World Journal of Pharmacology

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DOI: 10.5497/wjp.v4.i1.96

World J Pharmacol 2015 March 9; 4(1): 96-116
ISSN 2220-3192 (online)
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REVIEW

Hepatocellular carcinoma and multidrug resistance: Past, present and new challenges for therapy improvement

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Author contributions: Cuestas ML, Oubiña JR and Mathet VL contributed to this paper.
Conflict-of-interest: The authors declare no competing financial interests.
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Received: July 29, 2014
Peer-review started: July 29, 2014
First decision: September 18, 2014
Revised: November 13, 2014
Accepted: November 27, 2014
Article in press: December 1, 2014
Published online: March 9, 2015

Abstract

Hepatocellular carcinoma (HCC) is the most frequent form of liver cancer and the third most common cause of cancer-related death in the world. The main risk factor worldwide for this type of malignancy is chronic hepatitis caused by hepatitis B virus and hepatitis C virus infections. Advances in early detection and treatment have improved life expectancy of patients with HCC. However, this disorder remains as a disease with poor prognosis. In fact, epidemiological studies have revealed that there is an 8-mo median survival rate in patients, approximately 20% of whom survive one year while only 5% remain alive after three years. Additionally, HCC is particularly difficult to treat because of its high recurrence rate, and its resistance to conventional chemotherapy is due, among other mechanisms, to several members of the ATP-Binding Cassette protein family involved in drug transport being overexpressed. Fortunately, there is evidence that these patients may benefit from alternative molecular-targeted therapies. This manuscript intends to provide further insight into the etiology and molecular mechanisms related to HCC development and the latest therapeutic approaches to treat this malignancy. The development of effective delivery systems of antitumor drugs able to target the liver parenchyma is also assessed. Finally, the prospects in the development of more efficient drug therapies to overcome multidrug resistance are also examined.

Key words: Hepatocellular carcinoma; Therapy; Multidrug resistance; Drug delivery systems; Liver targeting

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Core tip: Hepatocellular carcinoma (HCC) is the most frequent malignancy of the liver. Despite the advances in early detection and treatment, this disorder still has a poor prognosis. This manuscript reviews the ongoing knowledge regarding the etiology and molecular mechanisms implicated in HCC development and the therapeutic strategies for the management of this malignancy. Finally, the development of effective delivery systems of antitumor drugs able to target the liver parenchyma as well as the prospects in the development of a more efficient drug therapy to overcome multidrug resistance are also examined.

Cuestas ML, Oubiña JR, Mathet VL. Hepatocellular carcinoma and multidrug resistance: Past, present and new challenges for therapy improvement. *World J Pharmacol* 2015; 4(1): 96-116 Available from: URL: http://www.wjgnet.com/2220-3192/full/v4/i1/96.htm DOI: http://dx.doi.org/10.5497/wjp.v4.i1.96

INTRODUCTION

Liver cancer, which is ranked fifth in frequency of occurrence worldwide and third in cancer mortality, is one of the most frequent types of cancer[1]. Hepatocellular carcinoma (HCC) represents 85%-90% of primary liver cancers and is the main subtype in terms of histologic origin. Its clinical course is aggressive, while frequent recurrence and metastasis are often associated with this malignancy. It is characterized by late presentation, fast progression, limited response to therapy and a very poor survival rate (6%)[2]. Asia and Africa are the countries where HCC is more prevalent; however, there has been a rising trend of HCC in Western countries[3]. Chronic liver diseases, such as chronic hepatitis B (CHB) and CHC[4] are among the major risk factors for HCC development. Other common causes leading to the development of this malignancy are: hemochromatosis, fatty liver diseases unrelated to alcohol consumption (non-alcoholic fatty liver disease), primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis, excessive alcohol use, ingestion of food contaminated with aflatoxin, vinyl chloride, and use of radioactive agents such as thorotrast[5,6].

The development of HCC involves several steps of a complex process characterized by both genetic and epigenetic changes that may activate cellular oncogenes, inactivate tumor suppressor genes and/or dysregulate multiple cell signal transduction pathways, such as the Wnt/β-catenin, the Ras-Raf-mitogen-activated protein kinase (MAPK), the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and the transforming growth factor-β (TGF-β) pathways[7-9].

Several potentially curative or palliative approaches to the treatment of HCC are available. The surgical approaches that are most commonly chosen are: surgical resection and orthotopic liver transplantation. However, preserved or adequate liver function is an essential criterion for surgical resection. In this regard, this surgical approach is not a feasible option for HCC patients[10] when the tumor is at an advanced stage, or is located in close proximity to important hepatic vessels within the liver preventing a negative-margin resection, or when there are tumors at multiple sites or there is inadequate remaining hepatic function. Furthermore, about 17%-69% of patients suffer from recurrence, thus limiting their long-term survival at 5 years postoperatively[11]. Orthotopic liver transplantation is considered to be the only curative solution for HCC that cannot be surgically removed. Candidates for this procedure are those patients having solitary HCCs of less than 5 cm in size or up to three nodules, each smaller than 3 cm[12,13]. Nevertheless, this procedure has limited availability due to the great difficulty in finding organ donors[10].

Non-surgical therapeutic approaches for HCC such as radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), transarterial embolization (TAE) and transarterial chemoembolization (TACE) are other therapeutic tools used to substitute first-line procedures; however, the probable course of the disease for the patients undergoing such procedures is still bleak. The annual recurrence rate is approximately 15%-20%, reaching 80%-90% within the 5-year post-treatment period[14,15]. A research study confirms that so far there are no adjuvant therapeutic postoperative regimens to successfully treat HCC.

A clinical investigation indicates that none of the adjuvant therapies is particularly effective in the treatment of HCC after surgery[16]. Systemic chemotherapy with doxorubicin, immunotherapy using interferon and hormonal therapy with tamoxifen, on the other hand, yielded poor results, with no significant survival benefits compared with symptomatic management[17-19]. One important limitation in the chemotherapy for HCC is the emergence of multidrug resistance (MDR) to conventional anti-tumoral agents[20]. This phenotype is commonly related to cancer cells that are able to overexpress drug transporter proteins belonging to the ATP-binding cassette (ABC) superfamily of proteins that move drugs out of cells, such as P-glycoprotein (P-gp), the multidrug resistance-associated proteins (MRPs) and the breast cancer resistance protein (BCRP)[21]. Additionally, current anti-tumoral drugs used in HCC treatment, also promote significant toxicities in other non-target organs affecting patient compliance and adherence to these therapeutic regimens[22]. Enhanced delivery[23] of these commercially available anti-cancer agents to liver parenchyma may provide an opportunity to selectively improve the efficacy of the current therapies and simultaneously reduce the adverse effects that often lead to treatment failure.

Up to now, no succesful systemic chemotherapy for patients with advanced and unresectable HCC is available. However, on November 16, 2007, the Federal and Drug Administration (FDA; United States) had approved sorafenib tosylate (Nexavar® tablets, made by Bayer Pharmaceuticals Corp.), as "a small molecule Raf kinase and VEGF receptor kinase inhibitor, for the treatment of patients with unresectable hepatocellular carcinoma (HCC)". Unfortunately, this promising treatment has demonstrated limited survival benefits with very low response rates[24,25]. Therefore, new approaches are urgently needed for: (1) improving the activity of prevailing antichemotherapeutic agents by targeting them to the liver using drug delivery systems designed with targeting moieties; (2) overcoming MDR by sensitizing tumor cells to conventional chemotherapeutics; and (3) improving the prognosis of HCC by

further development of the latest molecular targeting agents, such as sorafenib and rapamycin which - although limited - at present are deemed to be the most effective agents for managing unresectable HCC.

In this analysis we review the available information regarding the latest pharmacotherapy options for the treatment of patients suffering from advanced HCC, including molecular targeting agents. Prospects regarding a more effective pharmacotherapy for liver targeting and avoiding/preventing multidrug resistance in cancer cells are also addressed.

CURRENT THERAPIES FOR HCC

Unfortunately, owing to the asymptomatic nature of early HCC, in a majority of patients, HCC is usually diagnosed at an advanced stage, when most potentially curative therapies such as surgical resection, orthotopic liver transplantation and local ablation display a low efficacy. Moreover, in 60% to 80% of patients with liver cancer, the treatment is complicated by underlying liver cirrhosis and hepatic dysfunction[26]. In these advanced stages, systemic treatments are commonly used; however, they are also minimally effective, have severe side effects, develop high drug resistance, and most importantly, patient survival is not improved. HCC is rarely amenable to radiotherapy, leaving this disease with no effective therapeutic options and a very poor prognosis[27]. Through better understanding the molecular basis of hepatocarcinogenesis (*e.g.*, signal pathways and molecular alterations that promotes tumor growth and cell survival), new treatment modalities have recently emerged including molecular targeted therapy and gene therapy, such as antisense gene targeting.

Surgical therapies

At present, surgical resection and orthotopic liver transplantation offer the only chance for long-term cure of patients suffering from HCC. Surgical resection is an effective treatment for those patients with HCC that is not associated with liver cirrhosis or in patients whose hepatic function is well compensated. This means, that surgical resection is an option for only a small proportion of patients, less than 18%, since underlying chronic liver disease or cirrhosis accounts for about 85%-90% of HCC patients[28]. Thus, both tumor extent and hepatic function must be evaluated pre-operatively to avoid hepatic failure following resection, which is usually a fatal condition possibly requiring urgent liver transplantation. There is a 5-year post-operative survival rate of 40%-70% of duly selected candidates for liver resection; however, relapse takes place in near 70%, especially in patients with cirrhosis[29]. For this reason, orthotopic liver transplantation is considered to be the best choice for those patients suffering from HCC and cirrhosis, showing 5-year post-transplant survival rates of 65%-80% among well-selected candidates. Nevertheless, there is a limitation as to the use of this procedure, since the shortage of human donors is an unfortunate event these days[10]. Due to the strictness of Milan criteria regarding transplantation and the restrictions in finding available donors, scientists are now devoted to exploring other therapies for managing the disease in order to provide a solution for the disadvantages arising from transplantation or surgical resection[30].

Non-surgical therapies

Locoregional therapies: Percutaneous treatments: Percutaneous ablation (PA) is now the first alternative treatment when resection or orthotopic liver transplantation has been ruled out in patients suffering from early-stage HCC. PA can be thermal or chemical. The thermal ablation procedure destroys cancer cells by cryoablation or by heat using lasers, high intensity focused ultrasound, microwaves or radiotherapy. Chemical ablation consists on cancer cells destruction by injecting chemicals - *e.g.,* ethanol/acetic acid - introduced into the tumor mass by means of a very fine needle[10]. The ablative method as a treatment of choice will be based on the size of the tumor.

PEI was introduced in the 1980's, nowadays being the most prevalent kind of PA for treating HCC. Cohort studies and retrospective series analysis have shown that a five - year survival might be possible in 50%-80% of patients having a single tumor smaller than 5 cm in size or up to three nodules, lesser than 3 cm in size[31]. The main drawback of PEI is the high local recurrence rate (33%-43% at three years)[32]. RFA (radiofrequency ablation) is another ablative procedure initially outlined by Rossi *et al*[33], in 1993, and since then, it has become the favorite form of ablation for small tumors. When comparing RFA with PEI, the former showed to be better in relapse prevention and in improving tumor necrosis[30]. However, surgical resection is so far very superior to PA techniques.

Chemoembolization: TAE is another locoregional palliative treatment option in cases where surgical resection or other forms of treatment with curative potential are not advised for specific HCC tumors. The hepatic artery is responsible for the supply of blood to the tumor; therefore, the obstruction caused by TAE produces extended tumor necrosis as a result of ischemia, thus providing the rationale for its wide use in patients with HCC[34]. When this procedure is performed in combination with chemotherapeutic agents such as doxorubicin and cisplatin, usually mixed with lipiodol, it is termed TACE. The addition of chemotherapy aims to enhance the anti-tumoral action of ischemia. Usually, in TACE, anti-neoplasic drugs are mixed with lipiodol. By injecting the patient with a combination of anti-tumoral agents with the radio-opaque contrast agent lipiodol into the hepatic artery, drug delivery to tumor cells is expected to increase. Likewise, the chances of systemic side-effects related to chemotherapy are expected to decrease. Unfortunately, the use of

either TAE alone or TACE, remains a controversial treatment approach for patients with HCC, because some randomized controlled trials have failed to disclose a significant benefit in terms of survival of treated patients as compared with untreated patients[34,35]. Moreover, several studies demonstrated disappointing results, showing that TACE enhances intrahepatic and extrahepatic metastases, and even reduce survival[36]. Accordingly, anti-angiogenic therapy enhances the efficacy of transcatheter arterial embolization for HCC hepatocellular carcinomas[37].

Furthermore, severe side-effects produced by the arterial obstruction and by the toxicity of the injected anti-tumoral agents during the TACE procedure, counteract the anti-tumoral action resulting from arterial obstruction. It should be highlighted that the absence of effects due to chemotherapy is not the result of ineffective drug delivery but of the presence of MDR due to the over-expression of efflux pumps that belongs to the ABC superfamily of protein transporters, as well as to an abnormal p53 function that leads to an inhibition of apoptosis making tumor cells resistant to anti-tumoral treatment[38-40]. Dysfunctional p53 makes tumoral cells also less sensitive to hypoxia.

Radiation therapy

External beam radiation therapy: Before the 1990s, external beam radiation therapy (EBRT) has played a minor role in the primary treatment of HCC. However, EBRT was mainly used in the palliative setting for metastatic disease because of an intolerance of the adjacent normal liver to tolerate radiation that precluded a more intense use of radiation[41].

In 1987, the radiation therapy oncology group outlined the outcomes of a randomized clinical trial including radiotherapy of the whole-liver with a dose of 21 Gy in seven fractions or combined with the radiosensitizer misonidazole[42]. Although a whole-liver EBRT provided a significant palliative effect, the addition of misonidazole did not significantly improve the outcomes[43].

The dose-limiting complication of delivering EBRT to the liver is radiation-induced liver disease (RILD) a clinical entity characterized by the presence of anicteric hepatomegaly and ascites (associated with high levels in sera of hepatic enzymes) that may lead to liver failure and death[44]. Due to this reason, several approaches were designed by researchers at the University of Michigan to administer higher radiation doses to smaller liver portions, in order to produce greater tumor control rates without an increase in the damage to the liver parenchyma that is likely to be caused by radiation[45].

Based on the above, with the advent of intensity-modulated radiation therapy, image-guided radiation therapy and stereotactic body radiation therapy (SBRT; as described below, separately), higher doses could be delivered safely since the radiation dose can be distributed tightly into the tumor while preserving normal tissue in the liver from the effects of high doses of radiation[41].

Selective internal radiotherapy: Intrahepatic radiotherapy, better known as radioembolization or selective internal radiation therapy (SIRT), is a therapy based on the intrahepatic delivery of Yttrium-90 (Y-90)-labeled microspheres into the arteries that supply blood to the tumor, where the microspheres come into contact with tumor cells which are hit by radiation emitted by the radioisotope[46].

The microspheres are an implantable medical device consisting of resin-based or glass-based biocompatible microspheres loaded with Y-90[47,48].

The process of release of the microspheres occurs by using a flexible catheter inserted into the femoral artery which is moved forward by the radiologist until the hepatic artery is reached[47,49].

SIRT demonstrated an 89% treatment response with resin microspheres and 78% with glass microspheres, respectively, in patients suffering from HCC[49]. The median overall survival ranged 16.4-18 mo[50,51].

SIRT is a minimally invasive technique and a well-tolerated therapy. It is a new therapy for treating liver cancer and liver metastases originated from colorectal cancer.

Finally, SIRT represents a new therapeutic option for patients with unresectable HCC. Clinical studies showed an increase in terms of survival when this technique is used in combination with chemotherapy. Noteworthy, SIRT tends to reduce the size of the tumor and allows some patients to become eligible for surgical resection[52].

SBRT: As a means to ablate primary or metastatic liver tumors, technical advances in tumor localization and motion management were achieved.

SBRT has become an optimistic approach for the treatment of liver cancer as a result of the complex character of liver tumor motion along with the priority of decreasing the volume irradiated to the minimum to reduce the probability of RILD.SBRT[45].

Focal, high dose SBRT delivers ablative doses in fewer fractions and highly conformational radiotherapy volumes[43].

To avoid damaging nearby critical structures and organs, doses are minimized using tight margins. A robust immobilization device is thus crucial to achieve a reproducible and accurate setup. Image guidance can be accomplished by using a megavoltage/kilovoltage cone beam computed tomography (CT) or stereoscopic X-rays[43]. The local tumor control of SBRT turns out to exceed that of conventional fraction EBRT[42].

A retrospective analysis carried out by Choi *et al*[53], demonstrated that a dose of 50 Gy of SBRT in 5 or 10 fractions for primary liver tumor produced a median survival of 20 mo**.** Another study carried out by Tse *et al*[54] using SBRT at a dose of 24-54 Gy in 6 fractions, demonstrated that the median survival rate turned out

to be 13.4 mo.

Although liver metastasis is not the subject of the present review, it is noteworthy to point out that survival outcomes are better in patients with liver metastasis than with HCC. In both groups, there appears to be a dose-response for local control. For HCC, the dose of SBRT should be based on the cirrhotic status. For patients with Child-Pugh A cirrhosis, 48 Gy or higher distributed in 3 fractions is recommended. For patients with Child-Pugh B cirrhosis, more fractionated schemes are suggested (5 fractions of 40 Gy, for example). For liver metastases, doses greater than 48 Gy divided into 3 fractions or 14-26 Gy in one fraction is recommended[43].

Finally, with the use of innovative tools combined with radiotherapy such as advanced imaging and immunotherapy, further advances in liver cancer could be achieved. Research is under way to analyze the way of optimizing radiation delivery by using other procedures such as TACE and sorafenib administration[45].

Systemic treatments

Hormonal therapy: Since 15%-39% of HCC express estrogen receptor (ER), and overexpression of the progesterone receptors was detected in up to 39% of tumors, in the last decades there have been clinical trials with tamoxifen for patients with HCC[55,56]. However, later studies have shown that patients suffering from HCC and receiving tamoxifen did not have a survival benefit, reaching the conclusion that this anti-estrogen drug, either alone or in combination with other chemotherapy agents to treat advanced HCC is ineffective. According to Di Maio *et al*[57], a possible explanation for these unfavorable results resides on the selection of the patients in the clinical trials, since none of them had selected patients based on the expression-status of the hormonal receptor. Therefore, this constitutes a significant problem. It should be pointed out that in breast cancer, for example, it is well known that the adequacy of hormonal treatment is pertinent; however, it is only restricted to those patients having tumors with expressed hormone receptors. Moreover, in some HCC patients, a variant form of the ER alpha (vER) transcript derived from an exon 5-deleted transcript lacking the hormone-binding domain of the receptor, yet having an intact DNA-binding domain keeps constitutive transcriptional activity. These tumors with vER, which account for an important percentage of HCCs, have a bleaker prognosis characterized by faster doubling time and shorter survival[57]. Tamoxifen is ineffective in the treatment of tumors with vER because tamoxifen is not able to bind to the receptor. Thus, by choosing anti-hormonal treatment according to the presence of wild-type or variant ERs in the tumor, a significant improvement to the response rate to tamoxifen is observed[58]. Efficacy of megestrol acetate has been tested in HCC tumors expressing vER in a randomized study of 45 patients with advanced HCC. Although in this study it was observed that megestrol notoriously increases survival in this reduced group of patients (untreated patients: 7 mo; patients treated with megestrol: 18 mo)[59], an adequately powered randomized trial should be carried out to confirm these results.

As in the case of estrogens, it has been proved that androgens positively influence HCC growth; thus, androgens or luteinizing hormone-releasing hormone agonists (nilutamide, goserelin acetate, triptorelin, flutamide, leuprorelin) will possibly play a part in treating HCC. However, no benefit in terms of survival was found with anti-androgenic treatment in male patients with advanced HCC[60,61].

Finally, hormonal compounds have proved to be totally ineffective as regards patient survival. Although tamoxifen and anti-androgen drugs failed to prolong survival in advanced HCC cases, somatostatin -whose receptor is expressed in HCC - and its synthetic analogs like octreotide may play a role in prolonging survival in patients with advanced disease[57]. However, the results obtained so far are conflicting; therefore - as in the case of megestrol - further studies are required.

Systemic chemotherapy: Many patients seek systemic chemotherapy and for more than 50 years, conventional systemic cancer chemotherapy has been developed with the so-called anti-tumoral agents. However, in patients with HCC, the role of chemotherapy is quite limited due to inefficacy and toxicity of these antineoplastic drugs[62]. Single chemotherapy with cytotoxic agents such as cisplatin or 5-fluorouracil showed a low response rate (< 10%) without a clear benefit in overall survival[63]. In a recent clinical trial involving a large number of HCC patients, systemic administration of doxorubicin has provided a very low response rate (4%)[64].

Combination therapy is broadly regarded as a treatment option and used in oncology practice to enhance the efficacy of systemic chemotherapy. Moreover, it is the only treatment choice for those patients in whom unresectable HCC is not feasible for intra-arterial treatment. Although many regimens have not proved to be efficient for HCC patients, the combination of doxorubicin with paclitaxel (a microtubule stabilizer deemed to be one of the leading anti-tumoral agents in the past 10 years) showed a synergistic anti-tumor activity *in vitro* and *in vivo*[65].

A randomized phase Ⅲ trial assessing doxorubicin combination chemotherapy (cisplatin, interferon, doxorubicin and 5-fluorouracil, PIAF) revealed a higher overall response rate and better survival rates than those of patients receiving doxorubicin; unfortunately, these differences were not statistically significant. Moreover, increased toxicity was also related to PIAF[66].

The result in a double-blind phase Ⅱ multinational study assessing the treatment using sorafenib plus doxorubicin was greater median time to progression, overall survival and progression-free survival than doxorubicin monotherapy with treatment using sora-

fenib[67]. However, the combination therapy of sorafenib and doxorubicin is not yet indicated for routine clinical use.

The poor response nature of HCC to systemic chemotherapy is mainly due to its extreme chemo-resistance. Overexpression of several members belonging to the ABC-transporters superfamily leads to its MDR phenotype. At present, there is an intense search of agents for overcoming MDR, as it is discussed in the last section.

Immunotherapy: Immunotherapy is considered to be a possible treatment choice for those suffering from HCC, mainly as a second-line treatment to prevent relapse. In accordance with previous studies, there is direct correlation between patient survival and the the type and number of immune cells infiltrating the tumor, which indicates that there is a direct effect of immune responses on the disease evolution[68].

Immunotherapy represents an attractive alternative tool based on sensitivity, specificity against tumor cells, on the immune system capacity to renew itself, and its potential to eradicate residual tumors after conventional treatment. Therefore, results from several clinical trials have shown that immune-based therapy can improve outcomes in patients with HCC[69].

A randomized clinical trial demonstrated that there were statistically significant improvements in relapse time and relapse-free survival with the administration of interleukin 2 (IL-2) and anti-CD3 activated peripheral blood mononuclear cell in HCC patients that underwent surgical resection[70].

Interferons (IFNs) have immunomodulatory and anti-proliferative activities on tumor cells, and are widely used as therapy for neoplasias and viral diseases[71]. A randomized study carried out by Lai *et al*[63], reported that recombinant IFN-α turned out to be superior to doxorubicin in terms of survival, tumor response and toxicity in patients with unresectable HCC, both in prolonging survival and in inducing tumor regression.

One area of active research is immunotherapy with cytokine-induced killer cells (CIK)[71]; unfortunately, its efficiency is limited because of its low specificity to cancer cells. Another approach is the tumor-associated antigen (TAA)-pulsed dendritic cells (DC) therapy, but the outcomes remain unsatisfactory due to the poor immunogenicity of TAA that make tumor cells to fail to adequately stimulate DCs for effective presentation to immune cells[72]. A possible method for increasing the uptake of TAAs by DCs is to complex them with an IgG antibody, so that the resulting immune complexes may bind to Fcγ receptors (Fcγ-Rs) on DCs and induce phagocytosis of TAAs, leading to an effective immune response against the tumor cells[73]. Such targeting strategy was achieved by complexing the tumor cell membranes expressing α-Gal epitopes (Gal-α1, 3Gal-β1, 4-GlcNAc-R, α-Gal) with the anti-Gal IgG antibody (a natural antibody comprising 1% of IgG in humans)[74]. This opsonized binding complex may be phagocyted by DC and then enhance TAA presentation to naïve T or CIK cells, which are then activated and attack the remaining tumor cells *in vivo*[75]. In this study, the authors demonstrated that this anti-tumor vaccine could significantly increase the tumor-specific immune responders in circulation and the survival of advanced HCC patients (17.1 mo *vs* 10.1 mo in control groups) with no serious side effects.

In addition, results from a larger trial testing infusion of antigen-presenting cells that included 31 HCC patients receiving autologous tumor lysate-pulsed DC, showed an important 1 year survival (63% *vs* 10%), which supports the idea of immunotherapy for HCC based on DC[76].

Immunotherapy was also supported by rat models, since it was shown that there was a reduction in HCC relapse when administering DC in combination with IL-12 activated T and NK cells[77].

Antigen-specific immunotherapy and Treg ($CD25^+$ T-cells) depletion are worth mentioning as promising plans of action in physiologically important HCC preclinical models[68]. For example, immunization with a DNA-based synthetic vector (DNAmAFP/704) as an antigen-specific approach for targeting α-fetoprotein (AFP) proved to considerable reduce (65%) the tumor burden in an autochthonous model of a chemically produced hepatocarcinoma. Similarly, $CD25^+$ T-cell depletion by injecting the PC61 antibody significantly protected against tumor growth in an orthotopic HCC model[68]. Treg-depleting reagent Denileukin diftitox (Ontak) targets the constitutively expressed molecule CD25, thus producing the elimination of circulating Tregs without coordinating depletion of activated CD25-expressing T effector cells[78].

Another research work reported that the *ex vivo* treatment of $CD8^+$ T cells isolated from HCC patients with CTLA-4 blocking antibodies (ipilimumab) produced an expanded antigen-specific T cell repertoire, suggesting that this monoclonal antibody is likely to be highly effective in the treatment of HCC[79].

Direct reactivation of hyporesponsive tumor-specific T cells by providing T cell growth factors (IL-15, IL-7) or costimulatory agonists (anti-4-1BB, anti-OX40)[80,81] is another possible approach to successfully deal with tumor-mediated immunosuppression.

Furthermore, the use of therapeutic reagents inducing chemokine and adhesion molecule expression through blood vessel activation is also an interesting strategy for HCC treated with immunotherapy, since this kind of strategy may help restore T cell infiltration of the tumor[82].

Finally, it is expected that chemoimmunotherapy, that is, immunotherapy in combination with conventional therapy or other types of immunotherapies will elicit synergistic anti-tumor activity.

It has been earlier suggested that during or immediately following ablative therapy, immunotherapy will have its highest observed efficacy when tumor cells are about to die and the immune response has begun

its activity. In HCC, combined therapy of TAE with intratumoral DC infusion produced higher frequencies of AFP-specific T cells in comparison with TAE alone[83,84].

Advantageous therapeutic approaches in HCC will probably include combinations of immunotherapy involving several immune effector mechanisms, such as vaccines and T cell immune-modulators, along with immunotherapy supplemented with molecularly targeted inhibitors of tumor signaling pathways[84].

MOLECULAR TARGETED THERAPY

In the last decades, research on the molecular pathology of HCC has uncovered a plethora of molecules that are critical in the onset and progression of this human disease. With regard to cancer investigation, in order to target key molecules involved in cancer genesis and growth, several compounds for disease treatment were developed. The present section summarizes the status of the different therapeutic compounds developed for the targeting members of different signaling pathways that are crucial in the pathogenesis of HCC, *e.g.*, inhibitors of the epidermal growth factor receptors (EGFR) and the vascular EGFR (VEGFR), families, as well as inhibitors of the TGF-β and the mTOR signaling pathways (Table 1 and Figure 1).

Anti-angiogenic therapy

HCC is one of the most vascularized solid tumors, having high vascular endothelial growth factors (VEGF) and microvessel density levels. In addition, other relevant angiogenic factors involved in HCC pathogenesis are: VEGFs, fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs).

VEGF seems to be primary a mediator of angiogenesis in HCC. Moreover, a higher level of VEGF is associated with a more aggressive disease evolution and possible poor treatment response[85]. Therefore, VEGF/VEGFRs and PDGF/PDGFRs signaling pathways are prime targets for the development of anti-angiogenic treatments for cancer. The anti-VEGF antibody bevacizumab and the multi-targeted tyrosine kinase inhibitors (TKI) sunitinib, sorafenib and pazopanib, which inhibit VEGFRs and other receptor tyrosine kinases are agents approved by the FDA to directly aim at the VEGF pathway[86]. So far, the only agent that has been proven to be effective in terms of survival of patients with HCC is sorafenib, which has become the current standard for palliative treatment[86].

Unfortunately, resistance to anti-angiogenic therapy was described (Figure 2). Hypoxia-Inducible Factor-1α and -2α (HIF-1α and HIF-2α) may be caused by the use of anti-angiogenic agents due to constriction of tumor blood vessels, decrease in blood flow and intratumoral hypoxia[87]. HIF-1α and HIF-2α transactivate genes causing tumor angiogenesis, tumor cell growth and energy metabolism, therefore causing anti-angiogenic drugs to become resistant and leading to poor prognosis[88]. It was reported that HCC overexpress HIF-1α and that this overexpression is triggered by tissue hypoxia, aberrant growth factor receptor signaling and mutations in oncogenes and tumor supressor genes[87].

Furthermore, previous cancer experiences have shown that the expression of other angiogenic factors such as the FGF are up-regulated upon anti-VEGF treatment as an alternate escape mechanism. Thus, inhibitors of the FGF pathway such as brivanib were recently investigated for the treatment of advanced HCC as an option for patients with HCC following failure of sorafenib.

Sorafenib: Sorafenib is a multitargeting small molecule that exerts its anti-angiogenic effect through inhibition of VEGFR-1, VEGFR-2, VEGFR-3, CD135 or Fms-like tyrosine kinase-3 (Flt-3), PDGFR-β, and FGF receptor-1 (FGFR-1) promoting the formation of new blood vessels[89]. Sorafenib also acts blocking cellular proliferation mediated by the Raf/MAPK/ERK signaling pathway[89] and inducing both apoptosis and autophagy in human hepatoma cells[90,91]. As previously mentioned, the FDA has approved sorafenib for treating both HCC and renal cell cancer in 2007, and is the first systemic therapy to show some survival advantage. In 2008, a promising prospect for sorafenib monotherapy in the treatment of advanced HCC had been provided by a multicenter double-blind Phase Ⅲ trial (the Sorafenib HCC Assessment Randomized Protocol) which demonstrated a 44% increase in the median overall survival (10.7 mo in the sorafenib group and 7.9 mo in the placebo group)[24]. In the following year, an Asia-Pacific trial corroborated sorafenib efficacy reporting a median overall survival of 6.5 mo, whereas in the placebo group the reported median overall survival was 4.2 mo[92]. However, problems of drug-toxicity have been reported; among the most frequently observed drug-related adverse events, fatigue, anorexia, diarrhea, rash/desquamation, and hand - foot skin reactions were described[93]. Furthermore, other studies have shown that patients with severe liver dysfunction had a limited life expectancy after treatment with sorafenib (1.5 mo)[94]. In a meta-analysis of five randomized controlled trials encompassing 1462 patients with unresectable HCC, Shen *et al*[95], have recently shown that sorafenib use - as compared with placebo - improved the disease control rate (RR = 1.85, 95%CI: 1.55-2.20, $P < 0.001$), decreased tumor progression (HR = 0.61, 95%CI: 0,51-073, $P < 0.001$) and reduced mortality (HR = 0.71, 95%CI: 0.56-0.89, $P < 0.001$). Interestingly, further subgroup analyses demonstrated that results obtained were not modified by HCC etiology, performance status nor Barcelona Clinic Liver Cancer-stage[95] (Figure 3). Sorafenib has also shown benefit when combined with doxorubicin. In a phase I study combining sorafenib/doxorubicin, all four patients with metastatic HCC maintained stable disease state for more than 1 year of treatment[96]. In a randomized, double-blind, phase Ⅱ trial, the sorafenib/doxorubicin combination prolonged the median overall survival and progression-free survival when compared with doxorubicin alone[97].

Table 1 Molecular targeted therapy

Type of drug	Drug	Target	Stage of use (for HCC)
Inhibitors of angiogenesis	Sorafenib[1]	VEGFR members	Approved
		PDGFR-β	
		Flt-3	
		FGFR-1	
		Raf/MAPK/ERK signaling pathway	
	Bevacizumab	VEGFR members	Phase Ⅱ
	Sunitinib	VEGFR members	Phase Ⅱ
		PDGFR-α	
		PDGFR-β	
		Flt-3	
		c-Kit	
		RET kinases	
	Pazopanib	VEGFR members	Phase Ⅰ
		PDGFR-α	
		PDGFR-β	
		c-Kit	
	Brivanib	VEGF signaling pathway	Phase Ⅱ
		FGF signaling pathway	
	Axitinib	VEGFR members	Phase Ⅱ/Ⅲ
		PDGFR-α	
		PDGFR-β	
		c-Kit	
	Linifanib	VEGF	Phase Ⅱ
		PDGFR-α	
		PDGFR-β	
	TSU-68	VEGFR-2	Phase Ⅱ
		PDGFR-α	
		PDGFR-β	
		FGFR-1	
		c-Kit	
		Flk-1	
	Foretinib	VEGFR-2	Phase Ⅰ/Ⅱ
		c-Met	
	Dovitinib	VEGFR members	Phase Ⅰ/Ⅱ
		PDGFR-β	
		FGFR members	
		Flt-3	
		c-Kit	
	Ramucirumab	VEGFR-2	Phase Ⅱ
Inhibitors of EGFR	Erlotinib	EGFR/HER-1	Phase Ⅱ
	Lapatinib	EGFR/HER-1	Phase Ⅱ
		HER-2/NEU	
	Gefitinb	EGFR/HER-1	Phase Ⅰ
	Cetuximab	EGFR/HER-1	Phase Ⅱ
Inhibitors of the mTOR pathway	Rapamycin	PI3K/Akt/mTOR pathway	Phase Ⅰ/Ⅱ
	Everolimus	PI3K/Akt/mTOR pathway	Phase Ⅰ/Ⅱ

[1]Sorafenib also induces apoptosis and autophagy. HCC: Hepatocellular carcinoma; VEGFR: Vascular endothelial growth factor receptors; Flt-3: Fms-like tyrosine kinase-3; FGFR-1: FGF receptor-1; MAPK: Ras-Raf-mitogen-activated protein kinase; EGFR: Epidermal growth factor receptors; FGF: Fibroblast growth factor; PDGFR: Platelet-derived growth factor receptors; RET: Rearranged during transfection; HER-1: Human epidermal growth factor receptor-1; mTOR: Mammalian target of rapamycin.

A phase Ⅱ multicenter study of combined sorafenib/octreotide showed a higher disease control rate than sorafenib monotherapy (76% *vs* 43%, respectively) achieving an overall survival of 12 mo[98] sorafenib combined with TACE is currently under clinical investigation[99].

Inhibition of autophagy with specific pharmacological inhibitors such as chloroquine, produced more pronounced tumor suppression in HCC *in vivo* and *in vitro*[24]. Thus, the combination of sorafenib and autophagy modulation is a promising therapeutic option in unresectable HCC[91]. Moreover, up-regulation of HIF-2α induced by sorafenib contributes to drug resistance by activating the TGF-α/EGFR pathway in HCC cells[100], overcoming the negative modulation exerted by HIF-1α (Figure 2).

Bevacizumab: The FDA also approved a recombinant monoclonal anti-VEGF antibody to be used in advanced breast, non-squamous non-small cell lung and colorectal cancers in combination with chemotherapy. In Siegel's Phase Ⅱ study it was shown that bevacizumab as a single agent was effective, showing a 13% rate of objective tumor response and a median overall survival of 12.4 mo in patients suffering from non-metastasized HCC unable to be resected[101]. However, its use was

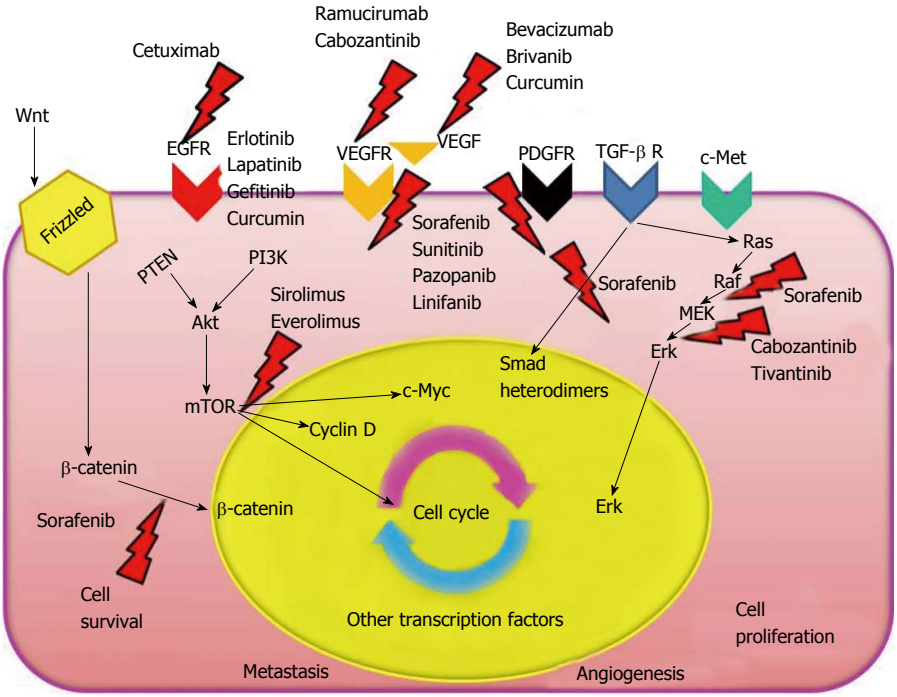

Figure 1 Hepatocellular carcinoma pathogenetic pathways. Main molecular targets of the major anti-tumoral drugs are indicated. VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptors; TGF-β: Transforming growth factor-β; Erk: Extracellular signal-regulated kinase; EGFR: Epidermal growth factor receptors; PDGFR: Platelet-derived growth factor receptors; TGF-β R: TGF-β receptor; RAS: Rat Sarcoma; RAF: Rapidly accelerated fibrosarcoma; MEK: Mitogen-activated protein kinase kinase; c-Myc: Myelocytomatosis cellular oncogene; PTEN: Phosphatase and tensin homology; PI3K: Phosphoinositide 3-kinase.

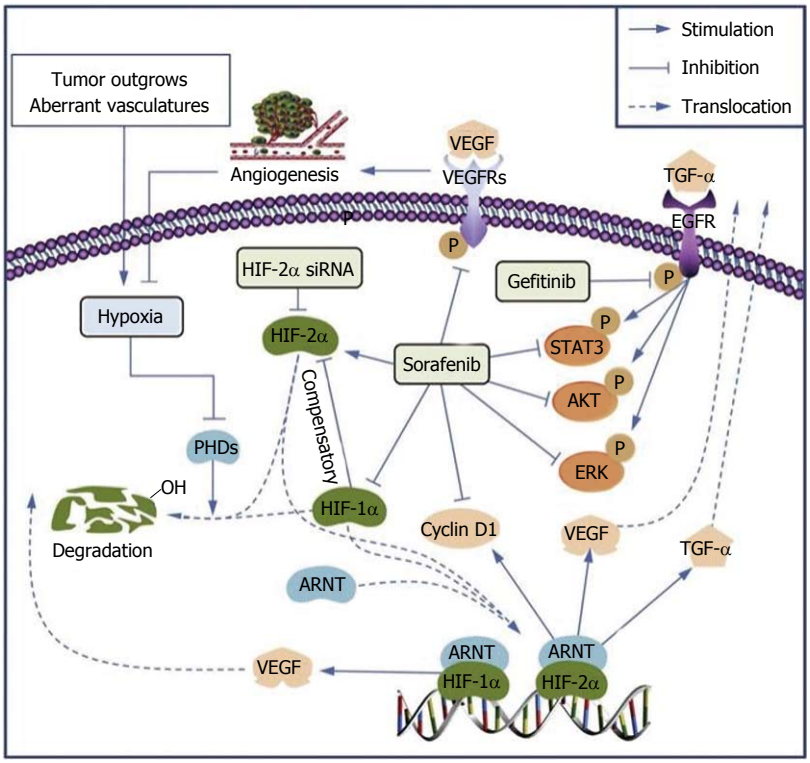

Figure 2 Proposed mechanisms by which upregulation of hypoxia-inducible factor-2α induced by sorafenib contributes to the resistance by activating the transforming growth factor-α/epidermal growth factor receptors pathway in hepatocellular carcinoma cells. ARNT: Aryl hydrocarbon receptor nuclear translocator; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; HIF-1α: Hypoxia-inducible factor-1α; HIF-2α: Hypoxia-inducible factor-2α; PHD: Prolyl hydroxylase; STAT3: Signal transducer and activator of transcription 3; TGF-α: Transforming growth factor-α; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor. Reprinted from ref. [100] with permission from Elsevier.

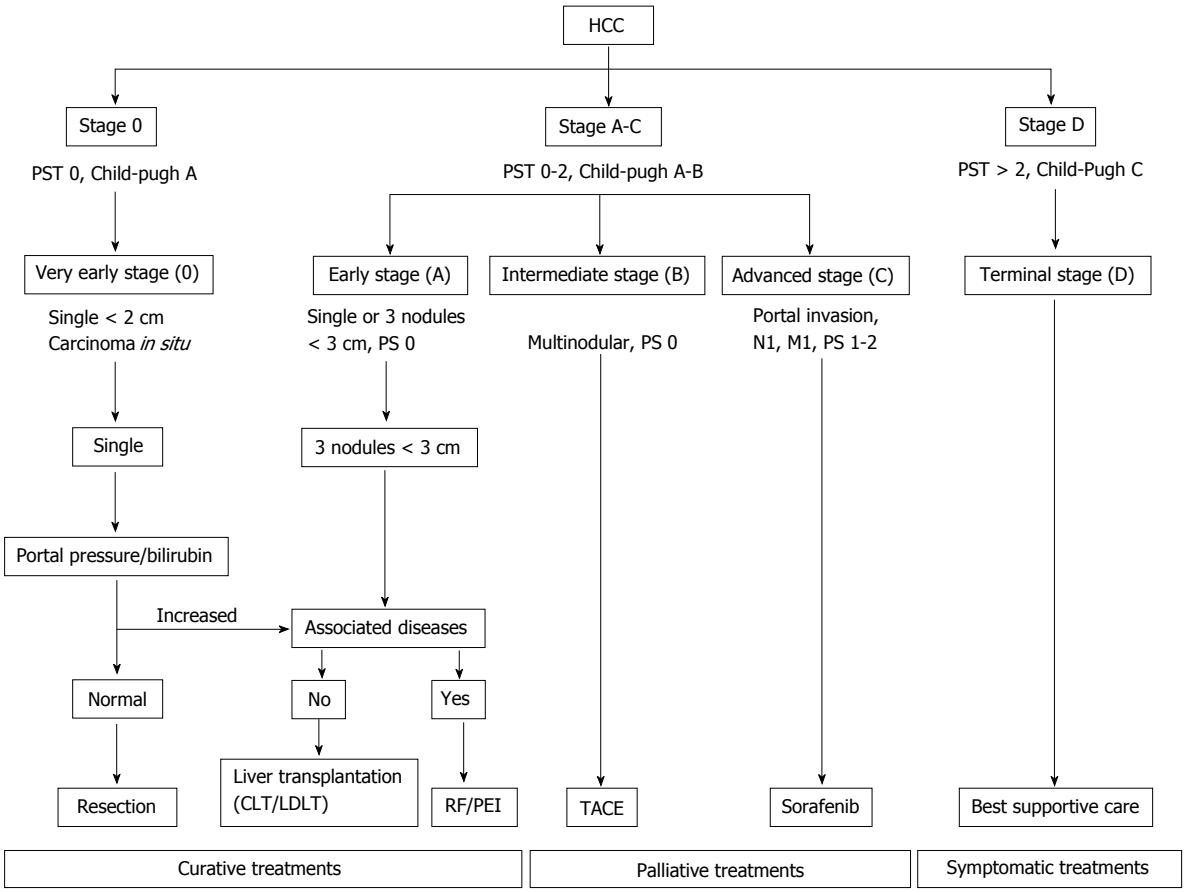

Figure 3 Barcelona Clinic Liver Cancer staging system and treatment strategy (2011). HCC: Hepatocellular carcinoma; CLT: Cadaveric liver tansplantation; LDLT: Living donor transplantation; RF: Radiofrequency; PEI: Percutaneous ethanol injection; TACE: Transarterial chemoembolization; PST: Performance status test; PS: Performance status.

associated with considerable bleeding in 11% of cases and thrombosis in 6% of the patients, therefore it is prone to drug-related complications. Patients receiving the combination of bevacizumab with gemcitabine-oxaliplatin (GEMOX)[102] or capecitabine-oxaliplatin[103] responded in up to 20% of cases, however the overall survival rate was 9.6 mo. The administration of these drugs also resulted in considerable toxicity associated with the treatment, causing leukopenia, transaminitis, hypertension and fatigue. To summarize, it has been proved in previous clinical studies that bevacizumab was relatively effective in HCC; therefore, since some severe drug-related complications such as thrombosis, hemorrhage and even death have been reported, further studies are needed to clarify its efficacy and safety.

Sunitinib: Sunitinib is an oral multi-targeted TKI that inhibits VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β, c-kit, Flt3, and rearranged during transfection (RET) kinases. Sunitinib has been approved by the FDA to treat renal adenocarcinoma and gastrointestinal stromal tumors. In a phase Ⅱ clinical trial to analize the efficacy of sunitinib as monotherapeutic agent in advanced stages of HCC, it was reported that this drug shows modest antitumor activity with a very low rate response and a median overall survival between 8 and 9.8 mo[104]. It is worth mentioning that sunitinib had been negative for its primary overall survival endpoint and proved to have greater toxicity than sorafenib. Thus, based on these results, the use of sunitinib as first line treatment in advanced HCC was not supported, being sorafenib monotherapy the standard of care in these cases. However, when sorafenib fails, sunitinib might be chosen as second-line treatment[105].

Pazopanib: Pazopanib, a synthetic indazolyl-pyrimidine is an oral angiogenesis inhibitor, recently approved by FDA for the treatment of patients with renal cell cancer[106]. This novel multitargeted TKI acts inhibiting VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β and c-Kit. It is still being assessed as potential treatment for HCC[107]. Phase Ⅰ clinical studies established that since the toxicity level of pazopanib is acceptable, it might be a possible option for advanced HCC treatment[108].

Brivanib: Brivanib, a TKI, is the first oral selective dual inhibitor of VEGF and FGF signaling pathways, that has showed encouraging antitumor activity in preclinical and phase Ⅰ studies. In a phase Ⅱ open-label study of brivanib as first-line therapy in patients with unresectable, locally advanced, or metastatic HCC,

brivanib demonstrated promising antitumor activity with a median overall survival of 10 mo. Furthermore, this anti-angiogenic drug showed a manageable safety profile, being fatigue, diarrhea, anorexia, vomiting, hypertension, constipation and nausea the most frequent reported adverse events[109]. In another phase Ⅱ, open label study of brivanib, which this time was assessed as a second-line drug treatment for HCC patients not responding to the administration of anti-angiogenic therapy, showed encouraging results in this group of patients for whom no approved treatment is currently available[110]. Recently, a multinational, randomized, double-blind, phase Ⅲ trial compared brivanib with sorafenib as first-line treatment for HCC. Results demonstrated that both drugs displayed a similar anti-tumor activity, based on secondary efficacy end points, although brivanib was less well-tolerated than sorafenib[111]. Finally, brivanib, as an adjuvant therapy to TACE in patients with HCC, failed to improve overall survival[112].

Axitinib: Axitinib is another multi-targeted TKI with activity against VEGFR-1, VEGFR-2, VEGFR-3, VEGFR-4, PDGFR and c-Kit. This drug has shown promising results for renal cell cancer and thyroid cancer. Phase Ⅱ/Ⅲ trials assessing this medication for HCC are still being planned[113,114].

Linifanib: Linifanib is an innovative and potent selective inhibitor aimed at inhibiting angiogenesis, tumor growth and metastasis. A phase Ⅱ clinical trial in patients undergoing the advanced stages of HCC showed that linifanib is clinically active for unresectable HCC with an acceptable safety profile. The median overall survival was 9.7 mo. A phase Ⅲ trial for comparing linifanib with sorafenib is currently under way[115].

TSU-68: TSU-68 is an oral compound which inhibits VEGFR, PDGFR and FGFR. A phase Ⅰ/Ⅱ clinical trial in patients with advanced HCC has shown promising efficacy with a median overall survival of 13.1 mo and a high safety profile even in patients who had been heavily pre-treated[116].

Foretinib: Foretinib is a novel receptor TKI that targets VEGFR-2 and c-Met that demonstrated significant anti-tumor activities in preclinical models of HCC. At present, phase Ⅰ and Ⅱ clinical trials are under way[117].

Dovitinib

Dovitinib potently inhibits receptor TKs, showing specificity for VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-β, FGFR-1, FGFR-2, FGFR-3, Flt-3 and c-Kit. Several phase Ⅰ/Ⅱ studies have been carried out to assess the efficacy, pharmacokinetics, pharmacodynamics and safety profile of this drug. In xenografts models of human HCC it was reported that this compound reduced angiogenesis and cell proliferation, inducing apoptosis of tumor cells[118].

Ramucirumab: Ramucirumab is a recombinant human monoclonal antibody that binds to the extracellular domain of VEGFR-2. A phase Ⅱ study of ramucirumab as first-line monotherapy in patients with advanced HCC showed that this monoclonal antibody has been well tolerated and has conferred a moderate disease control[119].

Inhibitors of the human EGFR

It has been broadly accepted that the role of growth factors and their receptors is crucial for several cancers to develop and progress, HCC among them[120]. In fact, inhibitors of the human EGFR-1 (HER-1) is usually overexpressed in chronic hepatitis, fibrosis, cirrhosis and HCC cases[121]. EGFR/HER-1 ligands such as epidermal growth factor (EGF), hepatocyte growth factor, TGF-α, TGF-β, and insulin-like growth factors (IGF) were shown to be mitogenic for hepatocytes, therefore contributing significantly to hepatocarcinogenesis[122]. Furthermore, hypomethylation of the EGFR/HER-1 gene was also described to be associated with the development of HCC[123]. Since drugs targeting EGFR have proved to increase survival rates in patients in whom cancer has metastized to the lungs and pancreas[124,125], there is a logic for analyzing the effectiveness of this novel class of compounds in patients with unresectable HCC. Regarding HER-2/NEU and its significance in HCC, the international literature shows conflicting data. Some studies have demonstrated that HER-2/NEU is rarely overexpressed in HCC and might not play a role in this kind of cancer[126], whereas the opposite has been shown by other studies[127].

Erlotinib: Erlotinib is an orally active selective inhibitor of the EGFR/HER-1-related thymidine kinase (TK) enzyme that inhibits its autophosphorylation process[128]. Erlotinib blocks the EGF-dependent growth of tumoral cells at submicromolar concentrations and arrests cell-cycle progression in the G_1 phase[129]. FDA has approved this selective inhibitor of the EGFR/HER-1-related TK enzyme for treating advanced lung and pancreatic cancers. A phase Ⅱ study of the single-agent erlotinib in patients with unresectable HCC reported that tolerance to this drug was good but had a modest benefit in controlling HCC, which was evidenced as a 13-mo discrete prolonged overall survival[130]. However, another phase Ⅱ study demonstrated a median overall survival of 10.75 mo[129]. Interestingly, it was also demonstrated in this study that overall survival between the group of patients that showed high EGFR/HER-1 expression and those with low EGFR/HER-1 expression was not significantly different[129]. This means that there was no correlation with EGFR/HER-1 expression and overall survival. The toxicity to erlotinib was mainly cutaneous and similar in profile to other drugs that target the EGFR/HER-1-related TK activity. A phase Ⅱ, single-arm, open-label trial of erlotinib in combination with bevacizumab obtained encouraging results and a favorable toxicity profile. The

best response showed minor tumor shrinkage, decreased tumor vascularity or increased necrosis. Adverse effects consisted on transaminases elevation, hyperkalemia, diarrhea, proteinuria, gastrointestinal bleed, fatigue and hypertension[130]. Further studies with erlotinib as a single agent or in combination with other agents are needed.

Lapatinib: Lapatinib is a dual inhibitor of EGFR/HER-1 and HER-2/NEU by docking into the ATP-binding site of the two receptors, thus inhibiting their autophosphorylation and the corresponding downstream signaling with consequent down-regulation of MAPK, AKT and p70S6 kinase, inhibiting tumor growth[131]. Clinical studies using lapatinib demonstrated that this drug was well-tolerated and displayed anti-tumor activity in heavily pretreated patients with several solid tumors. The most common adverse effects reported were rash and diarrhea. Lapatinib was recently approved by the FDA for use in metastatic breast cancer[132]. A phase II study of single agent lapatinib in patients with advanced HCC demonstrated that this drug was well-tolerated but revealed a minimal anti-tumoral activity based on the lack of objective responses and an overall survival of 12.6 mo[133]. The use of single-agent lapatinib in advanced HCC was tested in another phase Ⅱ study which revealed a lower median overall survival of 6.2 mo. Authors reported that this low median survival might be due to the small sample size. Anyway, they concluded that treatment with lapatinib failed to meet predefined efficacy standards and did not have significant activity on HCC[134].

Gefitinib: Gefitinib, an adenosine triphosphate mimetic anilinoquinazoline is an orally active EGFR-TKI that reduces EGF-stimulated tumor cell growth[135]. Results from the Eastern Cooperative Oncology Group's Study E1203 had shown modest activity in advanced HCC with a median overall survival of 6.5 mo[136]. Interestingly, combination of gefitinib and sorafenib has demonstrated synergistic effects to inhibit cell proliferation by promoting apoptosis *in vitro* and tumor growth suppression *in vivo*[99].

Cetuximab: Cetuximab is a chimeric (human and mouse) monoclonal antibody directed against EGFR, approved by the FDA for the treatment of squamous cell carcinoma of the head and neck and metastatic colorectal cancer. In phase Ⅱ clinical studies in patients with advanced and unresectable HCC, the use of cetuximab - as single agent therapy, as well as in combination therapy with GEMOX - demonstrated modest activity[137,138].

Inhibitors of the mTOR pathway

The PI3K/Akt/mTOR signal pathway is crucial in promoting protein synthesis and is implicated in various cellular functions such as proliferation, differentiation, tumorigenesis and apoptosis. In approximately 15%-41% of HCC patients, activation of this signaling pathway has been reported[139]. This event is implicated in metastasis, invasion and poor prognosis[140]. Blocking the mTOR pathway confers anti-cancer, anti-angiogenic and immunosuppressive properties. Preclinical data have shown that mTOR inhibitors were effective in both cell growth and tumor vascularity suppression in HCC cell lines and HCC tumor models[141]. According to this, rapamycin - the naturally occurring inhibitor of mTOR - and a number of recently developed rapamycin-analogues inhibit the growth of cell lines derived from multiple tumor types *in vitro* and tumor models *in vivo*. LY294002 is a PI3-kinase inhibitor that decreased the viability of HCC cells by inhibition of Akt activation. Other Akt inhibitors include wortmannin and inhibitor Ⅷ[142].

In addition, cyclooxygenase-2 (COX-2) has been recently implicated in the pathogenesis of HCC through Akt activation. According to this, the level of COX-2 expression and Akt phosphorylation is positively correlated in cultured HCC cells and human liver cancer tissues[143]. In this regard, Leng *et al*[143] demonstrated that HCC cells treated with the COX-2 inhibitor celecoxib showed significant reduction of Akt phosphorylation and induced apoptosis.

Sirolimus: Sirolimus (Rapamycin) is a macrolide antibiotic and antifungal drug isolated from *Streptomyces hygroscopicus.* Since it has been proved to have both immunosuppressive and antiproliferative effects, it has been regarded as an adjuvant therapy designed to treat cancer[88]. This specific mTOR inhibitor exerts its action in association with its intracellular receptor FKBP-12. Sirolimus may both inhibit rejection in liver transplantation patients and prevent the recurrence of HCC[144].

Everolimus: Everolimus is an oral inhibitor of mTOR. A phase Ⅰ/Ⅱ study carried out in patients with un-resectable or metastatic HCC showed modest anti-tumor activity with a median overall survival of 8.4 mo and a disease control rate of 44%. Everolimus was well tolerated in patients with advanced HCC. The most frequent adverse effects reported were fatigue, hyperglycemia, diarrhea, anemia, leukopenia and lymphopenia, thrombocytopenia, hyponatremia, anorexia, stomatitis and rash[145].

Curcumin

Curcumin is a naturally occurring and biologically active compound extracted from the rhizomes of Curcuma longa. *In vitro*, it was shown that this natural compound was able to induce apoptosis of HCC cell lines. In this regard, Cao *et al*[146] reported that curcumin induced apoptosis in human HepG2 cells through mitochondrial hyperpolarization and damage.

Wang *et al*[147] also demonstrated that in HCC J5 cells, curcumin induced apoptosis *via* Ca^{2+}-regulated mitochondria-dependent pathway.

Furthermore, curcumin has also been shown to

inhibit several angiogenic biomarkers, including VEGF and COX-2 expression[148]. This means that curcumin could be used as a candidate for the combined drug therapy for HCC in the future.

Other drugs

Cediranib blocks VEGFR, PDGFR and c-KIT. Similarly, BIBF-1120 targets VEGFR, PDGFR and FGFR; E-7080 inhibits VEGFR, FGFR, PDGFR and c-KIT; XL-184 targets VEGFR-2, MET and RET; vandetanib targets VEGFR and EGFR; BIIB-022, AVE1642 and cixutumumab inhibits IGF-1R; CT-011 inhibits PD-1/2; MEDI-575 inhibits PDGFR; BAY73-4506 inhibits VEGFR, PDGFR, FGFR-1, Raf, RET, and c-KIT; GC33 inhibits Glypican-3, which is highly expressed in HCC; salirasib blocks ras and mTOR activation, and finally, PI-88, which targets heparanases as well as sulfatases is now in Phase Ⅲ clinical trials for the treatment of HCC[149].

DRUG DELIVERY SYSTEMS AND TARGETING STRATEGIES TO THE LIVER PARENCHYMA OF ANTI-TUMORAL COMPOUNDS

As described above, since HCC is asymptomatic at an early stage, most cases are often diagnosed when the disease has advanced and most of the potentially effective treatments such as surgical resection, orthotopic liver transplantation and local ablation demonstrate poor efficacy. In these advanced stages, systemic treatments are commonly used. However, the efficacy of the current anti-tumoral drugs used in advanced HCC treatment cause significant toxicity in other non-target organs, therefore influencing on the patients' willingness to comply with and adhere to these treatments. Therefore, the effectiveness of treatments using anti-tumoral drugs for advanced HCC significantly depends on their pharmacokinetics, particularly in, their distribution and accumulation in the liver. An interesting approach to enhance anti-HCC drug action is to direct them into the liver by drug delivery systems (DDS) that recognize hepatocyte surface receptors. Thus, those strategies targeting the drug to its site of action, - in this case, the liver - may cause an increase in drug efficacy and a decrease in possible collateral effects in other non-target organs[150]. Indeed, several studies discussed below, have attempted to target anti-tumoral drugs to the liver for the treatment of advanced HCC, using novel formulations including liposomes, polymeric micelles, polymeric nanoparticles, dendrimers, nanocapsules and microspheres.

As mentioned above, since HCC originates from liver parenchyma cells, it is desirable to deliver drugs selectively to hepatocytes. To this end, asialoglycoprotein receptors (ASGPRs) are usually used as liver target due to their high expression on the surface of hepatocytes and in HCC-derived cell lines. ASGPRs specifically recognize ligands with terminal galactose or N-acetylgalactosamine residues, and endocytose them through an intracellular degradation process. The use of their natural ligand (asialofetuin) or synthetic ligands with galactosylated or lactosylated residues, has achieved significant targeting efficacy to the liver[22,151]. In this regard, Xu *et al*[152] synthesized a lactobionic acid conjugate of dioleoylphosphatidyl ethanolamine (Lac-DOPE) for targeting of solid lipid docetaxel-loaded nanoparticles. Following this approach, other works used the synthesis of lactosylated liposomes for targeted delivery of doxorubicin to HCC as a possible strategy to treat the disease[153]. Other groups used a cleavable poly(ethylene glycol) (PEG)-lipid [methoxypolyethyleneglycol 2000-cholesteryl hemisuccinate, PEG (2000)-CHEMS] linked *via* an ester bond and a galactosylated lipid {(5-cholesten-3 beta-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate, CHS-ED-LA} to modify doxorubicin. Results demonstrated that modification of liposomes with PEG (2000)-CHEMS and CHS-ED-LA turned out to be a potentially advantageous strategy for HCC therapy[154].

Polymeric micelles also constitute a safe and effective delivery system. Bei *et al*[155] designed three novel polymers named palmitoyl-trimethyl-chitosan (TPCS)-1, TPCS-2 and lac-TPCS-2, that hold a great potential in the development of nanomedicine for the therapy of liver tumors, especially lac-TPCS-2. On the other hand, polymeric micelles self-assembled from amphiphilic block copolymers of PEGs and poly(D,L-lactide) (PDLLA) with folate as a targeting ligand attached to the distal ends of the PEG (Folate-PEG-PDLLA) were prepared. Such Folate polymeric micelle was demonstrated to selectively deliver the anti-tumoral drug doxorubicin to HCC cells, since they also overexpress surface receptors for folate[156]. Cuestas *et al*[157] reported the synthesis of galactosylated poly(ethylene oxide)-poly(propylene oxide) block copolymers, proposed for potential targeting to the liver.

The nanoparticle DDS, which uses polymeric material from natural or synthetic sources as a carrier in drug delivery to targeted tissues, has remarkable targeting, slow-release and biodegradable properties that also makes it a promising therapeutical option. Regarding this, Cheng *et al*[158] reported the use of chitosan and the hepatoma cell-specific binding molecule glycyrrhetinic acid to synthesize glycyrrhetinic acid-modified chitosan (GA-CTS). The anti-tumoral drug 5-fluorouracil (5-FU) was conjugated onto this newly synthesized nano-material, thus forming the corresponding GA-CTS/5-FU nanoparticles. Results demonstrated that these nanoparticles accumulated selectively in the liver blocking tumor growth in an orthotopic liver cancer mouse model[158]. Another group reported the preparation of nanoparticles composed of galactosylated chitosan oligosaccharide and adenosine triphosphate for HCC cell-specific uptake[159].

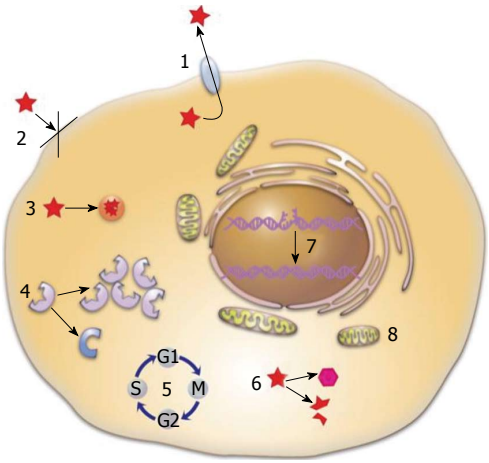

Figure 4 Mechanisms of multidrug resistance in cancer cells. (1) Active drug efflux by drug transporters, such as Pgp, multidrug resistance-associated protein, and breast cancer resistance protein; (2) Loss of cell surface receptors and/or drug transporters or alterations in membrane lipid composition; (3) Compartmentalization of the drug in cellular vesicles; (4) Altered/increased drug targets; (5) Alterations in cell cycle; (6) Increased drug metabolism/enzymatic inactivation; (7) Active damage repair; and (8) Inhibition of apoptosis. Reprinted with permission from ref. [181].

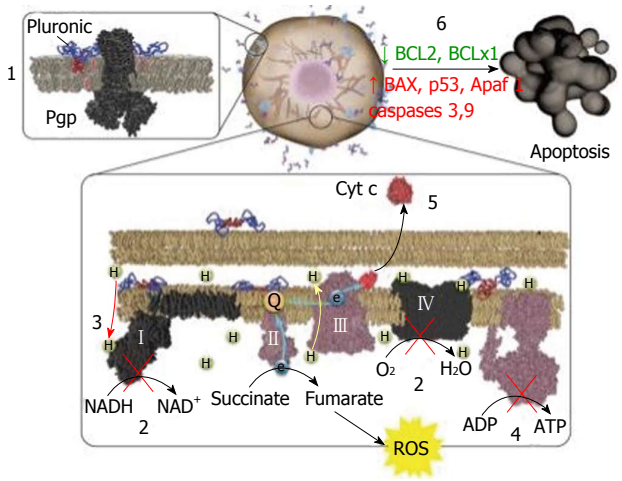

Figure 5 Summary of Pluronic effects in cancer cells. Pluronic binding with plasma membrane of multidrug resistance (MDR) cancer cells (1) induces membrane fluidization, disruption of membrane microdomains, and inhibition of drug efflux transporters' activity (Pgp shown as an example). Pluronic also reaches mitochondria where it (2, 3) inhibits complexes I and IV of mitochondria respiratory chain and (3) induces inner mitochondrial membrane depolarization. This (4) results in ATP depletion and (5) promotes cytochrome c release and ROS generation in MDR cells. Altogether, the MDR cells respond to a Dox/Pluronic combination by (6) an increased proapoptotic signaling and decreased antiapoptotic defense. Reprinted with permission from ref. [181]. ROS: Reactive oxygen species.

Poly(amidoamine) dendrimers are branched water-soluble polymers defined by consecutive generation numbers (Gn) indicating a parallel increase in size, molecular weight, and number of surface groups available for conjugation of bioactive agents. In this connection, Medina *et al*[160] targeted hepatic cancer cells with pegylated dendrimers displaying N-acetylglucosamine and SP94 peptide ligands. Lactosylated dendrimers were also used as a liver-targeting DDS[161].

An alternative strategy is to use microspheres[162] and nanocapsules[163]. For example, Kang *et al*[163] reported an innovative hepatoma-targeted gene delivery system which was prepared with a combination of a human liver cell-specific bionanocapsule and a tumor cell-specific gene regulation polymer that responded to hyperactivated protein kinase C in liver cells.

OVERCOMING MDR DUE TO OVEREXPRESSION OF ABC PROTEINS

As mentioned above, HCC is a molecular complex tumor with high intrinsic MDR (Figure 4). An increased cellular extrusion of chemotherapeutic drugs due to over-expression of MDR mediating ABC transmembrane proteins leads to a reduced effectiveness with response rates below 10%[164]. Actually, there are 49 known ABC transporters divided into 7 distinct subfamilies of proteins[165]. The most studied proteins were P-gp, MRP1 and BCRP.

A classic approach for overcoming MDR involves the use of low molecular mass ABC inhibitors co-administered with the pharmacotherapeutic agent, such as verapamil and valspodar. However, limited success has been achieved so far with these chemosensitizing agents that inhibit these efflux proteins. New advances to overcome MDR consists on the use of block copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) also known as poloxamers or Pluronics® and poloxamines or Tetronics® (Figure 5). Poloxamers consist of a central hydrophobic PPO molecule flanked on both sides by two hydrophilic chains of PEO. A slightly different structure is exhibited by poloxamines, which are tetrafunctional block co-polymers with four PEO-PPO blocks joined together by a central ethylene diamine bridge[166]. These surfactants have found a wide range of pharmaceutical, biomedical, clinical and nanotechnological applications. Some of them, such as Pluronic P85 were shown to sensitize MDR tumors refractory to many chemotherapeutic agents[167]. In addition, Cuestas *et al*[168] explored the prospective capacity of PEO-PPOs to overcome MDR in HCC-derived cell lines. Results demonstrated that there is multiple-inhibitory activity of poloxamines on P-gp, MRP1 and BCRP in two human hepatoma cell lines, Huh7 and HepG2[168]. Copolymers of intermediate to high hydrophobicity (*e.g.*, Tetronic® 304, 904 and 1301) inhibited P-gp and BCRP but not MRP1 in both cell lines[168]. This activity was related to both copolymer concentration and hydrophobicity. Conversely, there was no evidence of an inhibitory effect in Tetronic® 1107, a more hydrophilic counterpart[168]. Furthermore, the work by Cuestas *et al*[169], also analyzed for the first time the effect of branched PEO-PPOs on the expression of mRNA encoding for the main ABCs in a human hepatoma cell line and gave evidence of the down-regulation of mRNA levels corresponding to *p-gp* and *bcrp*[169].

All these technological strategies constitute a

positive starting point that will require further research to evaluate their potential efficacy in treating HCC.

FUTURE PERSPECTIVES

HCC remains a disease with poor prognosis despite recent advances in the knowledge of both its patho-physiology and therapy.

Since aberrant epigenetic deregulation events such as hyper-methylation (silencing) of tumor suppressor genes, hypo-methylation (activating) of proto-oncogenes, as well as abnormal expression of histone modifying enzymes and non-coding RNAs (microRNAs and long non-coding RNAs) have been associated with genetic instability and altered gene expression, this landscape should be analyzed as a complex network of crosstalk and cooperation (synergism) leading to HCC. Bearing in mind the potential reversibility of epigenetic changes, plausible next generation treatments might also consider the use of drugs that modify DNA methylation and/or those that promote histone modifications (such as DNA methyl transferases - or histone deacetylases - inhibitors to activate tumor suppressors), either as mono- or combined-treatment, together with conventional chemotherapeutic agents. Moreover, encouraging results obtained with the up-regulation of some anti-tumoral miRNAs (such as adenoviral vectored-miR-122[170], and adeno-associated-miRNA-26a[171], respectively) allow to consider this strategy as a candidate for the treatment of HCC[172].

Although only modest results have been thus far obtained with immunotherapy[173], a plausible use of immune-stimulating monoclonal antibodies (such as anti-CTLA-4/anti-programmed death ligand-1) together with inhibitors of the immune regulatory (suppressor) mechanisms exerted by Tregs and/or - as already demonstrated - locoregional conventional treatments intended to increase immunity and unmask TAA-specific T cell responses[174] might be envisaged as a next approach for HCC treatment. Moreover, the recent development of the calixarene compound OTX008[175] as an inhibitor of galectin 1[176] - a key regulator of extracellular matrix interactions, cell proliferation, invasion, angiogenesis and escape from the immune response by favoring the expansion of Tregs and the differentiation of tolerogenic dendritic cells, as well as by limiting T cell viability, and maintaining T cell anergy - promises a future view of tumor halting by selectively counteracting tumor immune escape[177]. A phase Ⅰ, first-in-man - study of OTX008 treatment to patients with advanced solid tumors is ongoing (Clinical trial NCT01724320). Treating patients suffering from advanced HCC and overcoming MDR still remain an important challenge. Since an association between miR-122 down-regulation and MDR has been established, and an *in vitro* therapeutic effect on MDR of HCC cell lines with adenovirus-vector miR-122 has been reported[178], it seems plausible that miR-122 treatment in human HCC might be worth to evaluate.

In this regard, a very recent report using cabozantinib (a VEGFR and MET inhibitor) demonstrated that patients with HCC with high level expression of phosphorylated-MET (activated by the hepatocyte growth factor) are associated with resistance to adjuvant sorafenib treatment. The dual blockade of VEGFR2 and MET by cabozantinib leads to significant anti-tumor activities in HCC by suppressing both tumor growth and metastasis[179]. Therefore, the use of this drug might help to overcome to some extent the resistance to sorafenib. Likewise, the oral use of tivantinib in a Phase Ⅱ placebo-control study demonstrated promising results in patients with HCC with high level of MET, which might be a second choice therapeutic in treating patients suffering from advanced HCC[180].

The challenge of the heterogeneous nature of HCC - and the corresponding biomarkers - needs the expedited discovery of novel chemotherapeutic and immunotherapeutic agents, in order to have multiple choices for therapy which can then be used alone, in combination and/or sequentially, as wellas the design of technological or pharmaceutical strategies for chemosensitizing HCC cells. Furthermore, despite the availability of several drugs for the treatment of advanced HCC, implementing liver-targeting DDS strategies in general and nanotechnologies in particular may result in future tools to: (1) enhance the efficacy and application of approved drugs to overcome and delay cellular resistance development; (2) limit systemic side effects by promoting selective accumulation in the liver; and (3) increase patient adherence to treatment by reducing administration frequency.

Finally, there are still many unknown technological drawbacks to be faced in the discovery and assessment of new drug candidates, which will demand the design of more suitable drug carriers to deal with their preliminary preclinical assessment.

ACKNOWLEDGMENTS

The authors are deeply grateful to Mrs. Victoria Illas who performed a superb task to improve the readability of the text.

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