drug interaction (DDI) [17] and immune-mediated idiosyncratic toxicity [18], often leading to drug-induced liver injury (DILI). This simplicity also overlooks the sequential process of disease development resulting from abnormal liver regeneration. Therefore, sequential drug delivery for combination therapy has the potential to avoid or reduce these side effects in the liver where the turnover and clearance of drugs are fast and to achieve more synergistic efficacy.

Sequential drug delivery, defined as the administration of two or more drugs with different mechanisms of action and given in a designed order, can attain synergistic efficacy owing to the optimal sequence of mode of action and can avoid DDI as in the case of simultaneous delivery [19]. The approach entails staggered delivery of multiple drugs by sequential administration through oral or parenteral routes or by the use of controlled- or triggered-release drug delivery systems. In addition to the technical challenges involved, lack of understanding of the liver physiology, pathology, and limitations of existing therapies also hinders the development of sequential drug delivery for liver disease treatments. In this review, we identify liver-specific anatomy and physiology features that may benefit from sequential drug delivery and expound on the possible applications in the treatment of liver diseases. We particularly focus on four major liver diseases: DILI, chronic hepatitis B (CHB) infection, NAFLD, and HCC. As there are limited examples of sequential drug delivery in the liver, the limitations of existing therapies for each disease are discussed and potential improvement by sequential delivery is proposed.

2. Abnormal regeneration leads to diseases

The liver performs essential functions such as nutrition metabolism and detoxification, as well as effective regeneration. Resumption of size and mass was observed in several rodent liver models [20,21]. For example, partial hepatectomy (PHx) in rats has shown that the liver can resume its size and mass even after resecting 70% of the liver [20]; this regenerative potential was unimpeded even after 12 sequential PHx [21]. Regeneration after PHx in normal and diseased livers has also been observed in humans [22]. Michalopous *et al.* and Miyaoka *et al.* have elucidated the precise orchestration of liver regeneration [23,24], which is a highly coordinated process involving the proliferation of different hepatic cell types and activation of various machineries in response to multiple signals. For instance, cellular hypertrophy mainly contributes to size compensation when the injury has occurred in less than 50% of the liver mass, whereas hepatocyte proliferates to increase cell number after a large injury. Upon hepatectomy, a rapid increase of mitogenic growth factors such as hepatocyte growth factor (HGF) and cytokines such as tumor necrosis factor (TNF) resets mature hepatocytes to proliferative status. In addition, the involvement of liver stem cell population, namely, the periportal oval cells [25] and the pericentral $Axin2^{+}$ stem cells [26], can also give rise to hepatocytes. If hepatocyte proliferation is impaired, then participation of biliary-based facultative stem cells is observed [27]. The proliferation is so tightly regulated that excessive hepatocytes produced will undergo apoptosis when cell number and function restore [28]. Transforming growth factor β1 (TGFβ1) is an important mito-inhibitory factor in terminating liver regeneration, which can maintain hepatocytes in the quiescent state. TGFβ1 is released from the existing extracellular matrix (ECM) at approximately 72 h after PHx. Although the hepatocytes are nonresponsive to the TGFβ1-mediated growth inhibition because of the absence of TGFβ1 receptors (TGFβR1) to transduce the inhibitory signals, the HSCs respond to TGFβ1 and produce ECM, which sequesters HGF. The sequestering of HGF leads to the absence of proliferative signals for the hepatocytes and terminates the regeneration. The sequestering of the HGF is the hallmark of hepatocyte cell cycle arrest and termination of liver regeneration [29].

The physiological responses of the liver to PHx or drugs critically involve provocative pro-inflammatory signals [30], growth factor signaling [31], metabolism [32], and matrix remodeling [33] (Fig. 1a). These responses coordinate the priming, proliferation, and termination of liver regeneration, and accurate temporal control of these events is critical for normal liver regeneration [34]. The loss of feedback mechanisms

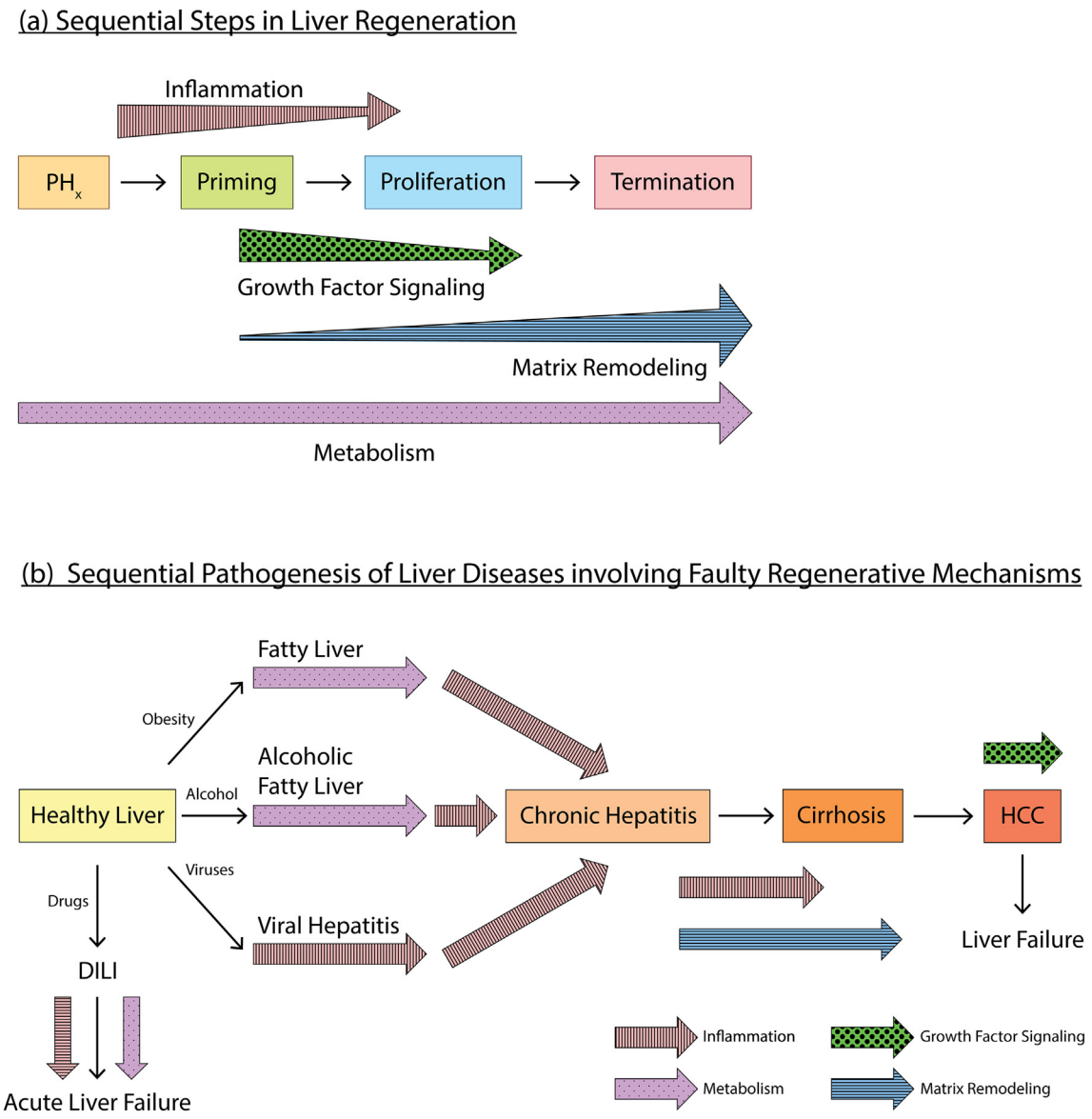

Fig. 1. Sequential patterns in liver regeneration and disease pathogenesis. (a) Four elements (inflammation, metabolism, proliferation and matrix remodeling) are tightly coordinating in the course of liver regeneration. (b) These elements attribute to diseases in a progressive manner when continuously disrupted by certain insults, implying that sequential removal of the current and preexisting pathologies would prevent disease development and recreate homeostasis for regeneration. DILI: drug-induced liver injury; HCC: hepatocellular carcinoma.

leads to the loss of temporal process control. The aberrant responses to injuries cripple liver regeneration and cause pathologies. For example, the acute metabolic reprograming in response to xenobiotic results in reactive metabolite and cell damage, which may stimulate inflammatory responses [35]. The inflammatory signals that are required to trigger proliferation in response to tissue damage need to be dampened once sufficient numbers have been achieved; sustained pro-inflammatory signals would cause extensive tissue damage [36]. Prolonged inflammatory response in nonalcoholic steatohepatitis (NASH) is a contributing factor highly correlated with the prevalence of cirrhosis [37]. The abrogation of HGF/*c-met* signaling impairs liver regeneration through persistent inflammation [38]. Similarly, matrix deposition, which sequesters growth factors and dampens proliferative signals, has to be tightly managed to avoid excess fibrous matrix deposition and impaired liver function [39]. Moreover, the timeframe of developing each pathological pathway varies from one disease to another. The metabolic reprograming in DILI generates numerous metabolites that elicit cytotoxicity in hours, while the onset of immune response is typically delayed (1-2 months) [35]. Such reprograming process could be chronic in NAFLD where steatosis gradually develops when the liver is constantly exposed to a lipogenic environment. These examples emphasize meticulous modulation of these pathways to promote liver regeneration under various pathologies.

In addition, conventional treatments for liver diseases may accompany the development of other pathophysiological events such as angiogenesis in cancer. Such event may compromise therapeutic efficacy by hampering focal drug penetration. Therefore, to benefit conventional treatment, sequential delivery should employ multiple drugs targeting different pathways according to their time-dependency and/or concurrent pathogenic roles. Details of pathological pathways that can be targeted are elaborated in the following section.

3. Sequential drug delivery for liver diseases

On the basis of the time-scale of disease development, DILI, CHB infection, NAFLD, and HCC were introduced accordingly. For each disease, the etiology is discussed in relation to the deregulated signaling events (Fig. 1b), and current treatment strategies and limitations are

summarized. Sequential drug delivery is demonstrated using real examples or extrapolated from proof-of-concept studies. Time/spatial scale of the sequential delivery of therapeutic agents is critical in the design and implementation of the sequential delivery scheme. If the need for switching drugs is global/systemic or in hours or days/weeks or longer, there is really no need to contemplate such a sequential delivery scheme. It suffices to only orally administer different drugs every few hours or days/weeks or longer in sequential treatments or sequential therapy. If the synergistic effects of sequential combination therapy require local or in the time scale of minutes or seconds to switch between drugs, then liver diseases are ideal targets for contemplating such a sequential drug delivery scheme.

Drug delivery systems such as hydrogels, micro/nanoparticles, layer-by-layer (LbL) assembly, and microdevices, as well as a combination of these systems, are potential candidates for sequential drug delivery in liver disease treatment. Hydrogels are physically or chemically crosslinked polymeric networks that can imbibe a large amount of water. The aqueous and porous environment within a hydrogel renders it most suitable for the encapsulation and delivery of proteins and cells. Hydrogels can be introduced into the body by either implantation or injection of gel precursors that crosslink *in situ*; the latter method is preferred, as it is less invasive. Micro/nanoparticles are widely used for the delivery of small-molecule drugs and proteins, and dual-drug release can be achievable by forming particles with a core-shell structure [40]. One strategy for sequential drug delivery is to pre-encapsulate one type of drug in micro/nanoparticles before incorporating the particles in a scaffold (e.g., hydrogels or nanofibers) containing a second drug [41,42]; drugs in the micro/nanoparticles are typically released at a slower rate than those in the scaffold. With the majority of materials accumulated in the liver after intravenous injection [43,44], nanoparticles may be advantageous as compared to other delivery systems in terms of efficiency in transporting drugs to the liver. LbL assembly refers to the method of forming multilayer coatings through alternate adsorption of oppositely charged polymers. Small-molecule drugs and biomacromolecules can be incorporated between the polyelectrolyte layers through electrostatic interactions and released sequentially as the polymeric layers degrade. Importantly, the entire LbL assembly process is performed in an aqueous environment, which protects proteins from denaturation. While the LbL assembly is a versatile method of forming a multilayer coating, the controlled release of incorporated drugs/proteins remains a challenge because of interlayer diffusion that leads to the mixing of polyionic species during the assembly process [45]. Microdevices such as micro-reservoirs, in which drugs are stored and released through active or passive pumping, not only are useful for sustained drug delivery but could also be designed with biodegradable materials and/or LbL assembly to achieve sequential delivery of multiple drugs. Potential applications of the above-mentioned drug delivery systems in the treatment of liver diseases are investigated in more detail in the following sections and illustrated in Fig. 2.

3.1. Drug-induced liver injury (DILI)

DILI has become a major cause of acute liver failure (ALF) and transplantation in Western countries [46] and accounts for most of the drug withdrawal from clinical trials or even postmarketing because of hepatotoxicity [47]. DILI may result from direct cell toxicity or toxic metabolites or through downstream events caused by the counterbalance of liver injury versus regeneration [48]. In terms of spatial distribution, the injury can be hepatocellular injury, cholestatic injury, or hepatocellular-cholestatic mixed injury. Variability of host factors including genetic polymorphism related to metabolism and immunity is highly associated with DILI; for instance, the HLA-B*5701 genotype is a determinant of flucloxacillin-induced liver injury [49]. DILI can be intrinsic (inDILI) or idiosyncratic (iDILI) in association with dose-dependency, predictability, and pattern of injury [50]. inDILI, such as APAP overdose or CCl_4-caused toxicity, exhibits a dose-dependent hyperacute injury pattern. The time scale is usually in hours, with a rapid onset and offset of massive zone-3 cellular necrosis, followed by activation of inflammatory response [50]. iDILI, however, refers to uncommon hepatotoxicity occurring in a minority of patients who do not follow a known pharmacological effect [51]. It is less predictable in its dose-dependent occurrence, with a relatively longer period (1–4 weeks) and rare prevalence (approximately 10 cases per 10,000 inhabitants) [52]. The immune system is substantially involved in the onset of iDILI [53]. DILI exhibits specific patterns in protective mechanisms and regeneration: particularly, the antioxidant glutathione (GSH) plays a key role for cell protection against DILI; thus, adjuvant or sequential therapy involving antioxidants have drawn some attention [46].

3.1.1. Current DILI treatments and limitations

The management of DILI involves the recognition of liver injury, identification of the suspected drug, and/or medical intervention [54]. Prompt withdrawal of the suspected insult is important for treating drug-induced liver damage [55]. Careful monitoring is required to minimize the risk of potential injury while controlling the clinical condition. In addition to the withdrawal of the suspected drug, the rest of the treatment is largely supportive. Among them, *N*-acetyl cysteine (NAC) is a clinically established antidote to treat APAP-caused liver toxicity. It can quench free radicals and restore hepatic GSH to detoxify *N*-acetyl-p-benzoquinone imine (NAPQI), the metabolite of APAP [56]. Currently, clinical studies have also supported NAC usage in DILI-related ALF, and early intervention in adults has shown to improve clinical outcome such as transplant-free survival rate [55,57]. However, high dose of NAC causes adverse effects such as nausea and vomiting. In addition to NAC, various anti-inflammatory and hepatoprotective agents (AIHPAs) are also used; however, large, prospective, randomized, controlled clinical evidence is still lacking. Examples of AIHPAs include corticosteroids [58], glycyrrhizic acid [59], silymarin [60], ursodeoxycholic acid [61], bicyclol [62], and *S*-adenosyl methionine [63] (Table 1), among which corticosteroids are most widely used and shown to be effective in ameliorating liver injury in recent clinical studies [64]. NAC and AIHPAs can be given either orally or as an intravenous infusion in the treatment of DILI. Despite the usage of NAC and empirically prescribed AIHPAs, the current treatment of DILI is not satisfactory, largely because of the complex spectrum of DILI in etiology, pathogenesis, and histology patterns.

In the event of ALF caused by DILI, orthotopic liver transplantation (LT) is the most essential treatment. However, LT is limited by the scarcity of liver donor. Transplantation of primary human hepatocytes is a surrogate for overcoming the accessibility issue of LT [76]. The limited proliferation ability of primary hepatocytes *in vitro* hinders this strategy from being widely used [77]. Recently, there has been progress in the long-term expansion of hepatocytes. Peng *et al.* demonstrated that by re-creating the inflammatory microenvironment during liver injury, primary murine hepatocytes could be expanded *in vitro* without losing their metabolic function [78]. A similar cocktail of small molecules and growth factors was used for the long-term expansion of mature murine and human hepatocytes as 3D organoids [79]. Functional hepatocyte derived from stem cells is an alternative to adult hepatocytes. Various types of stem cells, namely, mesenchymal stem cells (MSCs) [80], embryonic stem cells, and induced pluripotent stem cells [81], have been evaluated as alternative cell sources for transplantation [81,82]. Among stem cells, MSCs are favorable for cell-transplanting therapy owing to the excellent immunomodulating ability and well-elucidated mechanism of action [83]. MSCs have been prevalently used in preclinical studies showing improvement in liver functions and regeneration [84]. It is premature to anticipate similar findings in human patients.

3.1.2. Sequential drug/cell delivery in DILI

Where withdrawal of suspected drug failed to resolve DILI, patients who are susceptible to develop ALF may benefit from the sequential delivery of NAC followed by corticosteroids. An important consideration in

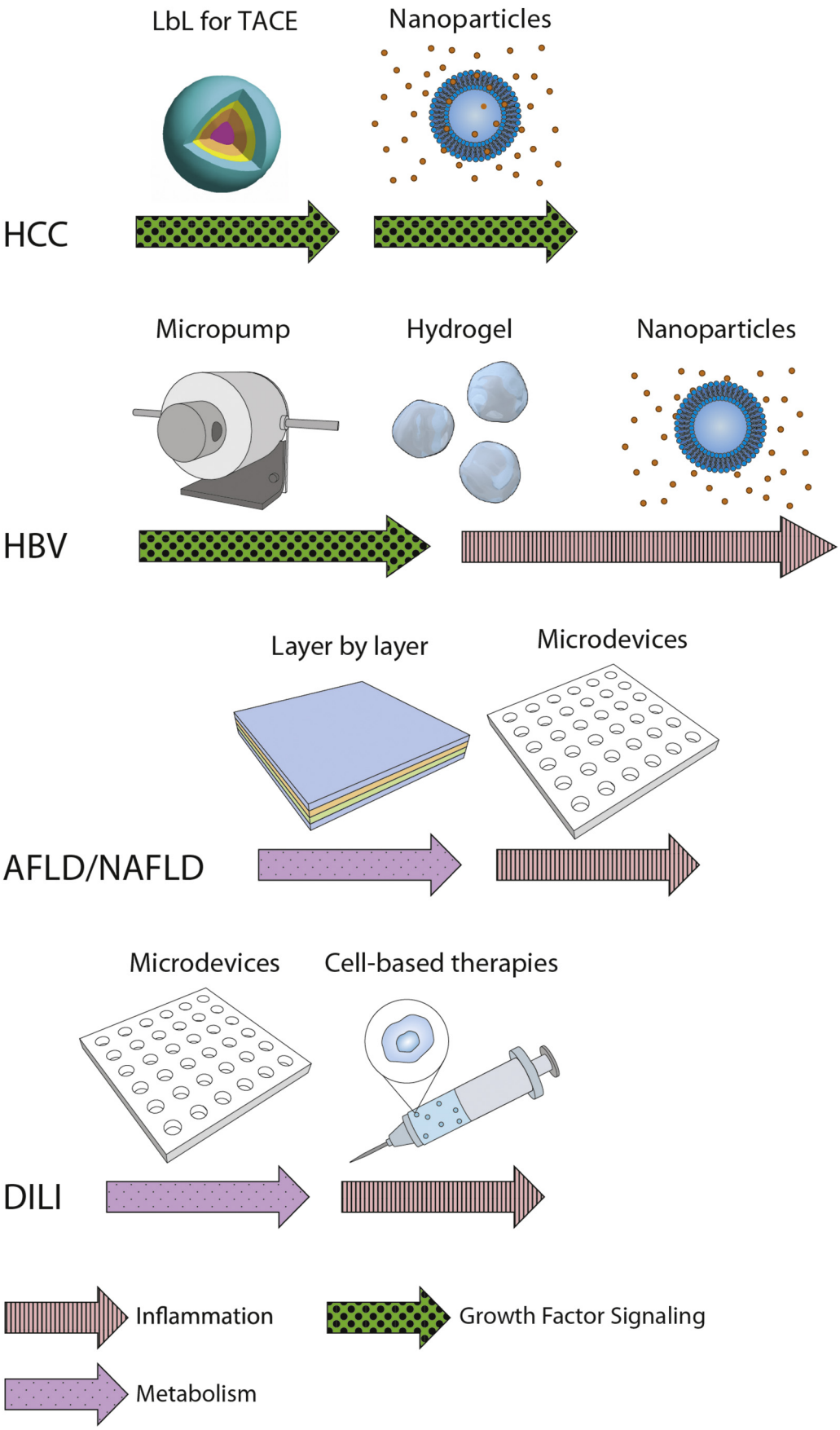
LbL for TACE
Nanoparticles
HCC
Micropump
Hydrogel
Nanoparticles
HBV
Layer by layer
Microdevices
AFLD/NAFLD
Microdevices
Cell-based therapies
DILI
Inflammation
Growth Factor Signaling
Metabolism

Table 1
Hepatoprotective agents used for different types of injury in DILI.

Drugs to be used	Mechanism of action	Targeted injury pattern	Ref
N-Acetyl cysteine (NAC)	Replenishment of the GSH pool	APAP-induced hepatotoxicity (mainly)	[65]
Corticosteroids	Regulation of glucocorticoid function to inhibit inflammation	Severe immune hepatitis-like features	[66,67]
Magnesium isoglycyrrhizinate (MgiG)	Attenuation of oxidative stress	Hepatocellular injury or mixed injury	[68]
Bicyclol	Scavenger of free radicals; inhibition of cytokine production	Mild-to-moderate hepatocellular and mixed DILI; severe inflammation	[69,70]
Glycyrrhizic acid	Inhibition of hepatic apoptosis and necrosis; inhibition of the NF-κB pathway	Mild-to-moderate hepatocellular and mixed DILI; severe inflammation	[71]
Silymarin	Prevention of apoptosis; enhancement of SOD and peroxidase.	Mild inflammation	[72,73]
Ursodeoxycholic acid (UDCA)	Regulation of bile acid pool; immunomodulation	Cholestatic DILI	[66,74]
S-Adenosyl methionine (SAMe)	Replenishment of the GSH pool	Cholestatic DILI	[75]

Abbreviations – DILI: drug-induced liver injury; GSH: glutathione; NF-κB: nuclear factor kappa B; SOD: superoxide dismutase.

designing formulations for sequential delivery is the onset (slow/fast) and the duration (short/long) of release for each drug. NAC is usually given for 72 h for DILI treatment [85], whereas corticosteroid therapy typically lasts several weeks, and abrupt withdrawal may cause relapse in liver injury [86]. Micro-/nanoparticles with a core-shell structure, such as microspheres consisting of a poly(lactic acid) (PLLA) core and a poly(lactic-co-glycolic acid) (PLGA) shell, are potential delivery systems [40]. As the PLGA shell degrades more rapidly than the PLLA core, incorporating NAC in the shell would facilitate rapid and short-term release for quenching of reactive metabolites. Superoxide dismutase (SOD) could also be used for the removal of reactive oxygen species, as targeted delivery of SOD to the liver has shown improvement in APAP-induced hepatotoxicity in an animal model [87]. Incorporating corticosteroid in the PLLA core would achieve prolonged release for the modulation of inflammatory response. Decorating the drug delivery particles with liver-targeting moieties such as galactose [88] may reduce side effects associated with the high dose of NAC through localized delivery. Sequential delivery of NAC and corticosteroid for DILI treatment is supported by a recent study, which reported that NAC (7 days) and prednisolone (2 weeks) treatment improved serum biochemistries of patients with severe flupirtine-induced liver injury [89]. It is worth investigating whether the long intervention window of DILI can benefit from convolution of or repeated cycles of much shorter window (minutes to hours) of sequential delivery of drug combinations.

Concerning MSC transplantation for ALF, immaturity of the differentiated hepatocyte is a major challenge. 3D culture systems can promote the maturity of stem cell-derived hepatocyte-like cells and maintain functions better than 2D monolayer culture systems [90,91]. Li *et al.* developed a PLGA scaffold coated with collagen for the differentiation of bone marrow MSCs to hepatocyte-like cells [92]. Compared to cell differentiation in 2D monolayer cultures, the cell numbers and metabolic functions were significantly higher in the scaffold. Sequential delivery of multiple growth factors can be synergized with a scaffold-based strategy to promote hepatic differentiation. Bishi *et al.* developed an *ex vivo* bioengineered liver tissue composed of bone marrow-derived MSCs grown on an electro-spun PLLA nanofiber mesh [93]. Gelatin was blended with the PLLA solution to form PLLA/gelatin nanofibers for improved wettability and cell adhesion through RGD–integrin interactions. Bone marrow MSCs were seeded on the PLLA/gelatin nanofiber mesh and subjected to endoderm differentiation, hepatic lineage specification, and hepatic maturation by sequential addition of growth factors. Higher cell proliferation and more hepatocyte-like cells were observed on the PLLA/gelatin nanofiber mesh than on the PLLA scaffold.

Hydrogels are potential candidates for local delivery of cells, as the porous structure and aqueous environment allow cell encapsulation and efficient exchange of nutrients and metabolites. For instance, a thermogel composed of poly(ethylene glycol)-*b*-poly(L-alanine) (PEG-L-PA) was developed for the encapsulation and hepatic differentiation of MSCs [94]. Tonsil-derived MSCs were encapsulated in PEG-L-PA thermogels by increasing the temperature of a mixture of cells and polymers to 37 °C to induce sol-gel transition. *In situ* differentiation of MSCs to hepatocytes in hydrogels could be carried out by incorporating growth factors such as epidermal growth factor, basic fibroblast growth factor, and HGF in the hydrogel. To prevent rapid diffusion of growth factors from the hydrogels, growth factors could be preencapsulated in microspheres before co-encapsulating with cells in the hydrogel [95]. The clinical benefits of these strategies that can promote MSC differentiation to hepatocyte-like cells for rescuing liver functions remain to be investigated.

3.2. Chronic hepatitis B (CHB) infection

CHB infection has become a global public health issue. Approximately 257 million people have been infected with HBV, which led to 887,000 deaths in 2015 [96]. Acute hepatitis B infection is defined as the first exposure to HBV. This could be effectively removed by intrinsic immunity of the patient within 6 months, with or without emergency treatment [64]. If the body fails to clear the virus and the infection persists for more than 6 months, then chronic hepatitis develops, requiring life-long medication to suppress the virus load and to reduce the risk of further liver damage [97].

HBV grows in the hepatocytes and mainly damages them. The life cycle of the virus has been well studied with the identification of key components such as entry receptor, polymerase, and assembly machinery [98]. The double-stranded DNA virion enters the hepatocytes in a receptor-dependent manner and releases the genome and polymerase into the host cytoplasm. After translocation into the host nucleus, the formation of a covalently closed circular DNA (cccDNA) relies on its own polymerase, whereas the transcription of viral RNA takes advantage of the cellular machinery. The presence of cccDNA attributes to the persistence and reactivation of the disease. More viral genomes and proteins are synthesized from RNA and enveloped in the host endoplasmic reticulum.

3.2.1. Current CHB treatments and limitations

Treatment for CHB is determined by the exposure to the virus and the period of infection. For exposure to the virus for a few days, emergency treatment is helpful to curb the infection; if infected for a few weeks or months (acute hepatitis B infection), then treatment is necessary to relieve the symptoms while the body fights off the infection.

Fig. 2. Sequential delivery strategy used in various diseases. The multifactorial pathogenesis requires precise control of drug release in a specific stage, or sustained release during the period. Due to the encapsulation ability of micro-/nanoparticle and lay-by-layer assembly, they benefit the delivery of anti-cancer, anti-viral and/or other small molecule drugs. Hydrogel is suitable for delivering peptide and/or protein drugs. The flexibility and capacity of microdevice allows it to deliver complex administration schemes for DILI and AFLD/NAFLD. Cell-based therapy is applicable for acute liver failure caused by DILI. AFLD: alcoholic fatty liver disease; DILI: drug-induced liver injury; HBV: hepatitis B virus infection; HCC: hepatocellular carcinoma.

When the virus is not cleared from the body and when the infection persists for more than 6 months (CHB), antiviral medication is administered to keep the virus under control and reduce the risk of liver damage [97].

Anti-HBV treatment involves targeting the HBV polymerase with designed nucleotide analogues (NAs), i.e., lamivudine, adefovir dipivoxil, tenofovir, and entecavir [99]. They are administered orally and show a rapid antiviral effect; however, drug resistance has been observed on prolonged treatment [99]. Other approaches to target viral replication, such as small interference RNA, capsid assembly inhibitors (heteroarylpyrimidines and sulfamoyl benzamide), and entry receptor inhibitors (i.e., Myrcludex B) showed equivalent or improved clearance compared to conventional NAs [100]. Despite sero-clearance of HBV, there is still a threat of liver-related complications and HBV reactivation because of the persistence of extrahepatic cccDNA. New approaches to eliminate this extrahepatic cccDNA have been investigated, including zinc finger protein, TALENs, and CRISPR/Cas9 [100]. On the other hand, improved host immune responses could enhance viral clearance; therefore, immunomodulating peptides (IMPs) have been developed [101]. Interferon α (IFN-α) and the pegylated analog (PEG-IFN) are the only approved IMPs in the market. The antiviral effect is based on two mechanisms: suppression of HBV replication in infected hepatocytes and activation of T cell-mediated HBV-specific immune response. However, severe side effects and high cost limit their prolonged administration [102].

3.2.2. Sequential drug delivery for CHB

Both NAs and IMPs have distinct complementary effects to host immunity, e.g., PegIFN depletes $CD8^+$ T cells [103], whereas long-term NA therapy restored HBV-specific T cells, which enhances the efficacy of IMP therapy [104]. Sequential delivery of IMPs and NAs could be administered for the treatment of HBV infection. Synergistic efficacy of NAs and IMP combination therapy with different strategies has been investigated and summarized by Su and Liu [105], and beneficial efficacy was reported when NAs and IMPs were administered sequentially. For example, in patients with chronic hepatitis B, sequential therapy of lamivudine (orally daily) for 8 weeks followed by combination with IFN-α (daily subcutaneous administration) for 16 weeks and then continuation of lamivudine until 52 weeks resulted in a higher seroconversion rate than lamivudine monotherapy [106]. The immunomodulatory effect of IFN therapy and the persistent antiviral effect of lamivudine contributed to the increased seroconversion rate. In Japan, sequential treatment with entecavir, followed by IFN, has become a recommended first-line therapy for selective patients [107].

Presently, there are no drug delivery systems designed for the sequential release of NAs and IFN in hepatitis B treatment. However, a number of delivery systems described in the literature may tackle the limitations of existing treatment regime. For instance, frequent injections of IMPs (e.g., IFN and PEG-IFN) in hepatitis treatment cause patient discomfort and lower patient compliance, which prompted the development of an injectable dextran hydrogel system containing PEG microdomains for the long-term release of pegylated IFN-α2a (PEG-IFN-α2a) [108]. PEG-IFN-α2a physically partitioned in the PEG microdomains was released for more than 3 months. In the context of sequential drug delivery for hepatitis B treatment, following NA therapy to inhibit viral replication, a hydrogel containing PEG-IFN-α2a may be given to patients who are deemed responsive to IFN therapy to achieve sustained viral suppression. Alternatively, it might be worth investigating whether repeated cycles of the local and short-duration (seconds to hours) sequential delivery of drug combination can achieve similar or better EPS than the longer intervention windows. For drug delivery systems that can release NAs and IFN sequentially, micro-electro-mechanical systems (MEMS)-based devices should be considered, as they can deliver a wide range of drugs including small-molecule drugs, mid-size peptide therapeutics, and larger protein-based therapeutics [109–111]. For instance, Kristy *et al*. used a dual-layered hydrogel system presented in microdevices with one reservoir to achieve sequential release of different-sized drugs, i.e., insulin and camptothecin [112]. Chirra *et al.* used multiple reservoirs microdevice that can be filled with multiple drugs using different hydrogels or biodegradable polymeric systems for independent rate-controlled delivery through oral administration with potential for sequential drug delivery [113].

3.3. Fatty liver diseases

Fatty liver diseases can be alcohol-induced fatty liver disease (AFLD) or NAFLD. AFLD is mainly caused by over-consumption of alcohol, while NAFLD has a close association with metabolic syndrome and/or obesity and diabetes [114]. Both diseases exhibit similar liver injury patterns caused by abnormal accumulation of lipid in hepatocytes greater than 5% [115]. In early stages of AFLD or NAFLD, a mild centrilobular steatosis is seen but reversible. The excessive fat, however, is cytotoxic to hepatocyte by exhaustion of redox balance. Hepatocyte damage and death result in orderly inflammatory and regenerative responses [116]. Both metabolic and inflammatory abnormalities are important mechanisms of liver injury progression in a severe form called steatohepatitis [117,118]. Repeated and cumulative hepatocyte injuries may elicit signals necessary to stimulate fibrogenesis, thus leading to cirrhosis and HCC [119].

As comorbidity to metabolic syndromes and diseases, the prevalence of NAFLD has been constantly increasing from 15% in 2005 to 25% in 2010 globally [120]. NAFLD is found in 40-80% of people who have type 2 diabetes and in 30-90% of people who are obese. Having noticed the substantial epidemiology and unique etiology of fatty liver diseases (combination of abnormal metabolism, inflammation, and matrix remodeling), NAFLD has become the leading risk factor of HCC [121]. The cirrhosis-independent development of HCC occurs more frequently in NAFLD than in viral infection [122]. The underlying carcinogenic factors include increased DNA damage and impaired cellular surveillance through metabolic and immune alteration as summarized by Yang *et al.* [123].

3.3.1. Current treatments for fatty liver diseases and limitations

For AFLD treatment, cessation of alcohol consumption can be effective. Herbal medicine such as lutein [124] and persimmon vinegar [125] is beneficial by reducing alcohol-induced oxidative stress and inflammation. For severe AFLD that has progressed to end-stage diseases, no direct cure is available other than LT [126]. Improvement by modifying lifestyle was relatively effective in a particular cohort [127], and any approved medicine is still scarce.

Metformin and pioglitazone have shown off-label, but controversial effects to improve steatosis in patients. Newer therapies target key regulators involved in lipid homeostasis to reduce lipid synthesis and increase catabolism. Approaches include agonism of the insulin pathway (thiazolidinedione), peroxisome proliferator-activated receptor (PPAR) (e.g., pemafibrate and elafibranor), and farnesoid X receptor (FXR) (e.g., obeticholic acid), as well as inhibition of *de novo* lipogenesis (aramchol). Intrahepatic or extrahepatic inflammation is a secondary target. Therapeutic effect reassessment has been applied to the antioxidant Vitamin E and some probiotics. Although Vitamin E significantly improved NASH activity score in the PIVEN trial (NCT00063622) compared to placebo, the medical use is still limited due to poor knowledge of the long-term effects. Toll-like receptor-4 is targeted, as it is a modulator of the classical activation pathway of Kupffer cells. Apoptosis signaling kinase 1 (ASK1) inhibitor is undergoing validation in a phase 3 trial. Fibrosis is the third target, including lysyl oxidase-like 2 (LOXL2) inhibitor, cysteine-cysteine motif chemokine receptor-2/5 (CCR2/5) inhibitor, and galectin-3 antagonist. More details are documented by Sumida *et al.* [128] and Younossi *et al.* [129].

There are no medications approved by the U.S. Food and Drug Administration for the treatment of NASH. Obeticholic acid, elafibranor,

and aramchol are the three drugs in the most advanced phase III trials [130]. In addition, combinatorial therapy showed potential for enhanced efficacy in preclinical and early clinical trials, prompting further investigation of this field. Gilead has initiated the ATLAS program to validate the combination of selonsertib and ACC inhibitor/FXR agonist (NCT03449446). More combinations include, to name a few, FXR agonist/caspase inhibitor, FXR agonist/cenicriviroc (TANDEM, NCT03517540), and so on.

3.3.2. Sequential drug delivery for NAFLD

It is worth investigating combination therapy and sequential delivery for synergistic efficacy in treating NFLAD and ultimately reducing the risk of HCC development. Because of the complex interplay between metabolic alterations and chronic inflammation [123], strategies to inhibit both processes are possibly beneficial for treating steatosis and NASH. To uncover additional complementary targets for therapeutic interventions, Friedman *et al.* suggested using transcriptomics to identify unaffected pathways in response to a known drug [131]. Whether repeated and local sequential delivery could improve EPS and reduce the course of treatment needs further evaluation. Further, supplementary therapy consisting of dietary and exercise could be considered to relieve metabolic syndromes.

Antifibrotic treatment may differ from those against steatosis and NASH. In fibrosis, the deposition of collagen not only distorts the area for hepatocyte regeneration [132] but also poses a barrier to drug transport and absorption [133]. Therefore, elimination of excessive collagen fiber by delivering interstitial collagenases such as matrix metalloproteinase-1 could be prioritized for the re-establishment of the regenerative sinusoidal microenvironment [134]. Subsequent administration of antifibrotic drugs to inhibit active HSCs is necessary to prevent the production of ECM. In addition to targeting HSCs directly, anti-inflammatory agents may be helpful, as HSCs are capable of sensing and mediating inflammatory responses [119]. This strategy may benefit from repeated and local sequential delivery, as better drug adsorption substantially depends on collagen resolving. In this case, nanoparticles with collagen-dissolving agents on the shell and HSC inhibitor in the core could become a prototype of a sequential delivery platform.

In addition, cell therapy has been used to improve fibrosis. Watanabe *et al.* injected MSCs and macrophages into rodents with fibrosis and showed synergistic pathological improvement [135]. M2 polarization of macrophages characterized with phagocytic ability was promoted when coexisting with MSCs *in vitro*. The migration and engraftment of macrophages were enhanced when co-delivered with MSCs. As MSCs play an auxiliary role to support the migration, polarization, and phagocytosis of macrophages, it is interesting to examine whether functional segmentation of these cells by sequential delivery would further improve liver function.

3.4. End-stage liver diseases

Cirrhosis is defined as the diffuse nodulation pattern of liver fibrogenesis in response to chronic liver injury [136]. It is the end-stage form of fibrosis. The hepatocytes lose contact with the central vein and become nonfunctional. It results from a range of causes from chronic viral hepatitis B (±D) or C, fatty liver diseases, autoimmune hepatitis, DILI, *etc.* The progression from chronic liver diseases to cirrhosis includes inflammation, active hepatocyte regeneration by proliferation of hepatocyte progenitor cells, activation of HSCs that contribute to the fibrosis with the help of portal fibroblasts, angiogenesis, and parenchymal extinction lesion caused by vascular occlusion [137–139]. TGFβ is an important anti-inflammatory agent but promotes fibrogenesis [140,141]. Upon successful removal of the insult, cirrhosis is reversible. Fibrogenesis is inactivated by growth factors and cytokines and counterbalanced by ECM clearance, modulated by enzymes such as matrix metalloproteinases [142]. This process called fibrosis/cirrhosis resolution occurs with a sequence of events leading to clinical cirrhosis regression.

HCC is the main cause of death in patients with cirrhosis and the third leading cause of cancer-related death [143,144]. It has a high incidence and mortality rates in most of the countries, particularly in eastern Asian regions such as China [145]. The most common causes of HCC are chronic liver diseases such as viral hepatitis (HBV, hepatitis C virus) [146], alcohol abuse, and NASH [122]. In spite of the high prevalence, the prognosis of HCC is so poor that approximately 70% of the time, it is detected and diagnosed at advanced, inoperable stages and results in a high morbidity rate [147]. HCC exhibits a complex pattern of heterogeneity in molecular pathogenesis, with various genetic/epigenetic mutations identified among the patients [148]. Tumor-specific and liver-specific characteristics also contribute to the extreme difficulty in our understanding and treatment of HCC [149].

3.4.1. Current HCC treatments and limitations

Several countries have similar clinical guidelines for the treatment of HCC [150–153]. It mainly includes resection, LT, ablation, transarterial chemoembolization (TACE), hepatic arterial infusion chemotherapy (HAIC), and systemic therapy depending on disease stages (Barcelona Clinic Liver Cancer staging) (Table 2).

TACE is used for patients with HCC in stage A or stage B. Taking advantages of the sole nutritional distribution to the tumor through the hepatic artery [154], the emulsion of iodinated oil (e.g., Lipiodol) and chemotherapeutic drugs are delivered using a catheter to this tumor-feeding arteries. The arteries are then occluded with additional embolic microparticles or microspheres such as Gelfoam or polyvinyl alcohol (PVA), which limit the blood supply to tumors, and high doses of cytotoxic drugs are released to the tumors. The integration of drug-eluting beads (DEBs) improves drug-releasing dynamics with lower cytotoxicity and for a prolonged releasing period.

Although initial treatment with TACE offers survival benefits to patients with unresectable tumors, the recurrence of the tumors is often observed [155]; the recurring tumors often have reduced doubling time [156], indicating accelerated growth of the neoplasms, which demands routine screening and timely intervention. It could be attributed to TACE-induced vascular endothelial growth factor (VEGF) secretion and local vasculature, both of which are beneficial for tumorigenesis [157]. Therefore, release of antiangiogenic agents from DEBs following chemotherapeutic drugs could be developed as sequential delivery.

In HCC with vascular invasion and extrahepatic metastasis (stage C), systemic therapy is favored to combat the proliferation and angiogenic activity of the tumor and the metastasized cancer cells. Thus far, there is only a handful of FDA-approved drugs for the treatment of liver cancer (sorafenib [158,159], regorafenib [160], nivolumab [161], pembrolizumab [162], and lenvatinib mesylate [163]), of which the current standard is oral sorafenib, which has shown only a three-month increase in overall survival [159]. Lack of effective systemic therapeutic drugs for stage C HCC warrants the need for further drug development and novel therapeutic strategies leveraging on combinatorial or sequential administration of different drugs.

An increasing number of studies have attempted combination treatment of sorafenib with other forms of treatment including TACE or sequential delivery of a second-line systemic therapeutic drug. The efficacy of combining TACE with sorafenib treatment varied widely, but assessment of the overall outcome of these trials suggests that this approach indeed leads to improvement in overall survival [164]. Furthermore, it is sensible to switch from TACE into systemic therapies when TACE refractory occurs during the intermediate stage, as the liver function is better preserved [165,166].

3.4.2. Sequential drug delivery for HCC

The fact that TACE induces local vasculature raises the possibility of sequential delivery of chemotherapeutic and antiangiogenic drugs. Initial release of chemotherapeutic drugs kills tumor cells, followed by a second pulse of antiangiogenic drugs to inhibit vasculature. Improved survival rate was observed in the sequential delivery of the

Table 2
Sequential drug delivery strategies for different stages of hepatocellular carcinoma

Stage[a]	Treatment	Current developments	Recurrence, limitations, and risks	Drug delivery strategies to overcome limitations
Stage 0	Resection	• Laparoscopic surgery to reduce surgical and postoperative complications • TARE to improve hypertrophy/-compensatory growth of the contraindicated lobe	• High recurrence rates	• N.A.
Stage A and Stage B	Resection, **TACE**, TARE, MWA, OLT, SBRT	**For TACE** • Various embolic agents: • Thermosensitive shape-memory hydrogels to adapt to complex vascular geometries [182] • Drug-eluting beads (DEB-TACE) • Radiopaque DEBs • Magnetic DEBs [183]	• Rapid release of drugs from DEBs [184] • No improvement in OS [185,186] • Recurrence of tumors in the same lesion or in new sites in the liver [155] • **TACE induces VEGF secretion and local vasculature** [157].	• LbL and core/shell microspheres for sequential drug release over long periods. • **Sequential delivery of chemotherapeutic and antiangiogenic agents (PTX/CA4 [167,168] and MTX/CA4 [171]).** • LbL, core/shell loaded with chemotherapeutic and antiangiogenic drugs may be used.
Stage C	• **Systemic chemotherapy:** sorafenib (1L), levatinib (1L), regorafenib (2L), cabozantinib (2L), nivolumab (2L) • **HAIT**	• Various delivery platforms [167,187] • **Nanomaterials with NIR dyes** [177]	• **Limited efficacy** • Systemic side effects [188] • High recurrence rates	• Combinatorial therapy with TACE • **Sequential delivery of PTX+RO3306 [172] PTX+TNF [173], epirubicin + κ-Selenocarrageenan [174]**
Stage D	OLT, BSC	TACE as a bridging therapy	• Scarcity of donor • High recurrence rates	• N.A.

[a] Several systems for staging and practical guidelines of HCC exist; this table refers to Barcelona Clinic Liver Cancer staging and practical guidelines of American Association for the Study of Liver Diseases [151]. Abbreviations – 1L: first line; 2L: second line; BSC: best supportive care; LbL: layer-by-layer; MTX: methotrexate; MWA: microwave ablation; NIR: near-infrared; OLT: orthogonal liver transplantation; OS: overall survival; PTX: paclitaxel; SBRT: stereotactic body radiation therapy; TACE: transarterial chemoembolization; TARE: transarterial radioembolization; TNF: tumor necrosis factor; VEGF: vascular endothelial growth factor.

chemotherapeutic drug paclitaxel (PTX) and the antiangiogenic agent combretastatin A4 (CA4) [167,168] when compared with the release of any single drugs. It is attractive, as monotherapy of PTX has limited efficacy for HCC in phase I [169] and phase II trials [170]. Similarly, improvement was shown in the combination of methotrexate (MTX) and CA4 [171].

Sequential delivery of PTX with another agent also showed synergistic efficacy. Xiao *et al.* reported that synergistic apoptosis was observed from the sequential treatment of PTX with a cyclin-dependent kinase 1 inhibitor (RO3306) on HepG2 cell line [172]. Mineroa *et al.* reported that the sequential use of PTX followed by TNF significantly induced apoptosis on HepG2 and mice models bearing HCC tumors when compared with PTX alone [173]. Ji *et al.* have reported the schedule-dependent effects of epirubicin and κ-selenocarrageenan on both *in vitro* and *in vivo* HCC models [174]. However, Ahn *et al.* reported that systemic therapy with sunitinib after sorafenib has limited effect for 18 patients with advanced HCC [175].

The implementation of sequential drug delivery for HCC could benefit from LbL or core-shell structures by compartmenting various drugs. A core-sheath nanofiber loaded with hydrophobic 10-hydroxycamptothecin (HCPT) and hydrophilic tea polyphenols (TPs) showed sequential release for HCC. The majority of HCPT was rapidly released in the first 4 days, whereas TP was dominant from the 4th day onwards [176]. Zhang *et al.* developed an amphiphilic Janus nanoparticle showing distinct release profile of the two drugs upon near-infrared (NIR) stimulation and improved tumor inhibition *in vitro* and *in vivo* [177]. Moreover, pH-sensitive pullulan conjugated with MTX could form a spherical shape. When loaded with CA4, a distinct release profile of MTX and CA4 was observed [171]. Although these nanoparticle-based platforms are not adaptable for TACE as appropriate embolic agents, proof-of-concept studies shed light on further investigation on the micron-graded sequential delivery system.

HAIC is also widely used in Asian countries for advanced-stage patients to improve both survival and quality of life, especially for patients with portal vein tumor thrombus who cannot accept TACE [178]. Although single or combined HAIC administrations of epirubicin, doxorubicin, 5-fluorouracil (5-FU), cisplatin (CDDP), miriplatin, zinostatin, and oxaliplatin have been clinically applied for HCC treatment, the optimal protocol has yet to be validated in a large-scale clinical trial [178]. Itamoto *et al.* reported sequential administration of HAIC (CDDP (1 h) + 5-FU (24 h) on days 1-5, cessation on days 6 and 7, repetition for more than three times to maximize the effect of 5-FU with pretreatment of CDDP [179]. Yamasaki *et al.* reported sequential drug infusion strategies (CDDP, 10 mg/1 h → lamivudine, 12 mg/10 min → 5-FU, 250 mg/5 h) through a subcutaneously implanted injection port [180]. Advances in the technology of implantable drug delivery systems have facilitated arterial infusion of repeated and multiple chemotherapeutic agents, as well as long-term continuous infusion. Celsite® Arterial Port Systems is a representative commercial product designed for arterial applications and permit loco-regional chemotherapy, which can directly target tumors [181]. The port is implanted at the base of the ribs, and the catheter is inserted into the gastroduodenal artery, with the tip lying in the hepatic artery. These implanted port systems show great potential for dose-controlled and sequential or combination therapy. Further, some wireless controlled and programmable techniques could also be incorporated with such implanted port system for precise controlled release, and a multireservoir design could be integrated for multiple drug delivery.

4. Future prospects and concluding remarks

The growing burden of liver diseases and the limitations of LTs worldwide warrant novel treatment strategies. In this review, we summarized the current treatment modalities and their limitations for four types of liver diseases and identified potential targets or combination of drugs for sequential drug delivery. While sequential delivery of drugs has shown enhancement in synergistic efficacy and reduced hepatotoxicity in cell-based and animal models, particularly in HCC treatment, these proof-of-concept studies remain to be validated by clinical trials, as animal models only partially recapitulate the pathological complexity and etiological heterogeneity [189].

In addition to identifying the most suitable combination of drugs, another challenge in sequential drug delivery is to determine the treatment duration of each drug to achieve optimal outcome. For example, in chronic hepatitis B treatment, various treatment regimens for involving NAs and IFNs have been reported, including the add-on strategy, in

which short-term NA monotherapy (several months) was followed by a combination of both NAs and IFN therapies for half a year, and the switch-to strategy, in which NA monotherapy lasting from several months to years was ceased before initiating IFN monotherapy [190]. In other cases, NAs were prescribed before and after IFN therapy, as in the study by Sarin *et al.*, in which lamivudine was continued after cessation of IFN [106]. More studies comparing the long-term therapeutic outcome between different treatment regimens are required to establish the guidelines for sequential therapy of NAs and IFNs/PEG-IFNs in chronic hepatitis treatment and likewise for other liver diseases.

Periodic on-treatment monitoring of biomarkers that are indicative of therapy outcome should be implemented to aid in the decision-making process of switching or terminating therapies in sequential drug delivery. For DILI, tracking hepatic enzymes such as alanine aminotransferase and aspartate aminotransferase and bilirubin is relatively indicative to the extent of hepatotoxicity [191,192]. For chronic hepatitis B, virologic response of NAs and IFN therapies could be evaluated by measuring the serum HBV DNA levels and hepatitis B surface antigen (HBsAg) [193]. For NAFLD, as biopsy is still golden standard for pathological assessment, development and application of noninvasive diagnoses are urgent for routine clinical setting [194]. For HCC treatment, imaging techniques such as CT and MRI could be used to evaluate treatment outcome, with MRI reported to be more sensitive than CT in detecting small lesions [195].

Through this review, we aim to highlight the needs of sequential drug delivery to encourage the development of innovative methods and technologies that could benefit patients suffering from liver disorders.

Declaration of Competing Interest

Hanry Yu holds equity at Invitrocue, HistoIndex, Osteopore, Vasinfuse, and Pishon Co. Ltd. There is no direct conflict of interest with the submitted work.

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