Abstract

Introduction: The combination of microbubbles (MBs) and ultrasound (US) is an emerging method for the noninvasive and targeted enhancement of intratumor chemotherapeutic uptake. This method showed an increased local drug extravasation in tumor tissue while reducing the systemic adverse effects in various tumor models.

Area covered: We focused on preclinical and clinical studies investigating the therapeutic efficacy and safety of this technology for the treatment of colorectal, pancreatic and liver cancers. We discussed the limitations of the current investigations and future perspectives.

Expert opinion: The therapeutic efficacy and the safety of delivery of standard chemotherapy regimen using MB-assisted US have been mainly demonstrated in subcutaneous models of digestive cancers. Although some clinical trials on pancreatic ductal carcinoma and hepatic metastases from various digestive cancers have shown promising results, successful evaluation of this method in terms of US settings, chemotherapeutic schemes and MBs-related parameters will need to be addressed in more relevant preclinical models of digestive cancers, in small and large animals before fully and successfully translating this technology for clinic use. Ultimately, a clear evidence of the correlation between the enhanced intratumoral concentrations of therapeutics and the increased therapeutic response of tumors have to be provided in clinical trials.

Keywords: digestive cancer, drug delivery, anti-cancer drugs, ultrasound, microbubble.

Article highlights box

- MB-assisted US is an emerging and promising method for non-invasive and targeted delivery of chemotherapeutics.
- MB-assisted US promotes the permeabilization of blood-tumor barriers, thus increasing the extravasation and the i.t. bioavailability of therapeutics; the enhanced i.t. accumulation of therapeutics increases their treatment efficacies, while reducing side effects to healthy tissues.
- Acoustically-mediated drug delivery is mainly developed and validated in s.c. animal models of digestive cancers. Further preclinical additional studies have to be performed in orthotopic and metastasis animal models of digestive cancers in small and large animals to confirm the potentiel therapeutic benefit of this strategy.
- Few Phase I/II clinical trials reported promising results for the treatment of inoperable pancreatic ductal adenocarcinoma (primary lesions and metastases) and hepatic

metastases; and new results are expected on the treatment of hepatic metastases from colorectal and pancreatic cancers.

- Careful evaluation of efficacity and tolerability of therapeutic scheme including the choice of chemotherapy regimen, US settings, choice of MBs is required to facilitate translation of this US technology to the clinic.
- Clinical investigations have to bring a clear evidence of the correlation between the enhanced intratumor concentrations of therapeutics and the increased therapeutic response of tumors to the therapeutics.

Abbreviation list

CRC: Colorectal cancer

CECT: Contrast-enhanced computed tomography

CT: Computed tomography

CTC: Common Toxicity Criteria

DC: Duty cycle

DOC: Docetaxel

DOX: Doxorubicin

ECOG: Eastern cooperative oncology group

FDG: Fluorodeoxyglucose

FOLFIRI: Calcium folinate, 5-fluorouracil, irinotecan

FOLFIRINOX: Calcium folinate, 5-fluorouracil, irinotecan, oxaliplatin

H&E: Hematoxylin and eosin

i.p.: intraperitoneal

i.v.: intravenous

i.t.: intratumoral

MB: Microbubbles

MRI: Magnetic resonance imaging

PC: Pancreatic cancer

PCNA: Proliferating cell nuclear antigen

PDAC: Pancreatic ductal adenocarcinoma

PET: Positron emission tomography

PNP: Peak negative pressure

PRF: Pulse repetition frequency

RECIST: Response evaluation criteria in solid tumors

s.c.: subcutaneous

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

US: Ultrasound

1. Introduction

The digestive system (also known gastrointestinal system) consists of the esophagus, stomach, small and large intestines, liver, pancreas and gallbladder. Altogether, these organs break down food into nutrients, which are absorbed by the blood circulation and transported to all of the cells in the organism. Digestive cancers refer to the set of malignant tumors that develop from these organs and glands. For the majority of them, these tumors are adenocarcinomas, which originate in the mucous membranes that line the walls of the digestive tract or excretory ducts of the glands [1]. In the world, about 4.8 million new cases of digestive cancers and 3.4 million related deaths were registered in 2018 [2,3]. Digestive cancers represented 26% of the global cancer incidence and 35% of all cancer-related deaths. The most common digestive cancers are colorectal and pancreatic cancers [2]. Treatments for digestive cancers depend on the location of the tumor, its local and distant extension and the general patient history [4]. Thus, primary lesions are removed most often using minimally-invasive surgical techniques. The loco-regional lesions are treated with chemotherapy, possibly combined with radiotherapy while metastases are mainly treated with chemotherapy. Using a combination of anti-cancer drugs, chemotherapy destroy or slow-down the growth of fast-proliferating digestive cancer cells, wherever they are in the organism. The chemotherapy is mainly delivered intravenously for the treatment of digestive cancer, but it can also be administered orally [4,5]. In addition, chemotherapy can be used in three different ways: (i) as neoadjuvant treatment; (ii) as adjuvant treatment and (iii) when surgery and/or radiotherapy is contraindicated for the patient [4,5]. This is ascribed to the tumor microenvironment, which restrains both effective drug extravasation and targeting of digestive cancer cells [6,7]. In addition, the systemic delivery of unspecific chemotherapeutics is also associated to major off-target effects. To overcome these limitations, the development of safe, efficient and targeted drug delivery systems is required to enhance the intratumor (i.t.) bioavailability of these chemotherapeutics, thus increasing their therapeutic efficacies, while minimizing side effects to normal tissues. Such drug delivery methods might have a great profit as neoadjuvant but also palliative care. They might decrease the tumor burden, thus either making it easier surgical resection or offering symptom relief.

Microbubble-assisted ultrasound (*i.e.*, sonoporation or sonopermeabilization) is an emerging method for the non-invasive and targeted delivery of chemotherapeutic drugs, immunotherapeutic agents, photo/sonodynamics agents, and nucleic acids into digestive cancers [8-10]. These therapeutics are either co-injected intravenously with microbubbles (MBs) or loaded on or into the MBs before their intravenous (i.v.) administration [11]. Subsequently, the volumetric oscillations of MBs under the action of ultrasound (US) generate

a number of local acoustic phenomena nearby the endothelial wall of blood-tumor barrier (BTB), which promote its transient permeabilization [12]. The BTB permeabilization increases the extravasation of therapeutics into the targeted tumor tissues through the stimulation of paracellular and transcellular pathways, thus increasing their i.t. bioavailability [13]. In addition, the permeabilization of endothelial cells increases the intracellular uptake of therapeutics through the formation of membrane pores, which promotes the destruction of tumor microvasculature and the reduction of nutrient supply [12,13]. As previously described [14], the efficiency of MB-assisted US depends on multiple factors: (i) sufficient accumulation of therapeutics and MBs into the tumor microvasculature, which is directly influenced by their pharmacological properties as well as the physiological properties of tumor tissues; (ii) US parameters, probes and devices; and (iii) treatment schemes, which is influenced by the pharmacological properties of therapeutics and MBs, and the duration of BTB permeabilization. The influence of some of these factors on the delivery of chemotherapeutics, has been investigated in murine models of digestive cancers but also in pilote clinical studies [14]. Both preclinical and clinical investigations mainly focused on colorectal, pancreatic and liver cancers [8, 15-17]. In this review, we provide a survey on the preclinical and clinical studies investigating the therapeutic efficacy and safety of delivery of chemotherapeutics using MB-assisted US for the treatment of colorectal, pancreatic and liver cancers. The limitations of these investigations and future perspectives are also discussed.

2. Methods

Pubmed®, Web of Science™ and ClinicalTrials.gov electronic databases were screened using pre-defined search dates (January 1995 – August 2021) and terms: The Pubmed® database search terms used were: (digestive cancer [MeSH terms]) AND (esophageal cancer [MeSH terms]) AND (stomach cancer [MeSH terms]) AND (intestine cancer [MeSH terms]) AND (gallbladder cancer [MeSH terms]) AND (colorectal cancer [MeSH terms]) AND (pancreatic cancer [MeSH terms]) AND (liver cancer [MeSH terms]) AND (microbubbles [MeSH terms] OR ultrasound contrast agents [MeSH terms]) AND (ultrasound [MeSH terms] AND Sonoporation [MeSH terms] AND microbubble-assisted ultrasound [MeSH terms]) AND ("English" [language]). The research terms ("digestive cancer") AND ("esophageal cancer") AND ("stomach cancer") AND ("intestine cancer") AND ("gallbladder cancer") AND ("colorectal cancer") AND ("pancreatic cancer") AND ("liver cancer") AND ("microbubbles OR ultrasound contrast agents") OR ("ultrasound" OR "microbubble-assisted ultrasound") were used on the Web of Science™. The ClinicalTrials.gov database search terms used were:

"digestive cancer" AND "esophageal cancer" AND "stomach cancer" AND "pancreatic cancer" AND "liver cancer" AND "microbubbles OR ultrasound contrast agents" AND "Ultrasound". The research term "ultrasound AND digestive cancer" were used on these three databases. The inclusion and exclusion criteria are summarized in **Table 1**. Thirty-three publications were identified.

3. Preclinical investigations

In this section, we focused specifically on these different aspects in the preclinical studies investigating the performances of MB-assisted US as a drug delivery method for the treatment of colorectal, pancreatic and liver tumors.

3.1. Colorectal cancer (CRC)

CRC is the third most commonly diagnosed cancer and remains the second leading cause of cancer death worldwide in 2020 [2,3]. In this context and as previously mentioned, many drug delivery methods have been designed to improve the therapeutic effectiveness of chemotherapeutics. Among these methods, the efficacy of MB-assisted US to deliver free chemotherapeutics as well as drug-loaded nanoparticles was mainly investigated in subcutaneous (s.c.) mouse models of CRC (**Table 2**) [18-29]. Among these therapeutics, doxorubicin (DOX) is one of the most powerful anti-cancer drugs prescribed on its own or in combination with other drugs for the treatment of solid tumors [21]. However, the current use of free DOX clinically is still rather limited because of its severe systemic side effects. To overcome these main issues, DOX has been encapsulated inside pegylated liposomes (Doxil® or Caelyx®) or polymeric nanoparticles [22-23]. Although these formulations strongly reduced the cardiotoxicity of DOX, other adverse effects and a limited i.t. DOX concentration have been reported [24]. One of the strategies to increase the therapeutic effectiveness of DOX and in particular in its liposomal form consists in its coadministration with MBs and then in exposing the tumor tissues to US. Thus, Lin *et al.*, investigated the therapeutic effectiveness of Doxil® delivery using MB-assisted US (lab-made US device; 1 MHz, 50 Hz PRF, 50% DC, 10 ms pulse length, 0.6 MPa, for 60 s; 100 µL/kg SonoVue® MBs) on the growth of early (15 mm^3) and medium (50 mm^3) in s.c. murine colorectal adenocarcinoma (CT-26) in mouse [25]. Mice with early-stage tumors received an intravenous (i.v.) injection of 10 mg/kg Doxil®. Then, MBs were intravenously administered and tumors were exposed to US pulses. Tumors were treated once a week for 3 weeks. The results showed that acoustically-mediated Doxil® delivery further and earlier inhibited the tumor growth as compared to Doxil® treatment alone. Similarly, a 6 mg/kg Doxil® dose was delivered once a week for 2 weeks using MB-assisted US in mice with

medium-stage tumors. The inhibition of tumor growth was more effective after acoustically-mediated Doxil® delivery than Doxil® treatment alone, and the tumors almost disappeared after acoustically-mediated Doxil® delivery. Using the same therapeutic scheme, the authors demonstrated that MB-assisted US significantly increased the therapeutic efficacy of a 4 mg/kg Doxil® dose, which did not inhibit the tumor growth when this dose was administered alone [25].

Moreover, Ingram *et al.*, investigated the therapeutic effectiveness of acoustically-mediated irinotecan delivery using VEGFR2-targeted and irinotecan-loaded MBs (thMBs) in the same murine model of CRC [28]. Seven days after tumor inoculation, mice received an i.v. injection of the thMBs (150-200 µL of 10^8 thMBs, *i.e.,* 2 mg/kg irinotecan) or free irinotecan (2 mg/kg). Then, tumors were exposed to US pulses (lab-made US device; 2.2 MHz, 260 kPa PNP, 1 kHz PRF, 10 µs tone burst US pulse, for 5 s) 4 min post-MB injection. Mice received 5 treatments in total over three weeks. The acoustically-mediated irinotecan delivery using thMBs inhibited significantly tumor growth compared to free irinotecan alone. Indeed, the percentage of tumor growth inhibition compared to the non-treatment was 38% for free irinotecan alone and 50% for acoustically-mediated irinotecan delivery using thMBs. In addition, this latter treatment exhibited a 19% tumor growth inhibition compared to free irinotecan alone. By assessing the concentrations of irinotecan and SN38 (active metabolite of irinotecan) in the tumors, the authors demonstrated that the therapeutic efficacy of acoustically-mediated irinotecan delivery using thMBs was directly correlated to a significant increase of i.t. irinotecan and SN38 concentrations. These results demonstrated that the combination of drug-loaded MBs with US is an efficient strategy to deliver chemotherapeutics in colorectal cancer.

It is questionable whether the exploitation of drug-loaded MBs for acoustically-mediated drug delivery is a promising strategy, since their clinical use will require additional pharmacological and toxicological evaluations, which are time consuming and expensive. To avoid this issue and to respect the clinical procedures for chemotherapy, our research group investigated the delivery of irinotecan using MB-assisted US in human CRC (HCT-116) liver metastases in mouse model. A 20 or 40 mg/kg irinotecan dose was intravenously administered once every 3 days for 9 days. Thirty minutes later, MBs (BG8214 ; Bracco Research SA, Geneva, Switzerland) were intravenously injected and the liver metastases were exposed to US pulses (lab-made US device; 1.3 MHz, 10 kHz PRF, 40% DC, 0.4 MPa PNP, for 3 min) using a dedicated 1.5-D US phased array probe connected to a modified Aixplorer US scanner (Supersonic Imagine, Aix-en-Provence, France). As shown in **Figure 1**, the results show that the combination of MBs/US with 20 or 40 mg/kg irinotecan dose both induced a significant

decrease in tumor growth compared to 40 mg/kg irinotecan treatment alone. No significant difference was observed between both mice groups treated with 20 mg/kg and 40 mg/kg irinotecan doses delivered by MB-assisted US. This result suggests an acoustically-mediated delivery of 20 mg/kg irinotecan dose is sufficient to induce a significant inhibition of tumor growth. To our knowledge, this is the first demonstration that MB-assisted US provide a synergetic effect for potentiating a targeted delivery of low irinotecan doses in CRC liver metastases in a mouse model. In a separate study, we also investigated the efficacy of MB-assisted US to deliver cetuximab in a subcutaneous CRC (HT-29) in a mouse model [29]. Cetuximab is a therapeutic monoclonal antibody, which binds to the epidermal growth factor receptor overexpressed at the surface of many CRC cells. To achieve this goal, we designed three US settings at 1 MHz frequency using a lab-made US device: (i) Setting 1 – 100 μs burst length, 40% DC, 600 kPa for 3 min; (ii) Setting 2 – 10 ms burst length, 20% DC, 500 kPa for 5 min; and (ii) Setting 3 – 1 s burst length, 5% DC, 350 kPa for 15 min. Mice received a i.v. injection of fluorescently AF750-labelled cetuximab. Subsequently, MBs (BG8214, Bracco Research SA) were intravenously administered and tumors were exposed to US pulses. The results showed that MB-assisted US significantly improved the i.t. accumulation of the fluorescently-labelled cetuximab in comparison with the i.v. administration of fluorescent-labelled cetuximab alone for all the US sequences (**Figure 2**). Additional experiments are required to confirm these encouraging results and to demonstrate the full potential of this method for the targeted delivery of therapeutic antibody in digestive tumors. Altogether, these preclinical studies show that MB-assisted US significantly improve the therapeutic effectiveness of chemotherapeutics and immunotherapeutics in CRC.

3.2. Pancreatic cancer (PC)

PC is responsible for almost as many deaths as cases due to its poor prognosis and represents the seventh leading cause of cancer death worldwide in 2020 [2,3]. The chemotherapy as a first-line treatment or in combination with the surgery is the core of treatment for PC. Drug resistance, high metastasis occurrence, poor prognosis and tumor relapse contribute to the complications in treating PC. Since 2010, many investigations have been performed to design therapeutic protocols for the acoustically-mediated delivery of chemotherapeutics [30-43]. (**Table 3**). Gemcitabine is one of the chemotherapeutic molecules that attracted a large number of drug delivery studies using MB-assisted US [35, 39-43]. Indeed, although this drug is considered as the most effective chemotherapeutics for the treatment of PC, the benefit remains relatively modest because of its pronounced systemic side effects [44-46]. Kotopoulis *et al.*,

reported the delivery of gemcitabine using MB-assisted US in an orthotopic PC (MIA PaCa-2) xenograft [41]. Three weeks after tumor inoculation, mice received an intraperitoneal (i.p.) injection of gemcitabine (125 mg/kg). Thirty minutes later, SonoVue® MBs (50 μL) were intravenously injected and tumors were exposed to US pulses (lab-made US device; 1 MHz, 40% DC, 0.2 MPa PNP) for 4 min total cumulated US time. This treatment was administered weekly for a total of 8 weeks. Tumor volume decreased by a factor of 4 following two treatments using MB-assisted US compared to gemcitabine treatment alone. Both gemcitabine alone or in combination with MB-assisted US significantly increased the survival rate in comparison with no treatment. However, no significant difference in survival rate was observed between both treatments. More recently, Bressand *et al.*, described the therapeutic benefit of the nab-paclitaxel (albumin nanoparticle of paclitaxel) delivery using MB-assisted US in s.c. human pancreatic adenocarcinoma (BxPC3) in a mouse model [36]. Nab-paclitaxel (5 or 20 mg/kg) was intravenously injected in tumor-bearing mice. Subsequently, a bolus of MBs (70 μL) was intravenously administered and the tumors were exposed to US pulses (lab-made US device; 1 MHz, 100 μs PRP, 40% DC, 400 kPa PNP) for 3 min. The treatment was performed twice a week until protocol endpoints were reached. The results showed that MB-assisted US potentiated the therapeutic efficacy of both low (5 mg/kg) as well as high (20 mg/kg) nab-paclitaxel doses in the s.c. PC mouse model. Interestingly, the acoustically-mediated delivery of nab-paclitaxel at the highest dose did not impact the animal well being as no body weight loss was observed which is commonly reported when using such high drug dose [36]. This result suggests that the delivery of nab-paclitaxel into the tumor tissue limits the non-specific drug accumulation into healthy tissues and its systemic toxicity. Altogether, these data demonstrated that MB-assisted US improves the therapeutic effectiveness of free or nanoformulated chemotherapeutics in s.c. and orthotopic PC mouse models [33, 34, 36, 41, 42].

Another approach that attracted much attention consists in loading or co-loading chemotherapeutics into MBs [35, 37, 38, 43]. Indeed, Gao *et al.,* described a new formulation of liposome-loaded MBs carrying irinotecan and oxaliplatin [38]. The two chemotherapeutics are usually associated with 5-fluoruracil for the treatment of advanced PC. As previously reported for other chemotherapeutics, the FOLFIRINOX regimen has shown a great benefit on patient survival, but it is only indicated for patients with good physical condition because of its severe off-target toxicity [44-46]. The efficacy of the acoustically-mediated codelivery of irinotecan and oxaliplatin using this formulation was evaluated in s.c. human PC (BxPC3) mouse model. The Ir/Ox-loaded MBs (irinotecan dose: 4.75 mg/kg; oxaliplatin dose: 0.91

mg/kg) were intravenously injected in tumor-bearing mice. Then, tumors were exposed to US pulses (Sonidel SP100 device; 1 MHz, 100 Hz PRF, 30% DC, 0.48 MPa) during and after administration of MBs for a total duration of 3.5 min. Mice were treated four times at one-day interval during one week. The tumors treated with this therapeutic protocol were 136% smaller than those treated with irinotecan/oxaliplatin-based chemotherapy conventionally delivered in a free form. In addition, no severe toxic effects or body weight loss were observed during the treatment, thus indicating the protocol was well tolerated. These data demonstrated that MB-assisted US induced a more effective delivery of irinotecan/oxaliplatin and by that enhancing the overall effectiveness of this drug combination. This preclinical study is the first to report on the acoustically-mediated codelivery of two chemotherapeutics using liposome-loaded MBs.

3.3. Liver cancer

Primary liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer death worldwide in 2020 [2,3]. As previously introduced, chemotherapy, in combination with radiotherapy and/or surgery, remains the most common therapeutic strategy for treating most types of primary liver cancer [47]. However, many biological barriers limit the i.t. bioavailability of chemotherapeutics, thus impairing their therapeutic effectiveness [6,7]. Several therapeutic procedures using MB-assisted US have been developed for the targeted delivery of various anti-cancer drugs [48-60] in s.c. and orthotopic liver tumors models (**Table 4**).

Thus, Zhu *et al.*, investigated the efficacy of MB-assisted US to deliver the Doxil® in hepatocellular carcinoma (H22) mouse model [55]. Four days after tumor transplantation, mice received an i.v. injection of Doxil® (10 mg/kg), then an i.v. injection of MBs (200 µL at 4×10^9 MB/mL). The tumors were insonified with US pulses (lab-made US device: 1.1 MHz, 50% DC, 2.06 W/cm^2) for a total exposure time of 150 s. This treatment induced a significant tumor inhibition (79.7% *vs* 62.4%) and hence a lower tumor volume growth (9.7% *vs* 26.1%) than Doxil® alone. The acoustically-mediated Doxil® delivery significantly increased the i.t. concentration of DOX compared with tumors treated with Doxil® on its own. These data clearly demonstrated a positive correlation between the acoustically-mediated enhancement of i.t. DOX bioavailability and the increase in its therapeutic effectiveness. Despite an increase in the i.t. DOX concentration after MB-assisted US, the DOX concentration in normal tissues has not decreased. Nevertheless, the assessment of liver function by dosing of alanine and aspartate aminotransferases showed that the acoustically-mediated enhancement of DOX concentration in the liver tissue did not induce hepatic side effects. In addition, the acoustically-mediated

Doxil® delivery significantly increased the mouse survival rate in comparison with Doxil® alone (30 days *versus* 26 days). This preclinical study clearly demonstrated that MB-assisted US improved the therapeutic effectiveness of Doxil®.

Docetaxel (DOC) is an anti-cancer agent of the taxane class currently used to treat a variety of advanced unresectable metastatic gastric carcinoma and hepatocellular carcinoma [61, 62]. However, its clinical use requires its solubilization in polysorbate 80 because of its poor water solubility. The use of such solvent usually caused adverse events. As previously reported, the encapsulation of DOC into nanoparticles could be the best strategy to overcome these issues and for a safe and efficient use in clinics [63]. In this context, different research groups designed DOC-loaded MBs for the treatment of s.c. [53, 55] and orthotopic [56] liver tumors. Kang *et al.,* explored the anti-tumor effects of DOC-loaded MBs combined with US on VX2 rabbit liver tumors [56]. DOC-loaded MBs were intravenously administered (2 mg DOC in 6 mL saline) US pulses were directed to the tumors (lab-made US device; 300 kHz, 50% DC, 2 W/cm^2) for 6 minutes. The treatments were performed thrice and every 3 days. Twenty-four hours after the last treatment, liver tumors treated with DOC-loaded MBs combined with US had the lowest tumor volume (621.7 ± 134.4 mm^3) than those received DOC treatment alone (797.2 ± 191.2 mm^3) or no treatment (897.4 ± 201.1 mm^3). The acoustically-mediated DOC delivery using DOC-loaded MBs induced a significant decrease of extensive metastatic rate (0%) in comparison with DOC alone (80%) or no treatment (100%). In addition, the acoustically-mediated DOC delivery using DOC-loaded MBs significantly prolonged the mice survival (36.8 ± 2.77 days) compared to the DOX treatment alone (28.4 ± 2.88 days) or no treatment (23.6 ± 3.05 days). The results demonstrate that DOC-loaded MBs combined with US inhibit the growth of liver tumor by deferring cell proliferation and promoting cell apoptosis, thus prolonging the mice survival. The therapeutic effectiveness of acoustically-mediated DOC delivery using DOC-loaded MBs has also been confirmed on s.c. liver tumor in mouse model [57, 58]. Nevertheless, the present study did not evidence a correlation between an enhanced therapeutic efficacy of DOC and an increase in DOC concentration in the tumor tissues as reported by Ren *et al.,* [57].

The acoustically-mediated delivery of multiple chemotherapeutics to treat liver tumors has not been extensively explored. Shen *et al.,* investigated the efficacy of MB-mediated US to deliver cisplatin, mitomycin and 5-fluorouracil for the treatment of s.c. HepG2 liver tumor in a mouse model [60]. Mice received an i.v. infusion of cisplatin (1 mg/kg), mitomycin (0.5 mg/kg) and 5-fluorouracil (5 mg/kg). Subsequently, a bolus of SonoVue® MBs was intravenously

administered and the tumors were exposed to US pulses (commercial US device from Jiangsu Han Mei Biotechnology, China; 20 kHz, 40% DC, 2 W/cm^2) for 1 min. The treatment was repeated once every other day for two weeks. One can already notice that the center frequency of US pulses, *i.e.*, 20 kHz, is far below the conventionally used frequencies in other preclinical scenarios (around 1 MHz). The authors showed that the acoustically-mediated delivery of this drug cocktail induced a significant increase in tumor cell apoptosis compared to the chemotherapy alone (79 ± 11% *versus* 47 ± 11%). In addition, they demonstrated that the acoustically-mediated delivery of the drug cocktail prolonged the survival time of mice in comparison to the chemotherapy treatment alone (68 ± 31 days *versus* 57 ± 18 days). Moreover, they confirmed these results in an orthotopic liver tumors in a mouse model, thus demonstrating for the first time that MB-mediated US can efficiently deliver multiple chemotherapeutics for the treatment of liver tumors.

4. Clinical trials

So far, few clinical studies have been conducted or are recruiting patients to investigate the effectiveness and the tolerability for the treatment of digestive cancers.

4.1.Treatment of pancreatic ductal adenocarcinoma, from primary tumor to metastases

At the Haukeland University Hospital (Norway), the therapeutic efficacy and tolerability of chemotherapeutic delivery using MB-assisted US were investigated for the treatment of inoperable pancreatic ductal adenocarcinoma (PDAC) [64-66]. Ten patients were included in the phase I study. Gemcitabine (100 mg/m^2) was infused intravenously over 30 min. During the last 10 min of chemotherapy, an abdominal echography was achieved to define the position of pancreatic tumor. At the end of chemotherapy, half milliliter of SonoVue® MBs followed by 5 mL saline was intravenously administered every 3.5 min to ensure their presence throughout the whole US treatment. Pancreatic tumors were insonated with US (1.9 MHz, MI 0.2, 1% DC, cumulative US exposure of 18.9 s) using a 4C curvilinear probe connected to a LOGIQ 9 scanner (GE Healthcare). Clinical data revealed that the acoustically-mediated gemcitabine delivery did not induce any severe side effects in comparison to the chemotherapy on its own. All patients tolerated well an enhanced number of gemcitabine cycles after acoustically-mediated drug delivery in comparison with chemotherapy alone from historical controls, thus resulting in improvement in the patient's health. In addition, tumor size and development were monitored using positron emission tomography (PET) and computed tomography (CT) imaging and characterized according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Eastern Cooperative Oncology Group (ECOG) performance status was used to assess

the therapeutic effectiveness of acoustically-mediated drug delivery. One patient showed a two fold decrease in tumor size after twelve treatment cycles. This patient was discharged from the clinical trial for radiotherapy followed by a pancreatectomy. The maximum tumor diameter was partially reduced in five of the ten patients from the first to last treatment cycle. These patients were further treated with a consolidative radiotherapy or received an i.v. infusion of 5-fluorouracil, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX scheme). The median survival was significantly increased after acoustically-mediated drug delivery compared to chemotherapy alone (17.6 *versus* 8.9 months; $p = 0.001$).

In collaboration with Haukeland University Hospital (Norway), the Thomas Jefferson University (USA) leads a phase I/II study on the delivery of standard chemotherapy regimen (*i.e.,* gemcitabine+nab-paclitaxel or FOLFIRINOX) using MB-assisted US for the treatment of unresectable, locally advanced but also metastatic PDAC (NCT 04821284). One hundred and twenty patients are under enrollment. In the arm I, the patients will receive either an i.v. infusion of gemcitabine and nab-paclitaxel over 60 minutes on days 1, 8 and 15, or an i.v. infusion of FOLFIRINOX regimen on days 1 and 2. At the end of chemotherapy, Sonozoid® MBs will be administered intravenously over 20 minutes and the tumors will be exposed to US. The doses of chemotherapeutics and MBs as well as US parameters, probe and scanner are not described. The treatments will be repeated every 28 days for up to 3 chemotherapy cycles for gemcitabine and nab-paclitaxel, and every 14 days for up to 7 cycles for FOLFIRINOX regimen in the absence of disease progression or intolerable toxicity. In the arm II, the patients will receive the same chemotherapy cycles as previously described in arm I but MBs will not be administered and US pulses not applied. All clinical data (*e.g.,* tumor volume, blood tests, *etc.*) will be compared between both groups of patients. The progression-free survival will also be monitored up to 3 years.

In phase 2 clinical trial (NCT 04146441), St. Olavs Hospital (Norway) investigates the therapeutic potential of MB-assisted US to increase the therapeutic efficacy of FOLFIRINOX regimen in the treatment of locally advanced PDAC. This clinical trial is recruiting 30 patients. The patients will receive an i.v. infusion of FOLFIRINOX regimen. At the end of chemotherapy, one milliliter of SonoVue® MBs will be intravenously administered 9 times at 3.5-minute intervals to ensure their presence throughout the whole US treatment. This treatment will last for 31.5 minutes every treatment day. This phase 2 study does not describe neither the US parameters used in this protocol. The primary aim of this study is to assess the efficacy of MB-assisted US to increase the therapeutic effectiveness of FOLFIRINOX regimen. To achieve this objective, a group of patients will be receiving an acoustically-mediated delivery of

systemic FOLFIRINOX regimen and their clinical data will be compared to those of patients treated with the FOLFIRINOX regimen only. The volume of primary PDAC tumors will be measured using CT scan before treatment and after 1 treatment cycle. The number of down-staged tumors from stage III to stage II will be also determined at 8 weeks and 1 year. Finally, the side effects will be registered and defined according to Common Toxicity Criteria (CTC).

4.2.Treatment of primary pancreatic carcinoma and hepatic metastases from digestive system

A safety study of chemotherapeutic delivery using MB-assisted US for the treatment of pancreatic carcinoma and hepatic metastases from gastrointestinal tumors was conducted at Beijing Cancer Hospital, China (NCT 02233205) [67]. The clinical trial enrolled twelve patients, including one patient with primary pancreatic carcinoma and eleven patients with hepatic metastases from pancreas (1), colon (9), and gallbladder (1) carcinomas. The therapeutic schedule was as follows: Chemotherapy was intravenously infused as monotherapy (*e.g.*, irinotecan) and combination therapy (*e.g.*, FOLFIRI+Cetuximab, FOLFIRINOX). Thirty minutes after i.v. infusion of chemotherapy, 1 mL of SonoVue® MBs was intravenously injected and was repeated five times in 20 min. A C1-5 abdominal convex probe (GE Healthcare, USA) was positioned on the tumor lesion and US were applied at different MIs (from 0.4 to 1) in contrast mode using a LogiQ E9 scanner (GE Healthcare). The primary objectives of this clinical study were to assess the tolerability of this protocol but also to explore the highest MI and US treatment time that patients can tolerate. Wang *et al.*, reported that all 12 patients exhibited no serious adverse events, which can be resolved with symptomatic medication. Moreover, they described that the increase in MI did not worsen the severity of side effects, thus suggesting MB-assisted US treatment is a tolerable procedure. The secondary objectives were to assess the effectiveness of this protocol by monitoring the tumor size using magnetic resonance imaging (MRI) and contrast-enhanced CT scan (CECT) according to the RECIST criteria. Six of the twelve patients had stable disease while only one patient exhibited a partial response after the first treatment cycle. The median progression-free survival was 91 days. This clinical study has major limitations: (i) it is difficult to make a clear conclusion and a comparison between patients as they did not receive the same chemotherapeutics, number of treatment cycles nor MI; (ii) the tolerability and efficacy results should be competed with those for patients receiving the chemotherapy alone in order to bring clear evidence of the therapeutic benefit of acoustically-mediated drug delivery.

4.3.Treatment of hepatic metastases from colorectal cancers

At Tours Hospital (France), a proof of concept study (NCT 03458975) has been implemented in 2017 to investigate the therapeutic potential of chemotherapeutic delivery using a dedicated 1.5-D ultrasound probe and a modified US scanner in patients with non-resectable hepatic metastases from colorectal cancer and eligible for antibody-based immunotherapy in combination with chemotherapy (FOLFIRI+bevacizumab). For each participant, 2 or 4 liver metastases are selected by the radiologist. For each pair of liver metastases, one will be randomized to receive sonoporation (US waves and SonoVue® MBs) used as optimized method for i.t. delivery of systemic chemotherapy and the two other metastases will receive only MBs and chemotherapy. A dedicated 1.5-D US probe has been designed and connected to a modified Aixplorer imaging platform that is able to generate therapeutic pulses. The primary aim of this clinical trial is to assess the objective response at two months for hepatic metastases treating optimized and targeted delivery of systemic chemotherapy regimen including 5-fluorouracil, leucovorin and irinotecan (FOLFIRI treatment) and bevacizumab (or cetuximab) in comparison with those receiving only this regimen. This response is defined as a decrease of at least 30% in the longer diameter of each selected liver metastasis measured using spiral CT scan. The secondary aim is to determine the safety and tolerability of this protocol during the three months of follow-up based on the Common Terminology Criteria for Adverse Events (US National Cancer Institute). In addition, the tumor density and vascularity are assessed using CECT, MRI and DCE-US. The serum concentrations of therapeutic antibody and cytokines are measured. This clinical trial is currently recruiting 15 patients.

4.4.Treatment of liver metastasis from breast and colorectal cancers

Since 2018, St. Olavs Hospital (Norway) leads also a phase I/II clinical trial to study the efficacy of the delivery of standard chemotherapy regimen using MB-assisted US in patients with hepatic metastases from breast cancers and CRC (NCT 03477019) on a total of 60 patients to be enrolled. CT imaging will be performed before the treatment starts in order to preselect two metastases in each patient liver, which will be randomized to be either the targeted and treated lesion (chemotherapy regimen + MB-assisted US) or control lesion (chemotherapy regimen + MBs). The patients will receive an i.v. infusion of standard chemotherapy regimen. As previously described, a bolus dose of 1 mL SonoVue® MBs will be intravenously administered every 3.5 minutes and repeated 9 times (total duration of treatment: 31.5 minutes). The targeted hepatic metastasis will be exposed to US pulses generated by a clinically-approved US scanner and probe. This treatment will be repeated maximally four times at 2- or 3-week intervals, depending on the patient's diagnose and therapeutic protocol. The authors did not provide a

detailed therapeutic scheme. The size of the treated and control hepatic metastases will be measured using CT scan up to 10-12 weeks after the baseline CT examination. The CTC will used for grading toxicity up to 8 weeks.

4.5.Treatment of hepatic metastases and abdominal wall tumors

Recently, Shen *et al.*, reported that the therapeutic response of hepatic metastases and abdominal wall tumors to chemotherapy regimen was significantly increased after acoustically-mediated drug delivery (Tumor Hospital of Nantong University, China) [60]. An iU22 US scanner (Philips, USA) was used to locate the tumors and to position the therapeutic probe on the patient surface. The patients received an i.v. infusion of laboratory made CO_2-loaded MBs, then the tumors were exposed to US pulses (20 kHz, 2W/cm^2, 40% DC) for 10 min. Ten minutes later, a second infusion of MBs was performed, then followed with an US treatment. Each patient was treated thrice a day for about 30 min. One treatment course was five days with two days of rest. For patient 1 (with hepatic metastases), chemotherapy regimen was composed of i.v. infusion of oxaliplatin (130 mg/m^2) on day 1 and oral administration of capecitabine (1000 mg/m^2) twice daily on days 1-14. Patient 2 (with hepatic metastases) received an i.v. infusion of oxaliplatin (130 mg/m^2) and calcium folate (200 mg/m^2), followed by a 24-h continuous i.v. infusion of 5-FU (500 mg/m^2). For patient 3 (with abdominal wall metastases), chemotherapy regimen was constituted of an i.v. infusion of epirubicin (50 mg/m^2) on day 1, a 2-h i.v. infusion of cisplatin (30 mg/m^2) on days 1-3 and followed by a 24-h continuous i.v. infusion of 5-FU (500 mg/m^2) on days 1-5. The size of tumor lesions in patients 1 and 2 was measured using CT scan while the size and the metabolic activity of tumor lesion in patient 3 were determined using ^{18}F-FluoroDeoxyGlucose PET/CT before and after treatment. No side effect was observed during and after MB-assisted US treatment. The acoustically-mediated drug delivery decreased the size of hepatic metastases from patients 1 and 2, and reduced the metabolic activity and the diameter of the abdominal wall tumor from patient 3. In addition, the symptoms of abdominal distention and pain in patients 1 and 2 were relieved. The current study suffers from major limitations. It is not clearly justified why 3 different drugs were used, so each patient was treated with a different chemotherapy, which makes the comparison between the results of the 3 patients not possible. In addition, efficacy results should be compared to a control group with patients receiving the chemotherapy alone in order to provide clear evidence that MB-assisted US improves therapeutic effectiveness of chemotherapy regimen.

Conclusions

Preclinical studies show that MB-assisted US enhances i.t. bioavailability of anti-cancer drugs, thus increasing in the therapeutic efficacy, while minimizing their non-specific accumulation in normal tissues and by that reducing side effects. The improvement in therapeutic effectiveness results in a decrease of tumor growth and a prolongation of animal survival. MB-assisted US efficiently delivers a single chemotherapeutics as well as a combination of chemotherapeutics in s.c. and orthotopic digestive tumors in small animal models. A growing number of clinical trials are registered to investigate the therapeutic effectiveness and the tolerability of MB-mediated US for the treatment of primary digestive tumors and hepatic metastases from digestive system. Some of them show promising results such as a decrease in tumor growth and an increase in patient survival. Nevertheless, firm conclusions cannot be made on the therapeutic benefit of this method in humans as only partial results are available now.

Expert opinion

Targeted delivery of chemotherapeutics using MB-assisted US has the potential to become a clinically-accepted strategy for improving local chemotherapy in digestive oncology. A growing number of preclinical investigations have successfully reported the therapeutic benefits of MB-assisted US in the delivery of chemotherapeutics in several animal models of digestive cancers. For 66 % of the preclinical studies on digestive cancers, the efficacy of this method has been evaluated on s.c. digestive tumors in rodent models. It must be noted that the preclinical studies on liver cancer were mainly performed on orthotopic tumor models, while those on colorectal and pancreatic cancers were carried out using s.c. tumor models. In addition, less than 5% of these studies have been achieved on animal models of metastasis. Subcutaneous tumor models are interesting as they allow to evaluate different protocols rather simply and rapidly. However, their clinical relevance is still questionable. Indeed, the physiology of the tumor and its response to chemotherapy vary strongly between s.c. tumors and orthotopic tumors and metastases, but also between tumors derived from a xenograft of immortalized cells of animal and human tumors. To overcome this last limitation, patients-derived tumors could be exploited in the validation of therapeutic protocols. In addition, the contribution of the host immunity to the tumor response to chemotherapy after acoustically-mediated drug delivery is still deficient in the available literature. Indeed, more than 60% of preclinical studies used digestive tumors in immunodeficient mouse model that did not allow this contribution to be studied. Large animal studies are still deficient and might face challenging and unpredicted physical limitations including US penetration depth and US

attenuation, but also biological limitations (*i.e.*, plasma life of MBs and chemotherapeutics). Future preclinical investigations should be achieved in more clinically relevant animal models including syngeneic or patient-derived orthotopic tumors and metastatic models in small and large syngeneic or immunodeficient animals, respectively, in order to replicate as much as possible the clinical physiological situation.

Moreover, preclinical and clinical investigations show that the coadministration or the successive administration of clinically-approved MBs and chemotherapeutics can be perceived as the fastest strategy to clinical translation, while greatest therapeutic benefit may lie in the design of drug-loaded MBs. The former approach is used in 37% of preclinical studies and 100% clinical trials on digestive cancers. This approach is likely to be the best option for a clinical translation as it combines clinically approved chemotherapeutics and MBs with the possibility to tune separately the volume and the concentration of both compounds before their i.v. injection. However, the efficacy of acoustically-mediated drug delivery using the coadministration of MBs and chemotherapeutics is strongly dependent on the sufficient accumulation of both agents in tumor microvasculature. Similar pharmacokinetics and biodistribution of MBs and chemotherapeutics are not guaranteed due to their different physico-chemical properties. Thus, drug-loaded MBs have been designed to overcome such issues. This approach was investigated in 63% of preclinical studies but none in the clinical trials. Drug-loaded MBs may hold the greatest therapeutic potential, as chemotherapeutics and MBs are colocalized at the same time in the tumor microvasculature and a local release of the drug is triggered upon US exposure. However, these MBs require extensive and costly characterization and evaluation before they can be clinically approved.

Furthermore, it not straightforward to directly compare the results of most preclinical studies because of the heterogeneity in US devices used and a lack of standardized US settings. Surprisingly, clinical US scanners are exploited in only 23% of the preclinical studies, while the use of these devices would appear to be relevant to facilitate the clinical transfer of this method. However, the US settings on such equipment are limited for safety reasons. Here lies the main reason why, over 50% of preclinical studies used commercial or lab-made US devices, which have been designed to control many US settings for subsequently being optimized for drug delivery. However, such devices are not clinically approved. We believe that a credible alternative to these devices would be the use of open clinical US scanners or modified therapeutic US scanners. Concerning US protocols, the majority of studies used a single set of US parameters to demonstrate the therapeutic potential of acoustically-mediated drug delivery. These studies did not describe whether US parameters are the results of a deep investigation of

their efficiency in delivering anti-cancer drugs or of an empirical analysis based on available literature. The former option is the most appropriate for safely optimizing US protocol, but it remains expensive and can come up against ethical considerations in animal experimentation due to the number of animals required for such investigations.

Most preclinical studies have clearly reported that MB-mediated US improves the therapeutic effectiveness of chemotherapeutics by monitoring the tumor size but also tumor perfusion in few cases. Unfortunately, the correlation between the improvement in therapeutic effectiveness of chemotherapeutics and its enhanced i.t. bioavailability is often lacking. Indeed, only 31% of preclinical studies explored and showed that an enhanced therapeutic effectiveness could be attributed to increased i.t. drug concentration. In addition, the published results of the first clinical trials did not investigate such a correlation. Future clinical trials apparently will not consider the assessment of i.t. drug concentration after MB-mediated US. We believe that in the context of phase 0/I clinical trials, this assessment can be planned for patients whose tumors are scheduled for surgical excision. Next to this consequence, another expected one of acoustically-mediated delivery of chemotherapeutics is the reduction of drug deposition in healthy tissues. This outcome is expected to be more significant for targeted release from drug-loaded MBs compared to the coadministration or the successive administration of chemotherapeutics and MBs where chemotherapeutics can freely penetrate in healthy tissues anyway, without US application. Less than 6% of preclinical studies investigated and reported a decrease in drug accumulation into the healthy tissues. Such investigation cannot be performed in humans due to the invasive nature of the biopsy procedures. However, the side effects related to a non-specific accumulation of drugs in healthy organs can be monitored by measuring body weight, by assaying serum biomarkers and by assessing the physiology of organs using imaging modalities.

Finally, one of the central questions in the development of MB-assisted US for drug delivery in digestive cancers is: What are the expectations of the clinicians in terms of therapeutic efficacy? Indeed, the objectives in terms of therapeutic efficacy are not clearly established in preclinical and clinical studies. Is a 30% reduction in tumor volume sufficient to perform surgical excision of the tumor? Is total eradication of the tumor desired? These objectives have to be clearly defined because the developments of US devices and the optimizations of therapeutic schemes will not evidently be the same to induce a partial or total reduction in tumor volume.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the materials. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Figure legends

Figure 1. *In-vivo* delivery of irinotecan using MB-assisted US in liver metastases from colorectal cancer (HCT-116) in mice. A 20 or 40 mg/kg irinotecan dose was intravenously administered once a week for three weeks. Thirty minutes later, MBs (BG8214 ; Bracco Research SA, Geneva, Switzerland) were intravenously injected and the liver metastases were exposed to US pulses (lab-made US device; 1.3 MHz, 10 kHz PRF, 40% DC, 0.4 MPa PNP, for 3 min) using a dedicated 1.5-D US phased array probe connected to a modified Aixplorer US scanner (Supersonic Imagine, Aix-en-Provence, France). As a control, mice received an i.v. injection of 40 mg/kg irinotecan dose only. Tumor volume was measured using anatomical US imaging. Data expressed as mean ± SD was calculated from 6-8 tumors.

Figure 2. *In-vivo* delivery of AF750-labelled cetuximab using MB-assisted US in s.c. colorectal (HT-29) cancer in a mouse model. In group group, mice received an i.v. injection of fluorescently AF750-labelled cetuximab only (Control). In treated groups, the i.v. injection of AF750-labelled cetuximab is followed by an i.v. administration of MBs (BG8214, Bracco Research SA) and the tumors were exposed to US pulses using three US settings at 1 MHz frequency: (i) 100 μs burst length, 40% DC, 600 kPa for 3 min (Setting 1); (ii) 10 ms burst length, 20% DC, 500 kPa for 5 min (Setting 2); and (ii) 1 s burst length, 5% DC, 350 kPa for 15 min (Setting 3). The i.t. biodistribution of AF750-labelled cetuximab was assessed *in-vivo* using whole-body fluorescence imaging. Representative images of control mouse (A) and treated mouse using the US setting 1 (B) are presented. Quantitative fluorescence analysis of

i.t. biodistribution of AF750-labelled cetuximab. Data expressed as mean ± SD was calculated from 5-6 tumors.

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Table 1. Inclusion and exclusion criteria used to select studies

Inclusion criteria	**Exclusion criteria**
Involving MB-assisted US	US without MBs
Delivery of chemotherapeutics and immunotherapeutics in digestive cancers	Delivery of nucleic acids and photo/sonodynamics agents in digestive cancers
Preclinical and clinical studies	*In-vitro* studies
Original articles	Review papers, comments, Letters
English	Other languages

Table 2. Preclinical studies on colorectal cancer

References	Drug	Drug-loaded MBs	Tumor model	Increase in therapeutic effectiveness	Enhancement of i.t. drug biodistribution	Reduction of drug concentration in normal organs	Reduction of side effects
[18]	Cisplatin	N	s.c.	Y	ND	ND	ND
[20]	Doxil®	N	s.c.	Y	ND	ND	ND
[21]	Camptothecin/fluoroxyuridine	Y	s.c.	Y	ND	ND	N
[22]	Doxil®	N	s.c.	Y	Y	ND	ND
[23]	Combrestatin	Y	s.c.	Y	Y	ND	ND
[24]	Irinotecan and SN38	Y	s.c.	Y	Y	ND	ND
[29]	Cetuximab	N	s.c.	N	Y	ND	ND
Unpublished data	Irinotecan	N	Liver metastases	Y	ND	ND	ND

Y, yes; N, No; ND, non-determined; s.c. subcutaneous tumor.

Table 3. Preclinical studies on pancreatic cancer

References	Drug	Drug-loaded MBs	Tumor model	Increase in therapeutic effectiveness	Enhancement of i.t. drug biodistribution	Reduction of drug concentration in normal organs	Reduction of side effects
[25]	Doxorubicin	Y	i.m.	Y	ND	ND	N
[26]	Doxorubicin	Y	s.c.	Y	Y	ND	ND
[27]	Doxorubicin	Y	s.c.	Y	Y	ND	ND
[28]	Gemcitabine	N	s.c.	Y	ND	ND	ND
[30]	Paclitaxel	Y	s.c.	Y	Y	ND	Y
[31]	Gemcitabine	Y	s.c.	Y	ND	ND	Y
[32]	Nab-paclitaxel	N	s.c.	Y	ND	ND	Y
[33]	5-Fluorouracil	Y	Orthotopic	Y	ND	ND	N
[34]	Irinotecan and oxaliplatin	Y	s.c.	Y	ND	ND	N
[35]	Gemcitabine	Y	s.c.	Y	ND	ND	ND
[36]	Gemcitabine	N	s.c.	Y	ND	ND	Y
[37]	Gemcitabine	N	Orthotopic	Y	ND	ND	ND
[38]	Gemcitabine	N	s.c.	Y	ND	ND	N
[39]	Gemcitabine	Y	s.c.	Y	ND	ND	Y

Y, yes; N, No; ND, non-determined; s.c. subcutaneous tumor; i.m., intramuscular.

Table 4. Preclinical studies on liver cancer

References	Drug	Drug-loaded MBs	Tumor model	Increase in therapeutic effectiveness	Enhancement of i.t. drug biodistribution	Reduction of drug concentration in normal organs	Reduction of side effects
[44]	Doxorubicin	Y	Orthotopic	Y	ND	ND	ND
[45]	Doxorubicin	Y	Orthotopic	Y	Y	Y	ND
[46]	Doxorubicin	Y	Orthotopic	ND	Y	Y	ND
[47]	Doxorubicin	Y	Orthotopic	Y	Y	ND	ND
[48]	Doxorubicin	N	Orthotopic	ND	Y	ND	ND
[49]	Doxorubicin	Y	Orthotopic	Y	ND	ND	N
[50]	Doxorubicin	Y	Orthotopic	Y	ND	ND	N
[51]	Doxorubicin	N	Orthotopic	Y	Y	ND	N
[52]	Docetaxel	Y	Orthotopic	Y	ND	ND	ND
[53]	Docetaxel	Y	s.c.	Y	Y	ND	N
[54]	Docetaxel	Y	s.c.	Y	ND	ND	ND
[55]	Resveratrol	Y	s.c.	Y	ND	ND	ND
[56]	Cisplatin, mitomycin and 5-fluorouracil	N	s.c. and orthotopic	Y	ND	ND	ND

Y, yes; N, No; ND, non-determined; s.c. subcutaneous tumor.