Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
MEDICINA MOLECOLARE E FARMACOLOGIA
CICLO XXXV

COORDINATORE Prof. Francesco Di Virgilio

miR-199a-3p enhances the anti-tumor activity of Palbociclib in *in vitro* and *in vivo* liver cancer model

Settore Scientifico Disciplinare MED/03

Dottoranda
Dott. Akanksha Samuel

Tutor
Prof. Massimo Negrini

Anni 2019/2022

Table of Contents

Table of Figures

ABSTRACT (English)

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide. Despite progress, HCC treatment remains a challenge with limited effective therapies. This study investigates the potential of combining Palbociclib, a CDK4/6 inhibitor with miR-199a-3p mimics to improve HCC treatment. While Palbociclib has shown promise in inhibiting HCC growth, it also activates AKT signaling, which limits its effectiveness. However, miR-199a-3p, a tumor suppressor miRNA, may help overcome this limitation. To evaluate the safety and effectiveness of this combined therapy we conducted experiments both, *in vitro* and *in vivo*.

Methods:

AKT inhibitors, MK-2206 or microRNA-199a-3p, were studied in combination with Palbociclib in the TG221 HCC transgenic mouse model and HCC cell lines. In in vitro studies, cell viability and apoptosis analyses were carried out. Signal transduction pathways (AKT, RB1, FOXM1) were evaluated by Western blots. In in vivo studies, the growth and number of liver tumors and weight changes during therapies were evaluated.

Results:

The combination of Palbociclib and MK-2206 proved to be highly effective but toxic to mice, as shown by dramatic weight loss. The combination of miR-199a-3p with Palbociclib was instead well tolerated and highly effective in inhibiting HCC progression in *in vitro* and *in vivo* models. At the molecular level, the combined treatment inhibits the phosphorylation of RB1 and FOXM1 (attributed to Palbociclib action) and AKT (attributed to miR-199a-3p). This suggests that the combination of Palbociclib and miR-199a-3p mimics may overcome the limitations of Palbociclib as a single agent and improve its antitumor activity.

Conclusions:

This study provides preclinical evidence for the combination of miR-199a-3p with Palbociclib as an effective anti-HCC treatment. The combination was well tolerated and

produced a significant reduction in the number and size of tumors compared to Palbociclib or miR-199a-3p mimics used as single treatments. Future studies should evaluate the safety and efficacy of this combination in clinical trials for the treatment of advanced HCC.

Keywords: Hepatocellular carcinoma (HCC); Palbociclib; miR-199a-3p; MK-2206; anti-tumor therapy.

ABSTRACT (Italian)

Il carcinoma epatocellulare (HCC) è una delle più frequenti cause di morte correlata a cancro al mondo. Sebbene ci siano stati progressi significativi nel trattamento del HCC, rimane un tumore maligno con prognosi infausta e terapie limitate. Palbociclib è un inibitore della chinasi ciclino-dipendente 4/6 (CDK4/6), che ha mostrato risultati promettenti nel trattamento di diversi tipi di cancro, in particolare il cancro al seno. In studi preclinici, palbociclib ha mostrato risultati promettenti nell'inibire la crescita del HCC prevenendo la fosforilazione mediata da CDK4/6 di RB1, una proteina oncosoppressoria. Tuttavia, palbociclib attiva anche la via di AKT, che può potenzialmente limitare la sua attività antitumorale. Nel presente studio, abbiamo studiato l'effetto di palbociclib come trattamento in combinazione con inibitori PI3K/AKT/mTOR per migliorare la sua attività antitumorale in modelli di HCC in vitro e in vivo.

Metodi:

Gli inibitori di AKT, MK-2206 o il microRNA-199a-3p, sono stati studiati in combinazione con palbociclib nel modello di topo transgenico HCC TG221 e in linee cellulari di HCC. Negli studi in vitro, sono state svolte analisi di vitalità cellulare ed apoptosi. Vie di trasduzione del segnale (AKT, RB1, FOXM1) sono state valutate mediante Western blots. Negli studi in vivo sono stati valutati crescita e numero di tumori epatici e cambi di peso durante le terapie.

Risultati:

La combinazione palbociclib e MK-2206 si è dimostrata altamente efficace ma tossica per i topi, come mostrato da una drastica perdita di peso. La combinazione di miR-199a-3p con palbociclib è stata ben tollerata e altamente efficace nell'inibire la progressione del HCC nei modelli in vitro e in vivo. A livello molecolare, il trattamento combinato inibisce la fosforilazione di RB1 e FOXM1 (dovuta a palbociclib) e AKT (dovuta a miR-199a-3p). Ciò suggerisce che la combinazione di palbociclib e miR-199a-3p mimics possa superare i limiti di palbociclib come singolo agente e migliorarne la sua attività antitumorale.

Conclusioni:

Questo studio fornisce un'evidenza preclinica della combinazione di miR-199a-3p con palbociclib come trattamento anti-HCC efficace. La combinazione è stata ben tollerata e ha prodotto una significativa riduzione del numero e delle dimensioni dei tumori rispetto a palbociclib o miR-199a-3p mimics usati come trattamenti singoli. Studi futuri dovrebbero valutare la sicurezza e l'efficacia di questa combinazione in studi clinici per il trattamento del HCC avanzato.

Parole chiave: carcinoma epatocellulare (HCC); Palbociclib; miR-199a-3p; MK-2206; terapia antitumorale.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer and the third most common cause of cancer-related deaths worldwide (Subramaniam, Kelley, and Venook 2013). HCC is likely found in people with cirrhosis, which is caused by conditions like chronic hepatitis B or C, alcohol abuse, or nonalcoholic fatty liver disease (NAFLD) (Global Burden of Disease Liver Cancer Collaboration et al. 2017). Other risk factors include chronic liver disease, exposure to certain chemicals, such as aflatoxins (toxins produced by mold), diabetes, obesity, and hereditary hemochromatosis. Symptoms of HCC include abdominal pain, weight loss, jaundice (yellowing of the skin and eyes) (Sun and Sarna 2008), weakness, fatigue, and loss of appetite. However, these symptoms may not appear until the cancer is advanced and may also be caused by other liver diseases. Hence, HCC can be difficult to diagnose in its early stages, as symptoms may not appear until the cancer has progressed. Once HCC has developed, it can grow and spread rapidly, invading nearby blood vessels, and spreading to other parts of the liver forming intrahepatic metastases or, at some time to other organs. As HCC progresses, it can cause life-threatening complications such as liver failure or the spread of cancer to other parts of the body.

PATHOGENESIS AND RISK FACTORS

The cell of origin for HCC is still the subject of debate since there are several possibilities, including liver stem cells, transit amplifying populations, or mature hepatocytes (Schneller and Angel 2019) (Sia et al. 2017). The presence of liver stem cells is still being debated and mature hepatocytes have been shown to retain significant proliferative potential in response to injury (Holczbauer, Wangensteen, and Shin 2022). Some studies support the idea that HCC originates from transformed mature hepatocytes (Mu et al. 2015), while others suggest that liver stem cells could be the source (Wu and Chen 2006). The mechanism or pathophysiology of HCC involves a complex interplay of genetic, environmental, and lifestyle factors. Chronic liver damage

is a major risk factor for the development of HCC. The accumulation of damage to liver cells over time can lead to the development of precancerous lesions, which can then progress to HCC if left untreated.

Gene mutations play an important role in the development and progression of cancer. They can lead to the activation of oncogenic pathways, altered cell cycle control, and decreased function of tumor suppressor genes. The identification of cancer-causing genes in HCC has become possible with high-throughput next-generation sequencing. The most common driver gene alterations found in HCC include activation of telomerase through TERT promoter mutations, viral insertions, chromosome translocation, or gene amplification (Guichard et al. 2012) (Schulze et al. 2015). Some of the most frequently identified genetic alterations associated with HCC include mutations in the TP53, CTNNB1, and AKT1 genes and changes in gene expression and amplification of oncogenes. Other genetic changes that have been linked to HCC include alterations in the tumor suppressor genes APC, SMAD4, and CDKN2A, as well as changes in the epigenetic regulation of gene expression.

The pathophysiology of HCC is indeed a complex and multistep process that involves multiple factors and mechanisms. Chronic liver damage and inflammation, as well as exposure to environmental factors like toxins, viruses, and chemicals, can all contribute to the development of HCC. The interaction between viral and non-viral risk factors, changes in the cellular microenvironment and immune cells, and the severity of underlying chronic liver disease all play critical roles in the development of HCC, being involved in every stage of malignant transformation. Various research has analyzed the primary oncogenic drivers and signaling pathways that lead to the initiation, development, and progression of HCC (Llovet et al. 2016).

Chronic hepatitis B virus (HBV) infection is a major risk factor for the development of HCC. Chronic HBV infection can cause genetic alterations in hepatocytes through viral DNA, leading to cellular proliferation and replication errors that produce premalignant cells. The viral-induced chronic inflammation and low activity of intrahepatic natural killer cells also contribute to the development of HCC. HBV infection is also associated with cirrhosis in many cases. Gender is also a factor, as there is an association between

high testosterone levels and HCC in early tumors (Yip et al. 2020). HBV vaccination has been effective in reducing HBV infection, as well as the risk of HCC development. Therefore, vaccination against HBV is a critical step in reducing the incidence of HCC associated with chronic HBV infection (Kaplan and Reddy 2003).

Likewise, *Hepatitis C virus (HCV)* infection has been recognized as a significant risk factor for the development of HCC. Studies have demonstrated a direct relationship between HCC incidence and advanced stages of hepatic fibrosis in chronic active hepatitis caused by HCV (Zein et al. 1996). The HCV-related nonspecific inflammatory process that induces hepatocyte proliferation, combined with a rise in alanine-aminotransferase (ALT) levels, can increase a patient's risk of progressing to HCC (Tarao et al. 2002). The HCV core protein can also inhibit apoptosis, which has a direct effect on carcinogenesis. Preventing the development of HCC includes anti-viral therapies for HCV infection.

Co-infection of hepatitis B virus (HBV) and hepatitis C virus (HCV) can increase the risk of developing hepatocellular carcinoma (HCC). An elevated risk is believed to be due to several factors, including enhanced fibrosis (a condition in which the liver becomes stiff and loses its ability to function properly) and increased inflammation (Zampino et al. 2015) (Konstantinou and Deutsch 2015). These factors can result in damage to the liver cells, leading to the development of HCC.

Aflatoxin is a potent liver carcinogen that is produced by certain strains of Aspergillus fungi, such as *Aspergillus flavus* and *A. Parasiticus (Magnussen 2013)*. These fungi commonly grow on crops like peanuts, and they can contaminate food and seed products if the crops are not properly stored. Exposure to aflatoxin can result in DNA damage to liver cells, which can lead to genetic alterations and an increased risk of HCC. The toxic metabolite of aflatoxin, aflatoxin B1-8,9-epoxide, has been shown to cause mutations in the p53 gene, (Bressac et al. 1991). This mutation can lead to the up-regulation of insulin-like growth factor II, a protein that promotes cell growth and survival, and a reduction of apoptosis (programmed cell death), which can contribute to HCC formation (Lee et al. 2000).

Hereditary hemochromatosis (HH) is an inherited disorder that affects the body's ability to regulate iron metabolism. In individuals with HH, excessive iron accumulates in various organs, including the liver, leading to organ damage. The accumulation of iron in the liver can lead to oxidative stress and the formation of free radicals, which can cause lipid peroxidation and damage to liver cells. This can result in cell death and fibrosis (the buildup of scar tissue), leading to cirrhosis and an increased risk of HCC. HH is a significant risk factor for the development of HCC. Studies have shown that patients with HH have a 200-fold increased risk of developing HCC compared to individuals without the condition and in addition, a 1.8-fold increased relative risk of HCC in patients with HH compared to those with non-HH chronic liver disease (Fracanzani 2001).

Alpha-1-antitrypsin (AAT) is a member of the serine proteinase inhibitor (serpin) family, which plays a critical role in regulating various enzymes involved in inflammation, the complement system, blood clotting, and fibrinolysis. Alpha-1-antitrypsin deficiency is a genetic condition that is inherited in an autosomal recessive manner (Potempa, Korzus, and Travis 1994). In individuals with AAT deficiency, the abnormal accumulation of AAT in the endoplasmic reticulum of hepatocytes (liver cells) can lead to dysplasia (abnormal growth) and cirrhosis of the liver. While many variants of AAT deficiency have been described, only two mutants, AAT Siiyama (53Ser->Phe) and AAT Mmalton (52Phe deleted), have been associated with plasma deficiency and hepatic inclusions (abnormal accumulations of AAT in the liver) (Parmar and Lomas 2000) (Mahadeva et al. 1999). These mutations can lead to an increased risk of liver disease, including cirrhosis and HCC.

Wilson's disease is a genetic disorder caused by mutations in the ATP7B gene, which is responsible for regulating the absorption, transport, and excretion of copper in the body. In individuals with Wilson's disease, there is an alteration in the regulation of plasma copper, leading to an accumulation of excess free copper in the body (Loudianos and Gitlin 2000) (Xu et al. 2007). The accumulation of excess copper in the liver can cause cytoplasmic injury and lead to cirrhosis and, in some cases, HCC (Reyes 2008). Therefore, it is important for individuals with Wilson's disease to undergo regular

monitoring and treatment to manage their copper levels and minimize the risk of liver damage and HCC development. This may involve taking medication to reduce the amount of copper absorbed by the body, and chelating agents to remove excess copper from the body. In some cases, a liver transplant may also be necessary.

Genetic alterations have been linked to an increased risk of HCC (Niu, Niu, and Wang 2016) (M. Liu, Jiang, and Guan 2014). Some of the genetic alterations that have been implicated in the development of HCC include mutations in genes involved in regulating cell division and growth, such as TP53, CTNNB1, and APC, as well as changes in genes involved in metabolism, such as HMGCR, HNF1A, and PIK3CA (Tornesello et al. 2013) (Friemel et al. 2016) (Willson et al. 2013).

DIAGNOSIS

Hepatic ultrasound is commonly used for the detection of hepatocellular carcinoma (HCC), but it can be limited in its ability to detect smaller lesions. This is the reason why computed tomography (CT) scans have become used for HCC detection, as they can better detect smaller lesions. Magnetic resonance imaging (MRI) is also a useful tool for HCC imaging diagnostics because it can differentiate between regenerative nodules and early HCC through T1-sequencing (Nadarevic et al. 2019). This ability to differentiate between different types of liver lesions makes MRI a valuable tool in the diagnosis and management of HCC.

Alpha-fetoprotein (AFP) is a protein produced during fetal development and has been used as a marker for the detection of hepatocellular carcinoma (HCC). Elevated levels of AFP are commonly found in patients with HCC; however, it can also be elevated in other gastrointestinal tumors and benign liver diseases like hepatitis and cirrhosis. The sensitivity and specificity of AFP as a marker for HCC depends on the serum levels of the protein, with higher levels (greater than 400 ng/mL) being more indicative of HCC (Masuzaki, Karp, and Omata 2012) (Zhang et al. 2020). However, low levels of AFP do

not exclude the possibility of HCC recurrence, as low AFP-producing metastasis can persist even after HCC has been completely resected.

Carcinoembryonic antigen (CEA) is a protein that is sometimes used as a marker for hepatocellular carcinoma (HCC). CEA testing is not widely used for the diagnosis of HCC, it is also limited by its poor specificity, as elevated CEA levels can be seen in many other conditions, including non-cancerous liver disease.

Liver biopsy is an important tool in the diagnosis of hepatocellular carcinoma (HCC). Liver biopsy can be performed using different methods, including guided or surgical ultrasound or computed tomography (CT). Percutaneous aspiration biopsy, in which a needle is inserted through the skin to obtain a tissue sample, is one of the most used methods for liver biopsy. However, there is a risk of tumor extension in the puncture zone, which is estimated to occur in approximately 1% of cases (Befeler and di Bisceglie 2002). The use of liver biopsy is reserved for cases where the diagnosis is unclear and cannot be definitively established through other means, such as imaging studies or blood tests.

Staging of HCC is important after a diagnosis is made to determine the most appropriate treatment approach and predict mortality. There are several staging systems that have been developed for HCC. *The Barcelona Clinic Liver Cancer (BCLC) system* is the most used staging system that divides HCC patients into four stages: early (A), intermediate (B), advanced (C), and terminal (D). This system considers the clinical significance of HCC in terms of the patient's ability to carry out normal daily activities. Various studies proved that BCLC works best among the current staging systems (PonS, Varela, and Llovet 2005) (Grieco 2005).

TREATMENT

The choice of treatment for HCC depends on several factors, including the stage of the cancer, the patient's overall health status, and the patient's liver function. Patients with early-stage HCC tumors are often the preferred candidates for surgical intervention,

such as resection, transplantation, or local ablation. For intermediate-stage tumors, trans arterial chemoembolization (TACE) is often the first line of treatment. Patients with advanced-stage HCC typically receive systemic therapies. The Barcelona Clinic Liver Cancer (BCLC) staging system is widely used to guide the management of HCC cases. According to the BCLC system, patients with well-preserved liver function (Child-Pugh A) and compensated disease have median survival times of 36 months for early-stage HCC, 16 months for intermediate-stage HCC, and 6 months for advanced-stage HCC (Llovet et al. 1999) (D'Amico et al. 2018). To minimize the risk of collateral liver dysfunction, certain therapies (such as resection and systemic therapies) are typically used only in patients with well-preserved liver function.

Surgical treatment is a type of therapy used for curative management of HCC (hepatocellular carcinoma). It includes two major procedures: *hepatic resection* and *liver transplantation*.

Hepatic resection is a procedure where a portion of the liver is surgically removed, and the remaining liver tissue regenerates over time to compensate for the loss. This procedure is performed in early-stage HCC tumors to achieve complete removal of the tumor with preservation of healthy liver tissue. It is the preferred treatment for patients with HCC (hepatocellular carcinoma) without cirrhosis, but it is associated with high morbidity in patients with non-cirrhotic NAFLD (nonalcoholic fatty liver disease). In patients with cirrhosis, Western guidelines restrict resection to those with a single tumor, well-preserved liver function, absence of clinically relevant portal hypertension, and preserved performance status. The Child-Pugh score, model for end-stage liver disease, and indocyanine green clearance are commonly used tests to assess liver function prior to resection. However, the albumin-bilirubin score (ALBI score) is a newer tool that has been shown to accurately stratify patients for resection with greater precision than the Child-Pugh score. The outcomes of hepatic resection are generally good, with a 5-year survival rate of around 70-80% (Marrero et al. 2018) (Galle et al. 2018). In conclusion, despite advances in surgical techniques and adjuvant therapies, the recurrence of HCC remains a major challenge in the treatment of this disease. The recurrence rate is as high as 70% at 5 years, and it can be either early or late, resulting

from micrometastases or de novo tumors (Roayaie et al. 2013). In cases of high risk for recurrence, pre-emptive liver transplantation may be considered.

Liver transplantation is a procedure where the entire liver is replaced with a healthy liver from a donor. This procedure is usually performed in advanced-stage HCC tumors or in patients with liver failure. Liver transplantation is a surgical procedure that involves removing a damaged or diseased liver and replacing it with a healthy liver from a donor. Liver transplantation may be considered as a treatment option for patients who meet certain criteria, such as having early-stage cancer that is limited to a small portion of the liver and has no evidence of metastasis (spread) to other parts of the body. The success of liver transplantation for HCC depends on several factors, including the stage of the cancer, the overall health of the patient, and the availability of a suitable liver donor. It is important to note that liver transplantation is a complex and major procedure that carries risks and side effects, including the risk of rejection of the new liver by the body's immune system, infection, bleeding, and complications from anesthesia. Patients who undergo liver transplantation also need to take immunosuppressant drugs for the rest of their lives to help prevent rejection of the new liver.

In summary, liver transplantation is an option for patients with cirrhosis and limited tumor burden who meet the Milan criteria (single tumor less than 5 cm or 2-3 tumors less than 3 cm without vascular invasion) (Lingiah et al. 2020). However, the scarcity of organ availability results in long waiting times and increased dropout from the waiting list due to tumor progression. Neoadjuvant therapies such as TACE or ablation have been explored to prevent tumor progression or reduce tumor burden within the Milan criteria. The response to neoadjuvant therapy has been proposed as a criterion for transplantation and a predictor of death after transplantation. The use of marginal donors and living donors has been advocated to expand access to transplantation, but this has been linked with a higher risk of tumor recurrence. No adjuvant treatment has been shown to prevent its recurrence after liver transplantation.

Ablation is a medical procedure used to treat small, early-stage hepatocellular carcinoma (HCC) tumors. Ablation is used in cases where the patient is unsuitable for resection due to liver dysfunction or multiple tumors and has contraindications for liver

transplantation. There are several methods of ablation including chemical, thermal, and electrical. Percutaneous ethanol injection is a seminal technique for local ablation. Currently, *radiofrequency ablation (RFA)* and *microwave ablation (MWA)* are the established thermal technologies.

Radiofrequency ablation (RFA) is a minimally invasive procedure that uses high-frequency electrical currents to heat and destroy cancer cells. The procedure is performed using a needle-like electrode that is inserted into the tumor, and the radiofrequency energy is delivered through the electrode to heat and destroy the cancer cells. It has been widely used for the treatment of early-stage hepatocellular carcinoma (HCC) and has been shown to be superior to percutaneous ethanol injection in terms of objective response rates and overall survival (Lencioni et al. 2003). The low risk of complications and a short recovery time, make it a popular treatment option for many patients with HCC. However, the success of RFA is dependent on the size of the tumor, with larger tumors being more difficult to treat. In some cases, RFA may be combined with other treatments, such as surgery or chemotherapy, to achieve the best outcomes for the patient.

Microwave ablation (MWA) is another minimally invasive technique that uses microwave energy to destroy cancer cells. The procedure is performed using a needle-like probe that is inserted into the tumor, and microwave energy is delivered through the probe to heat and destroy the cancer cells. Unlike RFA, the multiple needles can be used simultaneously, which allows for a larger ablation zone to be achieved. Several trials have compared the efficacy of RFA and MWA in treating HCC, and they have reported no significant differences in the primary endpoint or in local tumor progression at 2 years (Yu et al. 2017). However, there is a trend towards greater efficacy with MWA in tumors larger than 3 cm, but with higher complication rates compared to RFA (Sutter et al. 2017).

Trans-arterial chemoembolization (TACE) involves the delivery of chemotherapy directly to the liver tumor and blocking its blood supply. During TACE, a catheter is inserted into an artery in the groin and guided to the hepatic artery, which supplies blood to the liver. A combination of chemotherapy drugs and an embolic agent, such as a gel foam

particle or a metallic coil, is then delivered to the tumor. The embolic agent blocks the blood supply to the tumor, effectively cutting off its oxygen and nutrient supply, while the chemotherapy drugs attack the cancer cells. TACE can be used to treat intermediate-stage HCC that cannot be removed by surgery, as well as to control the growth and spread of more advanced HCC. It can also be used to relieve symptoms caused by the cancer, such as pain or bleeding. The benefits of TACE in HCC include its minimally invasive nature, its ability to deliver high doses of chemotherapy directly to the tumor, and its ability to block the blood supply to the tumor, effectively cutting off its oxygen and nutrient supply.

The potential side effects of TACE include nausea, vomiting, fatigue, abdominal pain, and liver function problems. In rare cases, TACE can also cause damage to the liver or other organs.

Systemic therapy refers to treatments that can reach cancer cells throughout the body, rather than just targeting the local tumor site. In the case of hepatocellular carcinoma (HCC), systemic therapies are used to treat advanced stages of the disease that have spread beyond the liver.

First-line systemic therapy refers to the first option treatments in systemic therapy. *Sorafenib* has been the only systemic therapy in advanced HCC for more than 10 years. It is a multi-kinase inhibitor that targets several signaling pathways involved in the growth and spread of cancer cells. It was the first systemic therapy approved for the treatment of advanced HCC. SHARP study (Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol) has had a significant impact on the design of clinical trials for the treatment of advanced hepatocellular carcinoma (HCC). This study demonstrated the efficacy of the tyrosine kinase inhibitor sorafenib in improving overall survival in patients with HCC, leading to its approval as the first systemic therapy for advanced HCC (Rimassa and Santoro 2009).

Lenvatinib is a small molecular tyrosine kinase inhibitor with potent activity against VEGF receptors and the FGFR family. The REFLECT study established the efficacy of lenvatinib as a first-line systemic therapy for advanced HCC. The study was a comparative study between Lenvatinib with sorafenib, the previous standard of care for

HCC. Lenvatinib resulted in a significantly improved median overall survival (13.6 months vs. 12.3 months), progression-free survival (7.4 months vs. 3.7 months), and objective response rate (24.1% vs. 9.2%) compared to sorafenib (Kudo et al. 2018) (Yamashita et al. 2020).

Second-line systemic therapy is used after first-line therapy has failed or is no longer effective. There are several drugs that have been approved by the FDA as second-line treatments for HCC, including regorafenib, cabozantinib, and ramucirumab.

Regorafenib is a multi-kinase inhibitor that targets multiple proteins involved in cancer cell growth and survival, including VEGFR1-3 and other kinases. It was the first drug to be approved in the second line setting for the treatment of advanced HCC after progression on sorafenib (Bruix et al. 2017). The approval of regorafenib was based on the results of a phase III clinical trial that demonstrated a significant improvement in overall survival compared to placebo, with a median survival of 10.6 months in the regorafenib arm versus 7.8 months in the placebo arm (HR 0.63, 95% CI 0.50-0.79; P < 0.0001) (Bruix et al. 2017). The trial also showed a significant improvement in progression-free survival (PFS) with regorafenib, with a median PFS of 3.1 months versus 1.5 months in the placebo arm (HR 0.46, 95% CI 0.37-0.56; P < 0.0001). The overall response rate (ORR) was also higher with regorafenib, at 11% compared to 4% with placebo. Side effects include hypertension, hand-foot skin reaction, fatigue, and diarrhea, which are the most common grade 3-4 events associated with the drug.

Cabozantinib is a multi-kinase inhibitor that targets several proteins involved in cancer cell growth and survival, including VEGFR2, AXL, and MET. The approval of cabozantinib for the treatment of advanced HCC after progression on sorafenib was based on the results of the CELESTIAL trial, which showed an improvement in median overall survival with cabozantinib compared to placebo. The median overall survival with cabozantinib was 10.2 months, versus 8 months with placebo (HR 0.76, 95% CI 0.63-0.92; P = 0.0049). The trial also showed a significant improvement in progression-free survival (PFS) with cabozantinib, with a median PFS of 5.2 months versus 1.9 months in the placebo arm (HR 0.44, 95% CI 0.36-0.52; P < 0.001) (Abou-Alfa et al. 2018) (Kelley et al. 2020) (Freemantle et al. 2022) NCT01908426. The overall response rate

(ORR) was low in both treatment arms, with single-digit ORRs observed. Like all treatments, cabozantinib can cause side effects, including palmar-plantar erythrodysesthesia, hypertension, increased aspartate aminotransferase levels, fatigue, and diarrhea, which are the most common grade 3-4 events associated with the drug.

Ramucirumab is a monoclonal antibody that targets the VEGFR2 receptor, and it was the first biomarker-guided therapy to be approved for the treatment of HCC. The REACH-2 trial showed that ramucirumab improved overall survival compared to placebo (8.5 months vs. 7.3 months) in patients with a baseline α-fetoprotein level of ≥400 ng/dl (Zhu et al. 2019). The trial also showed an improvement in progression-free survival (2.8 months vs. 1.6 months), but no significant difference in objective response rate between the two treatment arms. The most common grade 3-4 adverse effects were found to be hypertension, hyponatremia, and increased aspartate aminotransferase levels.

Immunotherapy is a rapidly evolving field in the treatment of HCC. This type of therapy has largely replaced the use of TKI in treating HCC, with some studies reporting durable responses in a significant proportion of patients. Atezolizumab, an anti-PDL1 antibody, is one of the drugs that has been used in combination with bevacizumab, an anti-VEGF antibody, to treat advanced HCC. The combination of these two drugs was the first to show improved overall survival compared to the standard treatment with sorafenib (Llovet et al. 2016). Additionally, other molecules such as CDK4/6 inhibitors, are in the early stages of clinical testing for the treatment of HCC. These inhibitors have shown promising results in other types of cancer, and ongoing research is being conducted to evaluate their safety and efficacy in treating HCC.

Investigation therapies

Palbociclib is a targeted cancer therapy that works by inhibiting the activity of certain enzymes called cyclin-dependent kinases (CDKs). These enzymes are involved in regulating the cell cycle and cell division and are often overactive in cancer cells. By

blocking the activity of CDKs, Palbociclib can slow down the growth and spread of cancer cells. Palbociclib is a CDK4/6 inhibitor that was first approved for clinical use in 2015 in combination with letrozole for the treatment of postmenopausal women with estrogen receptor (ER)-positive, HER2-negative advanced breast cancer (Beaver et al. 2015). In 2016, it was also approved in combination with fulvestrant for the treatment of women with hormone receptor (HR)-positive, HER2-negative advanced or metastatic breast cancer with disease progression following endocrine therapy (Cristofanilli et al. 2022) NCT01942135. Palbociclib is a selective CDK4/6 inhibitor that can manipulate the cell cycle progression by inhibiting the CDK4/6-mediated phosphorylation of retinoblastoma protein (RB1) (Bollard et al. 2017) *(**Figure 1**)*. The inhibition of RB1 phosphorylation leads to the accumulation of unphosphorylated RB1, which in turn results in the repression of E2F transcription factors and the inhibition of cell cycle progression (Fry et al. 2004).

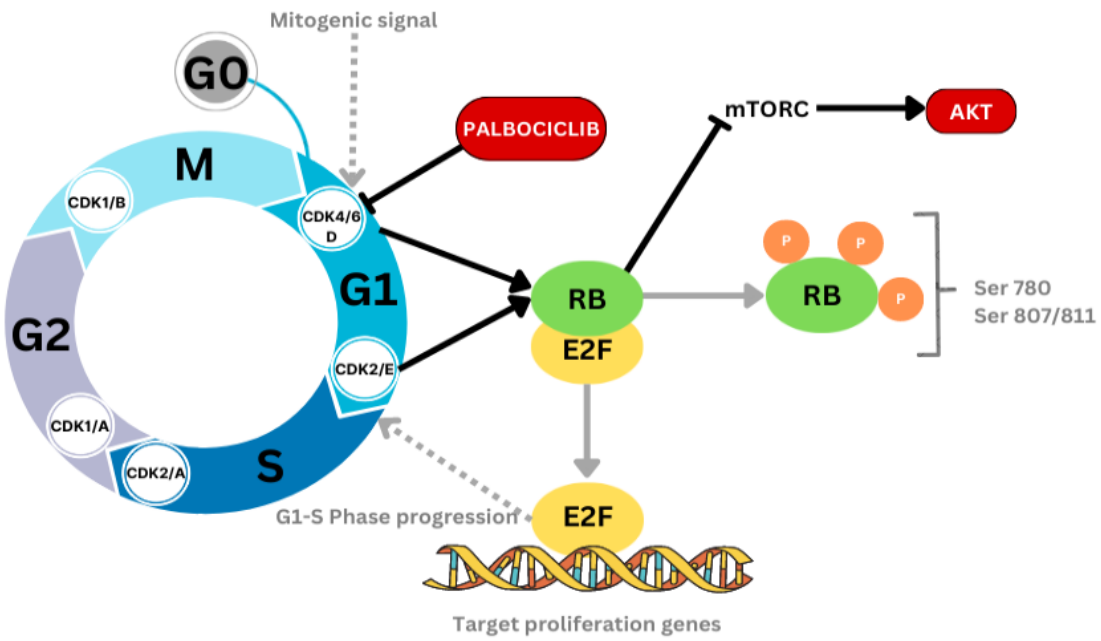


Figure 1. Mechanism of action of Palbociclib

MK-2206 is an orally available, small molecule inhibitor of the protein kinase Akt. Akt is a key player in the PI3K/Akt signaling pathway, which regulates a variety of cellular processes, including cell growth, survival, and metabolism (Xing et al. 2019). In many types of cancer, the PI3K/Akt pathway is activated, leading to increased cell survival and resistance to chemotherapy. MK-2206 is being developed as a potential treatment for various cancers, including solid tumors and hematologic malignancies (Molife et al. 2014, 1). By inhibiting Akt activity, MK-2206 may help to reduce the survival and proliferation of cancer cells and enhance the effectiveness of other cancer treatments (NCT01277757, NCT01307631). Clinical trials are underway to evaluate the safety and efficacy of MK-2206 as a single agent and in combination with other cancer treatments. The results of these trials will provide important insights into the potential role of MK-2206 in the management of cancer and the optimal ways to use it in combination with other treatments *(**Figure 2**)*.

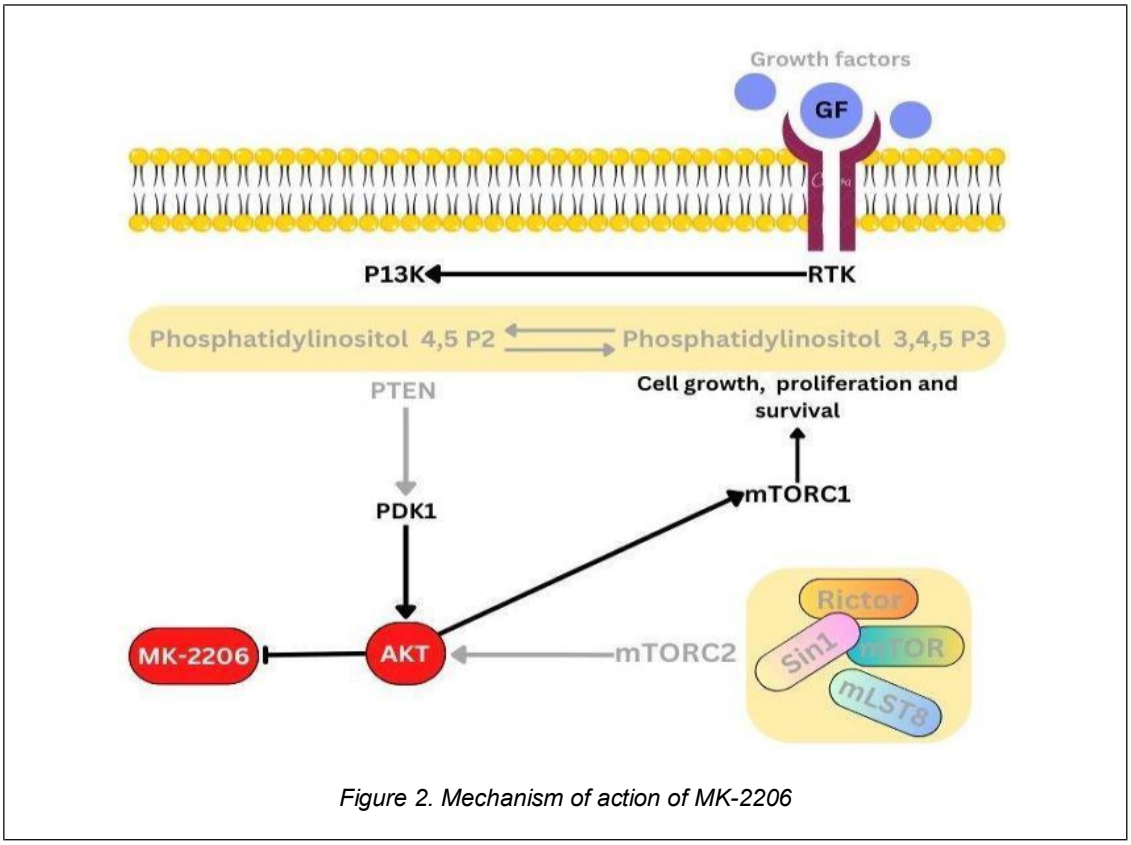


Figure 2. Mechanism of action of MK-2206

microRNA in HCC

MicroRNA (miRNA) are small, non-coding RNA molecules that play a crucial role in post-transcriptional gene regulation. There are approximately 22 nucleotides in length and are found in both plants and animals. MiRNAs regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target messenger RNA (mRNA) molecules, causing their degradation or inhibiting their translation into proteins (Xu et al. 2018). By doing so, miRNAs play important roles in a wide range of biological processes, including development, cell differentiation, proliferation, and apoptosis. MiRNAs are transcribed from DNA sequences in the genome and undergo a series of processing steps to generate the mature miRNA molecule. The mature miRNA then binds to the RISC (RNA-induced silencing complex) to regulate gene expression. Abnormal expression of miRNAs has been linked to numerous diseases, including cancer, cardiovascular disease, and neurological disorders. Dysregulation of miRNAs has been implicated in the development and progression of HCC.

microRNAs have emerged as promising targets for the development of new cancer therapies, including for HCC. Here are some potential strategies for using miRNAs in the treatment of HCC:

- *miRNA replacement therapy*: In this approach, synthetic miRNAs are delivered to cancer cells to restore the expression of a tumor-suppressive miRNA that is downregulated in HCC. This approach has been investigated in preclinical studies for miR-199a-3p and other miRNAs and has shown promising results in inhibiting HCC cell growth and inducing apoptosis (Giovannini et al. 2018) (Kobayashi, Sawada, and Kimura 2018). For example, miR-26a is frequently downregulated in HCC, and studies have shown that introducing synthetic miR-26a into HCC cells can inhibit tumor growth and induce apoptosis (Kota et al. 2009).

- *miRNA inhibition therapy*: The synthetic oligonucleotides are used to inhibit the expression of an oncogenic miRNA that is upregulated in HCC. This approach has been investigated for several oncogenic miRNAs in HCC and has shown potential as a therapeutic strategy. For example, miR-221 and miR-222 are frequently upregulated in HCC, and studies have shown that inhibiting these miRNAs can inhibit tumor growth and induce apoptosis (Rong, Chen, and Dang 2013) (Di Martino et al. 2022).

- *Combination therapy:* Given the complexity of cancer biology, combination therapy with miRNAs and other treatments, such as chemotherapy, radiation therapy, or targeted therapy, may be more effective than using miRNAs alone. miR-199a-3p has been shown to sensitize HCC cells to chemotherapy and radiation therapy, suggesting that combining miRNA therapy with conventional treatments may be a promising strategy for HCC (Fornari et al. 2010). Another example, combining miR-34a replacement therapy with sorafenib, a targeted therapy for HCC, which has been shown to enhance the antitumor effects of sorafenib (Yang et al. 2014).

- *Diagnostic and prognostic biomarkers:* miRNAs can also be used as diagnostic or prognostic biomarkers for HCC. For example, miR-199a-3p expression levels have been shown to correlate with tumor stage and patient survival in HCC, suggesting that it may be a useful prognostic biomarker (Yin et al. 2015) (Chen et al. 2016).

There are numerous microRNAs (miRNAs) that have been identified as responsible for tumorigenesis and hence potential targets for HCC therapy *(**Figure 3)**.*

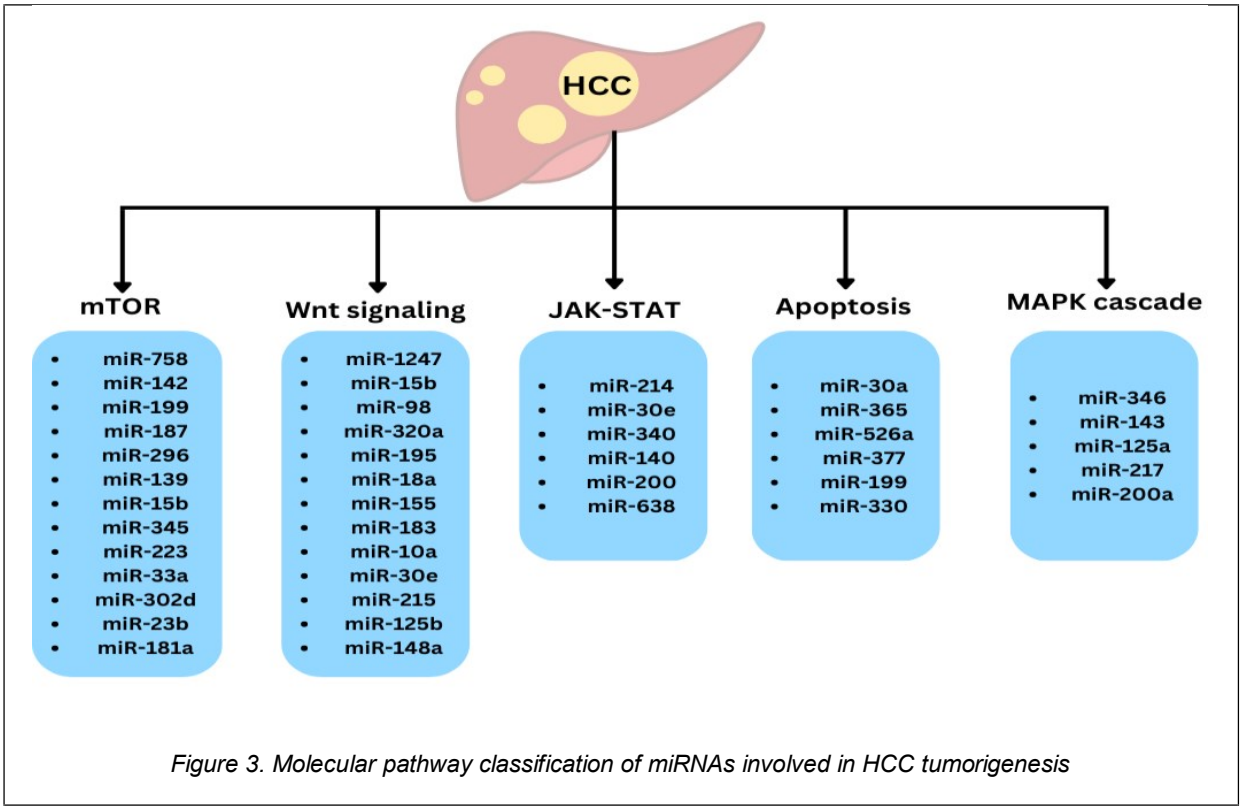


Figure 3. Molecular pathway classification of miRNAs involved in HCC tumorigenesis

miR-199a-3p is a microRNA, which is a small non-coding RNA molecule that plays a role in post-transcriptional regulation of gene expression. Specifically, miR-199a-3p is the mature form of the miR-199a gene, which is located on human chromosome 19. Research has shown that mir199a-3p is involved in a variety of biological processes, including cell proliferation, differentiation, and apoptosis (Callegari et al. 2018) (Ghosh et al. 2017). In terms of its mechanism of action, miR-199a-3p functions by binding to target mRNAs, which can lead to degradation of the mRNA or inhibition of translation. This ultimately results in decreased expression of the target gene. The specific target genes regulated by miR-199a-3p can vary depending on the cell type and context in which it is expressed.

miR-199a-3p is frequently downregulated in HCC tissues compared to adjacent non-cancerous liver tissues, suggesting that it may have a tumor-suppressive role in HCC. The tumor-suppressive effects of miR-199a-3p in HCC are mediated through its regulation of multiple target genes. For example, miR-199a-3p has been shown to target mTOR, a key regulator of cell growth and metabolism as well as the oncogene c-

Met (Fornari et al. 2010) and PAK4, a crucial component of the PI3K-AKT-PTEN pathway involved in cell survival and proliferation (Engelman 2009). Moreover, the ability of miR-199a-3p to suppress tumor growth, migration, invasion and angiogenesis in HCC by targeting vascular endothelial growth factor A (VEGFA) and its receptors, hepatocyte growth factor (HGF) and matrix metallopeptidase 2 (MMP2), has been shown (Ghosh, A., Dasgupta, D., Ghosh, A., Roychoudhury, S., Kumar, D., Gorain, M., Butti, R., Datta, S., Agarwal, S., Gupta, S., et al. (2017). MiRNA199a-3p suppresses tumor growth, migration, invasion, and angiogenesis in hepatocellular carcinoma by targeting VEGFA, VEGFR1, VEGFR2, HGF and MMP2. Cell Death Dis. 8, e2706). *(**Figure 4**).*

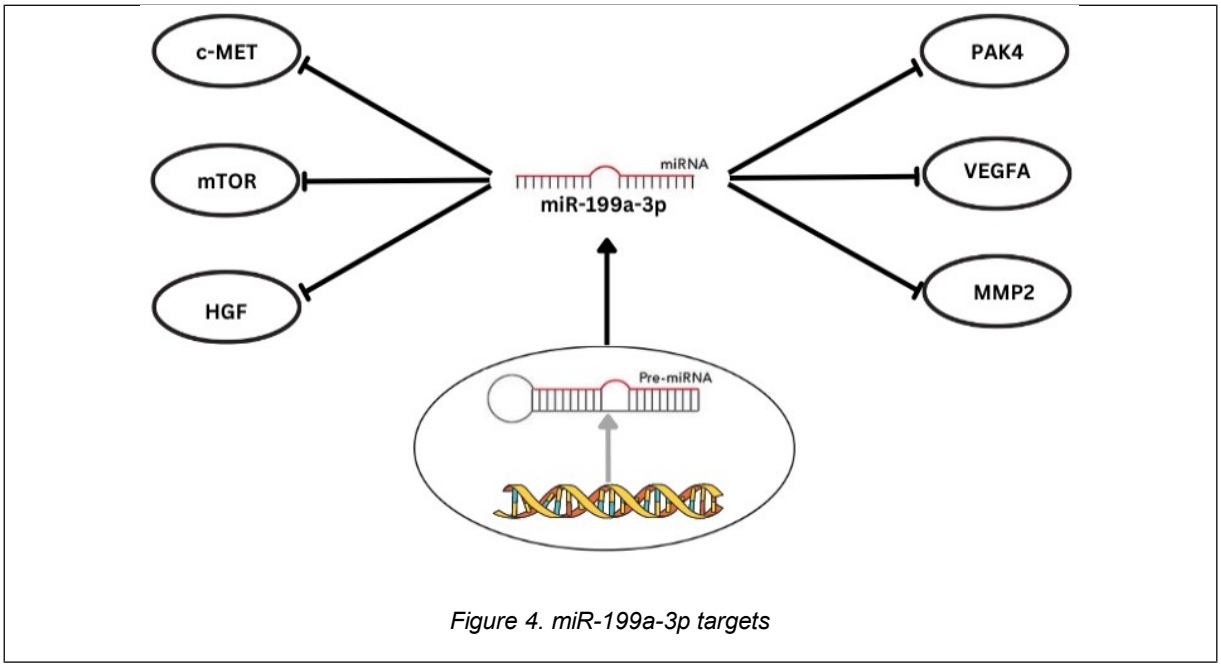


Figure 4. miR-199a-3p targets

AIMS AND OBJECTIVES

Palbociclib, is a cyclin-dependent kinase (CDK) inhibitor. CDKs play a critical role in regulating the cell cycle by controlling the progression from one phase to another. Palbociclib specifically inhibits CDK4 and CDK6, leading to the inhibition of cell cycle progression at the G1 to S phase transition (Bollard et al. 2017). However, Palbociclib was shown to induce a dose-dependent up-regulation of the target of rapamycin (mTOR) and AKT protein phosphorylation, which reduces its anti-tumor efficacy by activating the PI3K/AKT/mTOR signaling pathway (Cretella et al. 2018), suggesting that the combination of Palbociclib with inhibitors of the PI3K/AKT/mTOR pathway may represent a promising strategy for the treatment of certain cancers (Cretella et al. 2018) (M. A. Bonelli et al. 2017) (M. Bonelli et al. 2020) (Wong et al. 2019).

The present study is indeed aimed at investigating the use of Palbociclib in combination with AKT inhibitors to improve the efficacy of Palbociclib against HCC. We tested the combinations of Palbociclib with MK-2206 or with miR-199a-3p. MK-2206 is an allosteric inhibitor of Akt, a kinase involved in cellular signaling pathways that regulate cell growth, survival, and metabolism. By inhibiting the PI3K/Akt signaling pathway, MK-2206 reduces the activation of downstream targets that promote cell survival and growth (Hirai et al. 2010). miR-199a-3p is a small non-coding RNA molecule that can regulate gene expression by binding to complementary messenger RNA molecules and inhibiting their translation into protein. It has been shown to play a role in the regulation of cell proliferation and apoptosis in cancer cells (Callegari et al. 2018) (Ghosh et al. 2017).

The objective is to investigate the potential Akt inhibitors likely to increase the efficacy of CDK4/6 inhibitor, Palbociclib in HCC (hepatocellular carcinoma) models and the safety of this combination in treating HCC.

Aims covered in this thesis:

- Antitumor activity: The study to evaluate the ability of the combination therapy to inhibit the growth of HCC tumors and/or shrink existing tumors in preclinical models.
- Mechanism of action: The study explores the underlying mechanisms by which combination therapy affects HCC cells, such as through inhibition of cell cycle progression and regulation of gene expression.
- Safety: The study to assess the safety of the combination therapy, including the risk of side effects or toxicity.
- Efficacy: The study to evaluate the overall efficacy of the combination therapy in HCC, considering measures such as tumor response.

The goal of this study would be to demonstrate the potential benefits of combination therapy, which can increase the efficacy of Palbociclib without increasing the toxic effects and to provide a foundation for future research.

MATERIALS AND METHODS

Cell culture

The HCC cell lines Hep3B (HB-8064) and HepG2 (HB-8065) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were propagated and maintained in Dulbecco's modified Iscove's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 0.1% gentamycin, and 1% L-glutamine (Sigma-Aldrich, St. Louis, MO, USA). In vitro, cell transfections were performed using Lipofectamine 2000 (Invitrogen).

Determination of IC50, viability, and apoptosis

IC50 refers to the concentration of a substance that is needed to inhibit a particular biological process by 50% (Sebaugh 2011). The lower the IC50 value, the more potent the substance is. The Presto Blue Cell Viability Reagent was used (Thermo-Fisher, A13261). This reagent is a cell-permeable resazurin-based solution that functions as a cell viability indicator by using the reducing power of living cells to quantitatively measure the proliferation of cells. Specifically, Presto Blue Reagent uses the reducing power of living cells to convert resazurin to fluorescent resorufin (Martín-Navarro et al. 2014). The colorimetric analysis was completed with the help of a fluorescence plate reader.

The Muse® Count & Viability Kit was used to perform quantitative analysis of cell count and viability (MCH100102, Luminex Corporation, Austin, TX, USA). The Muse Count & Viability Kit is designed to determine the absolute cell count and viability of cell suspensions derived from various cultured mammalian cell lines. The kit works by using different stains on viable and non-viable cells based on their permeability to DNA-binding dyes in the reagent. The Muse Count & Viability Reagent contains two different DNA-binding dyes, each serving a specific purpose. One of the dyes is membrane-permeant and stains the nucleus of all cells with a nucleus. This parameter is known as NUCLEATED CELLS and is used to differentiate cells with a nucleus from debris and

non-nucleated cells. The Muse System then uses the size properties of the stained nucleated events to differentiate between free nuclei, cellular debris, and cells to provide an accurate total cell count. The other dye is used to stain cells that have lost their membrane integrity and are able to penetrate the nucleus of dead and dying cells. This parameter is referred to as VIABILITY and is used to distinguish between viable (live cells that do not stain) and non-viable (dead or dying cells that do stain).

The Muse™ Annexin V and Dead Cell Assay kit (MCH100105, Luminex Corporation) was used to measure viable, apoptotic, and dead cells. This assay is based on the use of Annexin V, which binds to phosphatidylserine (PS) that becomes exposed on the outer surface of the plasma cell membrane during the early stages of apoptosis, and the dead cell marker 7-Aminoactinomycin D (7-AAD), which distinguishes late-stage apoptotic cells that have lost membrane integrity. The procedure involves staining the cells with fluorescently labeled Annexin V and the 7-AAD viability dye, followed by analysis using a flow cytometer. Overall, the data obtained by this assay can be used to determine the percentage of live, apoptotic, and dead cells in the sample.

All assays were performed in triplicate and analyzed in a Muse® Cell Analyzer instrument (Merck Millipore).

Recombinant AAVV-199

Expressing miR-199a-3p was generated through a three-step process. First, the miR-199a-3p cassette was obtained from the pIRES-miR-199a plasmid and cloned into the pAAV-IRES-GFP vector using XbaI restriction sites. This resulted in the creation of a new vector, pAAV-199, that expressed the miR-199a-3p sequence upstream of the IRES-GFP sequence. Next, the pAAV-199 vector was transfected into 293FT cells along with two helper plasmids, pAAV-DJ and pHelper, in a 1:1:1 molar ratio. This step was done to generate the infectious recombinant AAV virus. Finally, the production of the recombinant AAV virus and its titration (determination of virus concentration) were performed. The resulting AAVV-199 expresses miR-199a-3p and can be used for

further studies of the regulation of gene expression by this microRNA (Callegari et al. 2018).

In vivo mouse studies

The study was performed according to the Guidelines for the Care and Use of Laboratory Animals of the Italian Ministry of Health. All animals were randomly assigned to different treatment groups at the start of the studies. The TG221 transgenic mouse used in the experiments was characterized by the overexpression of miR-221 in the liver and was predisposed to the development of liver tumors. was already described (Callegari et al. 2012). All mice were maintained in vented cabinets at 25°C with a 12-h light-dark cycle, with food and water ad libitum. To facilitate tumor development, 10-day newborn male mice received one i.p. injection of DEN (#N0756, Sigma-Aldrich) (7.5 mg/kg body weight). In Figure 5 the layout of the groups is shown. Mice were monitored for the presence of hepatic lesions using an ultrasound diagnostic device (Philips IU22) as previously described (Callegari et al. 2019). Mice were randomly enrolled for treatments at six months of age as shown in (***Figure 5***). At the end of all the *in vivo* experiments, mice were sacrificed, tumor tissues were collected, immediately frozen in liquid nitrogen, and stored at -80°C or fixed in 10% phosphate-buffered formalin for 12– 24 h and embedded in paraffin for histological analysis.

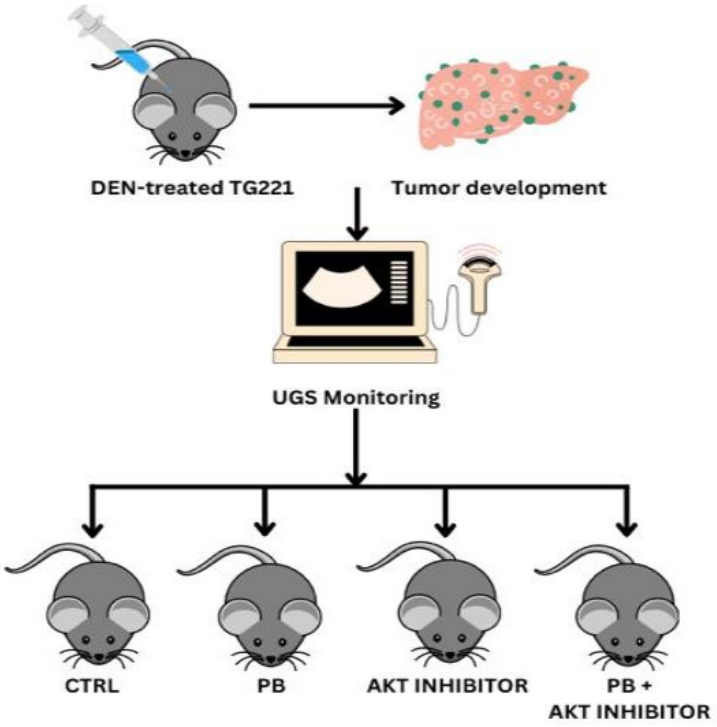


Figure 5. In vivo experimental treatment groups

TG221 male mice will be treated intra-peritoneum (i.p.) with the carcinogen diethylnitrosamine (DEN) at 10 days of age to accelerate the development of liver tumors. Tumor development will be monitored by ultrasonography. When the volume of tumors will reach about 2-3 mm3 (approximately 6 months of age), mice will be split into the following experimental groups: (1) CTRL (vehicle); (2) Palbociclib; (3) AKT-inhibitor; (3) Palbociclib + AKT-inhibitor. Drug efficacy will be assessed by measuring tumor nodule volumes by ultrasonography at the beginning and the end of the treatment.

Anti-tumor drugs

For in vitro experiments, all drugs were solubilized in dimethyl sulfoxide (DMSO). For all in vivo experiments, drugs were administered daily by oral gavage: sorafenib (S-8599, LC Laboratories, Woburn, MA, USA) was dissolved in a 50:50 Cremophor EL and ethanol solution; MK2206 (MK2206 dihydrochloride, Medchem Express., HY-10358-0002, NJ, USA) was dissolved in 15% Captisol (SBE-b-CD, Medchem Express, HY-17031-0731); Palbociclib (Palbociclib Isethionate Salt, CAS No.: 827022-33-3, BOC sciences, Shirley, NY, USA) was dissolved in sodium lactate buffer (50mM, pH 4.0)

RNA mimics and lipid nanoparticles

RNA mimics are synthetic RNA molecules that mimic the function of endogenous RNA molecules, such as microRNAs, messenger RNAs, or transfer RNAs. They were used to study the role of specific RNA molecules in regulating gene expression or protein synthesis. The miR-199a-3p mimics are designed to mimic the effects of the endogenous miR-199a-3p. miR-199a-3p mimics and scrambled unmodified single stranded RNA oligonucleotides were obtained from Axolabs GmbH (Kulmbach, Germany). The oligonucleotides sequences were as follows: (1) miR-199a-3p mimic sequence 5' –ACA GUA GUC UGC ACA UUG GUU A-3' (unmodified sequence); (2) scramble sequence 5'-UCA CAA CCU CCU AGA AAG AGU AGA-3' (unmodified sequence). For in vivo delivery, lipid nanoparticles were used as vehicles.

Lipid nanoparticles are small particles composed of lipids (fats or oils) that are used as carriers for the delivery of various substances, including drugs, nucleic acids, and vaccines. The lipid nanoparticles are a commonly used method for delivering RNA molecules to cells in vivo, as they can protect the RNA from degradation and help it reach target cells. They are typically composed of a mixture of lipids, such as phospholipids, and polyethylene glycol (PEG), which is used to stabilize the particles and reduce their recognition and clearance by the immune system. The lipid components of the nanoparticles were 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycerol, methoxy polyethylene glycol (DMGPEG, Mw 2,000; #15091, Cayman Chemical Company, Ann Arbor, MI, USA), and linoleic acid (#L1376, Sigma-Aldrich, St. Louis, MO, USA). The preparation of empty nanoparticles was performed as previously described (Huang et al. 2013).

Reverse transcription and droplet digital polymerase chain reaction (ddPCR)

Total RNA was extracted from cells or from frozen liver tissues using the automated Maxwell Rapid Sample Concentrator Instrument (Promega Corporation, Madison, WI, USA) with the purification kit Maxwell® RSC miRNA from Tissue (#AS1460, Promega) according to the manufacturer's instructions. Droplet digital polymerase chain reaction

(ddPCR) was used to measure the expression level of miRNAs. For quantitative PCR analysis, 5 ng of purified RNA was retro-transcribed using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and cDNA was used for amplification as previously described (Callegari et al. 2018). A TaqMan miRNA PCR probe set specific for miR-199a-3p (assay ID002304; Applied Biosystems) was used, while a TaqMan Assays for RNAs U6 (assay ID001973; Applied Biosystems) was used to normalize the relative abundance of miRNAs

Western blot

Used for detecting specific proteins in a sample of tissue or fluid, to determine the size and amount of a specific protein in a sample, to confirm the presence or absence of a protein, and to evaluate the effects of different treatments on protein expression. The method is based on separating the proteins by size through electrophoresis and then transferring them onto a solid support, such as a nitrocellulose or polyvinylidene difluoride (PVDF) membrane (Yang and Mahmood 2012). Before adding the proteins in an SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel, reducing agents like β-mercapto ethanol or dithiothreitol (DTT) is added, and the proteins are then mixed with a sample buffer. The sample buffer contains SDS, which is a detergent that denatures the proteins, unfolds them, and creates a uniform negative charge on the protein. The buffer also contains other components, such as glycerol, which increases the viscosity of the sample and helps to keep the protein in the well of the gel during electrophoresis, and a tracking dye, such as bromophenol blue, which helps to monitor the progress of the electrophoresis. Additionally, a loading buffer can also include a pH stabilizer, such as Tris-HCl, and a reducing agent to minimize any oxidative damage to the proteins during the electrophoresis. The proteins were denatured at 95 degrees for 5 minutes approx. This process involves breaking the non-covalent bonds that stabilize the native three-dimensional structure of the proteins, causing the proteins to unfold and become linear. Denaturation is often achieved by adding a strong denaturant, such as SDS, to the protein sample. The SDS helps to create a uniform negative charge on the protein, allowing it to migrate through the gel

under the influence of an electric field. The resulting separation of the proteins based on size can be visualized using a stain, such as Coomassie Brilliant Blue, that binds to the proteins and makes them visible. The membrane is then incubated with a specific antibody that recognizes the target protein, and the complex is visualized through detection methods such as chemiluminescence (Pillai-Kastoori, Schutz-Geschwender, and Harford 2020). Cell cultures were collected at the indicated time point and then washed with phosphate-buffered saline (PBS). Tissue samples were collected, immediately frozen in liquid nitrogen, and stored at -80°C until protein extraction.

All samples were dissolved in radioimmune precipitation (RIPA) Buffer (#R0278; Sigma-Aldrich) containing phosphatase and protease inhibitors (#P2850 and #P8340; Sigma-Aldrich). Lysates were centrifuged at 8,000x g for 10 min at 4°C to pellet the debris, and supernatants were collected. The protein concentration was measured using the Bradford protein assay and equal amounts (30µg) of protein extracts from all samples were applied to SDS–PAGE electrophoresis (4-15% Tris Glicyne Gel, #4561083, Bio-Rad) and then transferred to a PVDF membrane (#1704156, Bio-Rad). After blocking the membrane with 5% Blocking agent, the membrane was incubated overnight at 4°C with the following antibodies: Rabbit antibodies against p-RB (Ser780, D59B7, #8180), p-AKT (Ser473, D9E XP, #4060), p-FOXM1 (Thr600, D9M6G, #14655), RB (D20, #9313), FOXM1 (D12D5, #5436) and PAK4 (#3242) were diluted in 5% w/v BSA (A4503, Sigma-Aldrich), 1X Tris-buffered saline (TBS, Bio-Rad Laboratories, Hercules, CA, USA), and 0.1% Tween20 (Bio-Rad) and incubated at 4°C for 16 hr. Rabbit antibody against AKT (C-20, sc-1618) was diluted in 1% w/v milk, 1X Tris-buffered saline (TBS), and 0.1% Tween20 (Bio-Rad). The anti-glyceraldehyde-3-phosphate dehydrogenase 398 (GAPDH) monoclonal antibody (clone 2D9, TA802519; OriGene Technologies, Rockville, MD, USA) was used as a loading control. For chemiluminescent detection, horseradish peroxidase-conjugated secondary antibody (#7074; Cell Signaling Technology) was used in combination with Clarity Western ECL Blotting Substrate (#170-5060; Bio-Rad), and digital images were acquired using a Chemidoc (Bio-Rad). The signals were quantified using ImageJ software (https://imagej.nih.gov) and the protein expression levels were normalized according to GAPDH expression.

Immunohistochemical analysis

A technique used to detect specific proteins within tissues by using antibodies that specifically bind to the target protein. The tissue samples were taken from the liver at autopsy and fixed in 10% phosphate-buffered formalin for 12 to 24 hours and embedded in paraffin. These samples, two representative fragments of each lobe of the liver were taken at autopsy, were embedded in paraffin, and then serial 4 µm thick sections were processed. The slides are then treated with a blocking solution to reduce background staining, followed by incubation with the Caspase-3 (Asp175) and the Ki67 antibodies that specifically recognize the target protein. The bound primary antibody is then visualized using a secondary antibody conjugated to a marker, such as a fluorescent dye or an enzyme which in our case is a polymer-based HRP-conjugated detection reagent. Specifically, Cleaved Caspase-3 (Asp175) (D3E9) Rabbit mAb (#9579, Cell Signaling) and Ki-67 (D2H10) Rabbit mAb (#9027, Cell Signaling) were diluted in SignalStain® Antibody Diluent (#8112, Cell Signaling) and detected by the polymer-based, HRP-conjugated Signal Stain Boost IHC Detection Reagent (#8114) in combination with SignalStain® DAB Diluent (#11724, Cell Signaling) and Chromogen Concentrate (#11725, Cell Signaling), following the manufacturer instructions. After rinsing in distilled water, slides were counterstained with Leica Microsystem's hematoxylin (Fisher Scientific, Italy) and mounted with Micro mount mounting media (Diapath, Italy, SKU060500). For apoptosis and proliferation evaluation, separate areas of cleaved caspase-3- and Ki-67-stained tissues were analyzed. The percentage of cleaved caspase-3 or Ki-67-positive stained area was calculated per selected region and the results were quantified by Image J software. This information was used to assess the levels of apoptosis (cell death) and proliferation (cell growth and division) in the liver tissues of transgenic mice.

RESULTS

Combination therapy of Palbociclib and MK-2206 in *in vitro* HCC models

The effects of Palbociclib and MK-2206 were investigated *in vitro* either individually or in combination in the HepG2 and Hep3B cell models. Ic50 was measured in Hep3B and HepG2 cell lines, treated with Palbociclib, and MK-2206 individually, and in combination. MK-2206 was kept constant at 5 μM while the concentration of Palbociclib varied. The investigation showed that MK-2206 led to a reduction of the IC50 of Palbociclib from 7.0 μM to 2.8 μM in HepG2 cells (Figure 6A) and from 23 μM to 11.15 μM in Hep3B (Figure 6B).

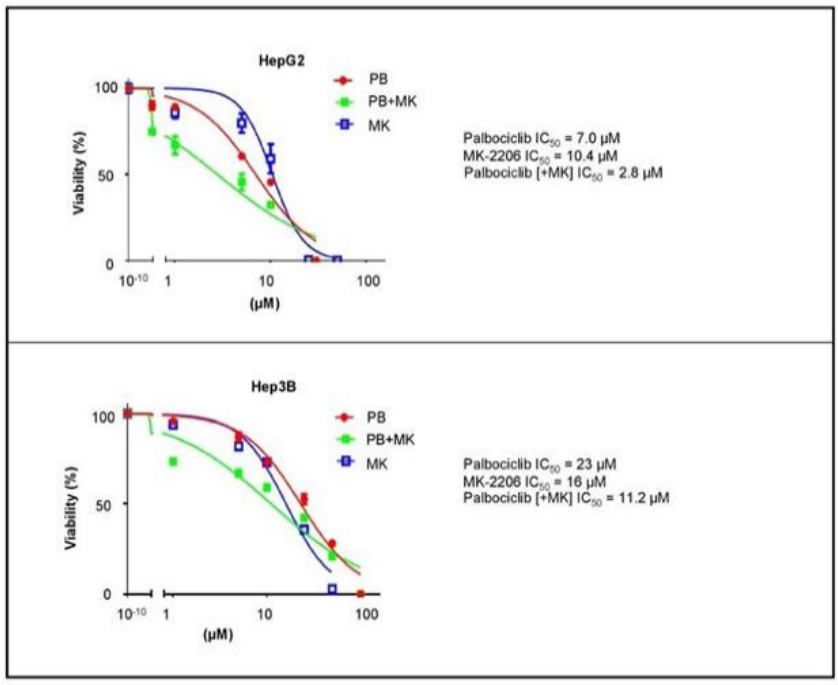


Figure 6. IC50 of Palbociclib, MK-2206 and their combination in HepG2 and Hep3B model.

The concentration of drugs used is expressed on a log10 scale, and the results are normalized to the average viability of untreated cells. The points represent the average percentage of viability at each drug concentration, and the error bars represent the standard deviation (SD). The combination therapy was able to induce a significant reduction of Palbociclib's IC50, suggesting an increased efficacy of the drug when used in combination with MK-2206.

The reduction in IC50 indicates that the combination is more potent than each of the drugs individually.

The biological effects of the drugs were also investigated individually or in combination by testing the viability and apoptosis after 72 hours from the beginning of the treatment. It indicates that the combination caused an increased apoptotic effect in both the HepG2 and Hep3B cell models in comparison with the individual drugs *(*Figure 7*).*

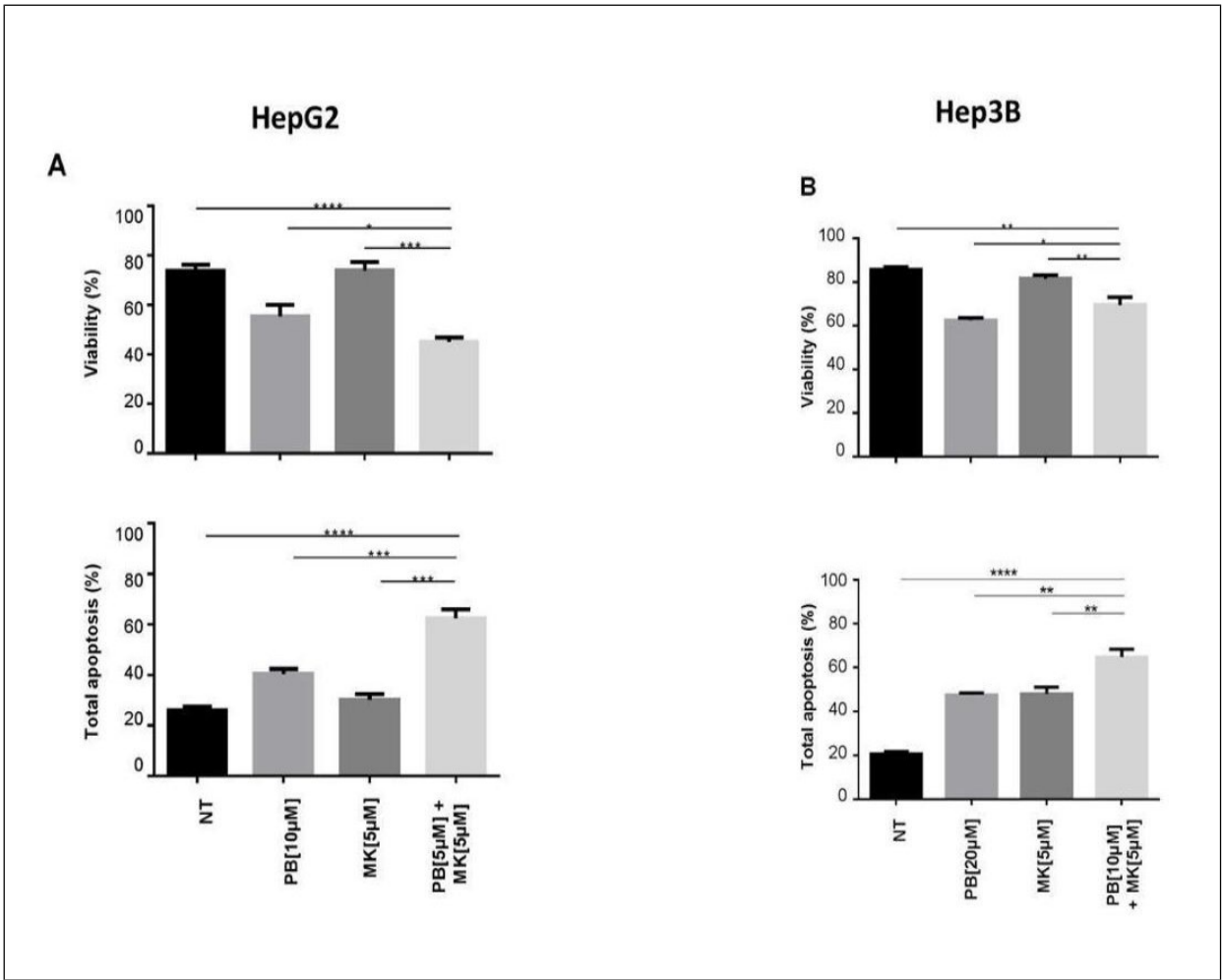


Figure 7. Biological effects of Palbociclib and the AKT inhibitor MK-2206 on HepG2 and Hep3B cells

(A) Apoptosis and viability of Palbociclib (10μM), MK-2206 (5μM) and their combination (PB 5μM +MK 5μM) in HepG2 cells.

(B) Apoptosis and viability of Palbociclib (20μM), MK-2206 (5μM) and their combination (PB 10μM +MK 5μM) in Hep3B cells. Data were represented as mean + SD. *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001.

Since we tested Palbociclib, a CDK inhibitor, and MK-2206, an AKT inhibitor, the expression of RB1, AKT, and their phosphorylated forms were investigated at the

molecular level in both cell lines. The status of these proteins can in fact provide evidence of the action at the molecular level of the employed drugs

Firstly, we assessed the basal expression of RB1 and Akt proteins in HCC cell lines. Analyses of these proteins can provide a better understanding of the molecular mechanisms involved in the regulation of cell cycle progression and survival in HCC, as RB1 and Akt proteins are key regulators of these cellular processes. Knowing the basal expression of RB1 and Akt proteins can also be useful in predicting the response of

HCC cells to specific therapies. For example, RB1 and AKT status can be predictive biomarkers for the response to the therapeutic agents used in the investigation. In Figure 8, it is evident that the expressions of the RB1 protein in HepG2 appear significantly higher than in Hep3B. This was expected since Hep3B cells carry a STOP codon in the RB1 gene (c.1727C>G, p. Ser576Ter), and so a very low expression of full-length RB1 protein can be found in these cells.). This finding suggests that inhibition of RB1 protein phosphorylation is unlikely a major mechanism responsible for the observed pro-apoptotic effects of Palbociclib on Hep3B cells.

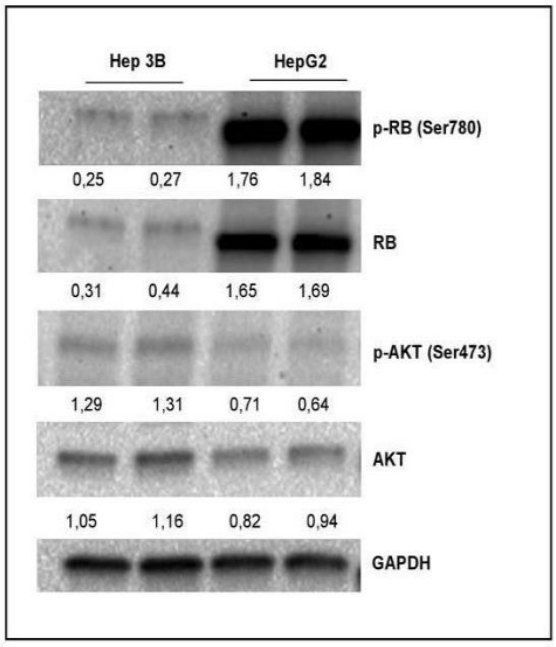


Figure 8. The basal expression of RB1 and AKT proteins in Hep3B and HepG2 cells.

The values were normalized on the GAPDH protein levels.

The inhibitory effects of Palbociclib and MK-2206 on the expression of RB and p-RB and AKT and p-AKT proteins were investigated on the HepG2 model, individually as well as in combination *(*Figure 9*)*. Palbociclib inhibited the expression of RB1 and p-RB1, while MK-2206 can inhibit p-AKT. The combination of the two drugs inhibited both p-RB1 and p-AKT, thus blocking both cell cycle progression and the AKT survival pathway.

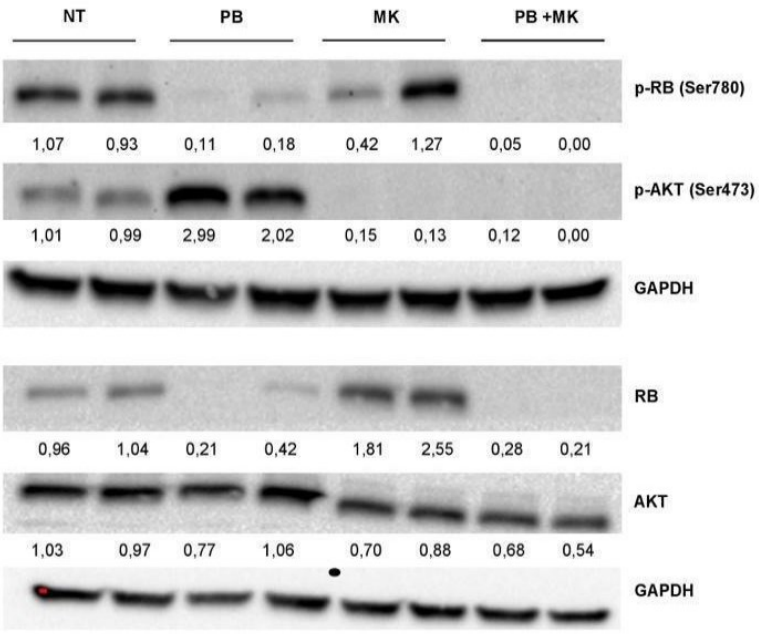


Figure 9. Molecular efficacy of Palbociclib, MK-2206 and their combination in HepG2.

Western blot analysis and quantification of RB1, AKT proteins and their phosphorylated forms in HepG2 treated cells. The groups NT (no-treatment), PB (treated with Palbociclib), MK (treated with MK-2206), and PB+MK (combination) each are in pairs as biological replicates. The values are normalized on the GAPDH protein and compared to the average levels detected in the untreated cells

Likewise, the expression of RB, AKT, and their phosphorylated forms was investigated in Hep3B cells. The reduction of the expression in combination treatment was significant enough to confirm the action of MK-2206.

Differently from HepG2, Forkhead box protein M1 (FOXM1) was also investigated in Hep3B cells, considering that RB1 expression was largely abrogated because of the presence of a STOP codon, and CDK4/6 can phosphorylate various proteins involved in cell cycle regulation, including FOXM1. FOXM1 is a transcription factor that plays a

critical role in regulating cell cycle progression, DNA damage repair, and apoptosis. CDK4/6 phosphorylates FOXM1 at specific sites, which leads to its activation and stabilization. This phosphorylation increases the DNA binding and transcriptional activity of FOXM1. Thus, CDK4/6-mediated activation of FOXM1 results in the expression of genes that promote cell cycle progression (Anders et al. 2011). In summary, CDK4/6 activation of FOXM1 plays a crucial role in promoting cell cycle progression. Therefore, measuring FOXM1 expression in Hep3B cells can serve as a biomarker for Palbociclib action, in the absence of RB1. Here we confirmed that Palbociclib induced an inhibition of FOXM1 activity mainly by reducing total FOXM1 protein in Hep3B cells (Figure 10*).* As FOXM1 activation is AKT-dependent (Yao, Fan, and Lam 2018) (Chesnokov et al. 2021), inhibition of FOXM1 was also sustained by MK-2206.

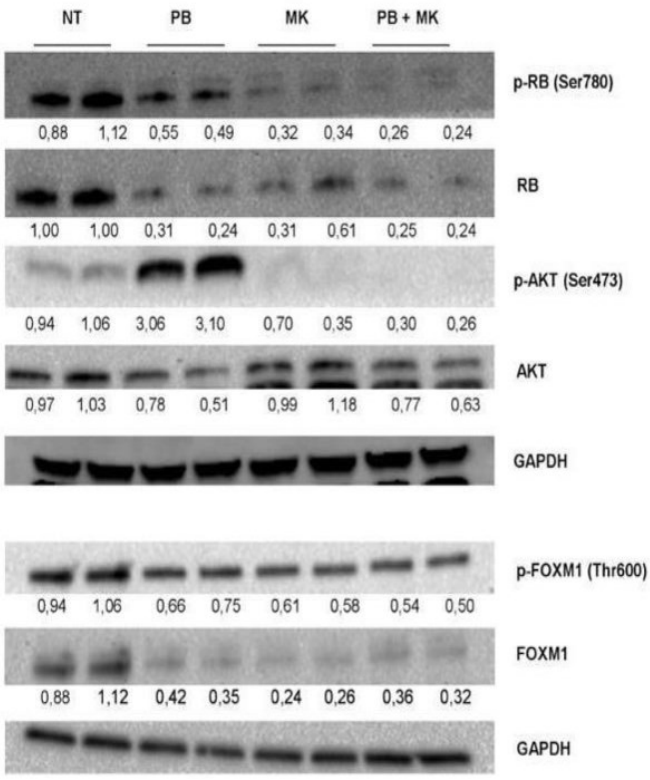


Figure 10. Molecular efficacy of Palbociclib, MK-2206 and their combination in Hep3B.

Western blot analysis for quantification of RB1, AKT and FOXM1 proteins and their phosphorylated forms. The values are normalized on the GAPDH protein and compared to the average levels detected in the untreated cells (NT) Hep3B cells exhibit low level of full length RB1 protein, hence the digital images of RB1 and p-RB1 were acquired with an exposure time of 300 seconds instead of 30 sec.

Combination therapy of Palbociclib and MK-2206 in *in vivo* HCC models

The in vivo anti-cancer activity of Palbociclib and MK-2206 was investigated in TG221 male mice with induced liver tumors. The tumors were induced in the pups by intraperitoneal administration of carcinogen N-diethylnitrosamine (DEN) at day 10. The early administration can accelerate the tumor development which is then monitored by ultrasonography. Once the mice reach 6 months of age or have a tumor volume approximately between 2-3 mm^3 they are separated into treatment groups i.e., 1. CTRL (vehicle) 2. PB (Palbociclib 100mg/kg), 3. MK-2206 (150mg/kg), and 4. Combination PB+MK-2206 (100mg/kg+150mg/kg) *(*Figure 11*).* Treatments were administered daily through oral route for 3 weeks. The tumor volume in each case was inversely proportional to the efficacy of the treatment. The tumors were measured with ultrasonography at the beginning and at the end of the treatment.

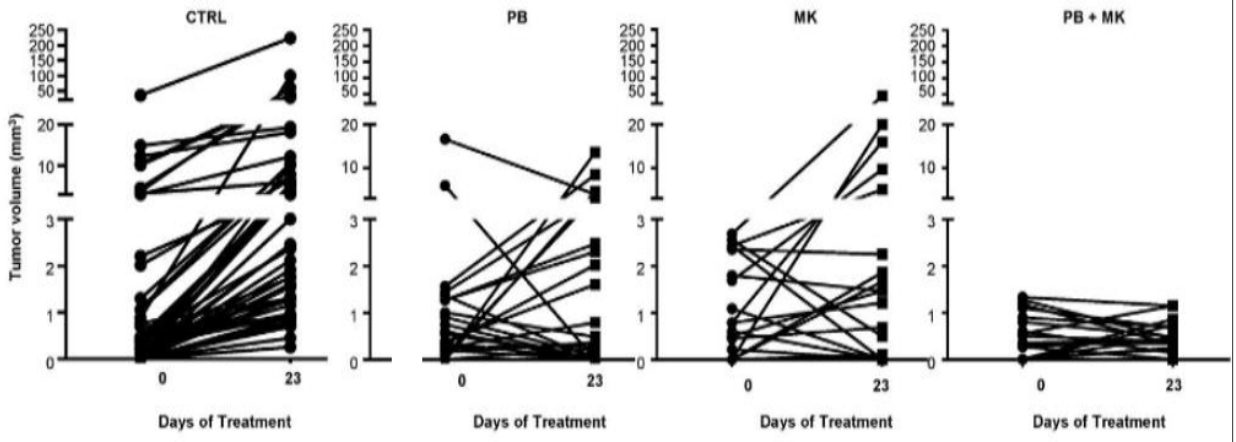


Table 1. Anti-cancer activity of Palbociclib, MK-2206 and their combination

CTRL = untreated; PB= Palbociclib; MK=MK-2206

	Number of mice	Tumor Volume difference between post and pre-treatment (mean ± SD, mm^3)
CTRL	13	9,80 ± 28,14
PB	6	0,55 ± 4,62
MK	6	4,63 ± 10,73
PB+MK	5	-0,01 ± 0,48

Figure 11. Anti-tumor effects of the therapy of Palbociclib and MK-2206 and combination.

Tumor nodules volumes in DEN-treated TG221 mice of the following groups are reported: (1) vehicle (CTRL) (n=13); (2) Palbociclib (PB) (n=6); (3) MK-2206 (MK) (n=6); (4) Palbociclib + MK-2206 (PB+MK) (n=5). Experimental therapies started at 6 months when all mice presented one or more tumor nodules in their livers. Single tumor nodules were monitored by ultrasound analysis at the beginning (Day 0) and end of treatment (Day 23) and the mean tumor size for the different treated groups was reported in the Table.

The investigation revealed a severe increase in tumor volume in the CTRL control group, a mixed response of the tumors in the PB group or in the MK-2206 group, and a highly significant growth inhibitory effect, very often shrinking of tumor, in the combination group. The combination evidenced a highly significant increased efficacy when compared to the other treatment groups. To assess the toxicity of treatments and the wellbeing of the various animal groups under treatment, the animals' weight was monitored every 5 days. In the combination treatment group, the drug toxicity resulted in severe weight loss in all the mice. The weight loss caused by individual drugs was doubled in the combination group which was too toxic to be continued for further studies *(*Figure 12*).*

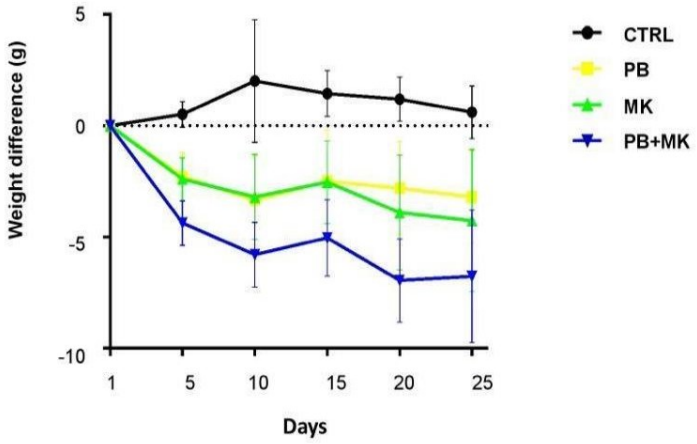


Figure 12. Toxicity evaluation of the combination therapy of Palbociclib and MK-2206.

Mice weight was monitored during treatment to check drug toxicity. Data are represented as mean +/- SD

Combination therapy of Palbociclib and miR-199a-3p in *in vitro* HCC models

To overcome MK-2206 toxicity, Palbociclib was investigated in combination with miR-199a-3p (Fornari et al. 2009) (Callegari et al. 2018) both *in vitro* and *in vivo* models. Firstly, the miR-199a-3p basal expression in the *in vitro* models Hep3B, HepG2 was tested. Since miR-199a-3p is expected to have a very low expression in liver cancer cells, HepG2/miR199 cells overexpressing miR-199a-3p were also analyzed as positive control (Callegari et al. 2013) (**Figure 13***).*

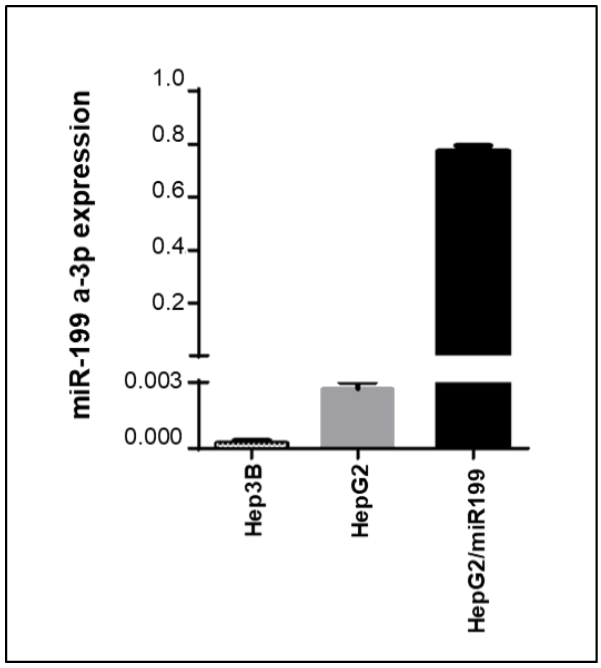


Figure 13. miR-199a-3p expression in in vitro experimental models.

Basal levels of miR-199 expression levels were assessed in human cell lines employed. As a positive control for miRNA expression, RNA extracted from HepG2/miR199 cells, a stable cell clone over-expressing miR-199a-3p. Data are represented as mean +SD

To evaluate the biological effects of Palbociclib and miR-199a-3p in HepG2 and Hep3B cells, the cells were transduced with an adeno-associated viral vector expressing miR-199a-3p (AAVV-199) at MOI (multiplicity of infection) = 100 and treated with Palbociclib (10uM in HepG2 cells and 20 uM in Hep3B). Specifically, Palbociclib was added to the cells 72 hours before evaluation. The miR-199a-3p expression was measured 120 hours (5 days) after transduction. The miR-199 expression was measured in the untreated (NT), PB treated (Palbociclib), AAVV-199, and AAVV-199+PB (). There was a

sharp increase in the miR-199 expression in HepG2 cells transduced with AAVV-199 *(**Figure 14*A**)*. There were similar increases in the Hep3B model as well *(**Figure 15*A**)*.

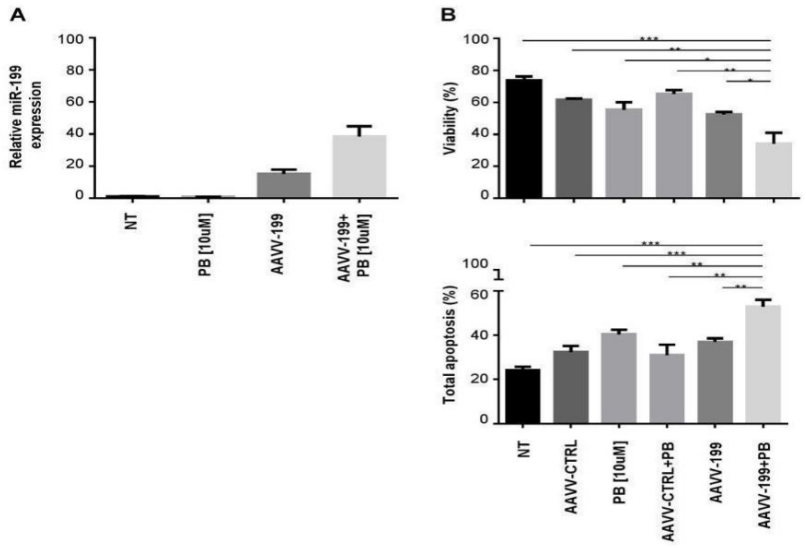


Figure 14. Biological effects of Palbociclib and miR-199a-3p on HepG2 cells.

miR-199 expression in HepG2 cells; (B) Evaluation of cell viability and apoptosis in HepG2 cells. Data are represented as mean +SD *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001

The combination resulted in a significantly decreased cell viability and an increased cell apoptosis compared to individual treatments or control AAVV (AAVV-CTRL) in both the cell models *(**Figure 14*B**), (**Figure 15*B**)*.

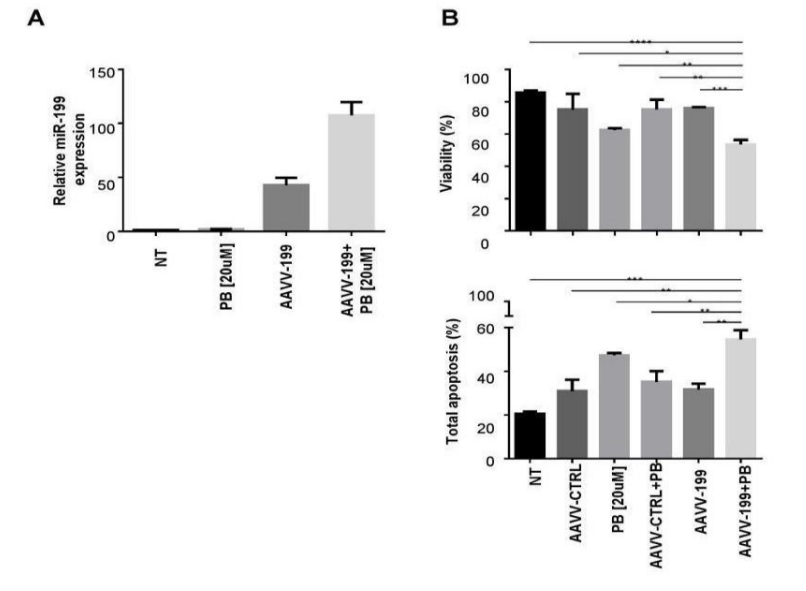


Figure 15: Biological effects of Palbociclib and miR-199a-3p on Hep3B cells.

(A) miR-199 expression in Hep3B cells; (B) Evaluation of cell viability and apoptosis in Hep3B cells Data are represented as mean + SD. *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001

The molecular effects of the combinations were investigated by testing the protein expression in each treatment group.

While Palbociclib led to a reduction of RB1 and p-RB1, the expression of miR-199a-3p led to a reduction of phosphorylated AKT (S473), decreasing AKT activation, in HepG2 cells *(***Figure 16*A*)**.

In Hep3B cells, the combined treatment reduced the phosphorylated AKT (S473), decreasing the activation of AKT as well as phosphorylation and amount of FOXM1 protein, which is also AKT-dependent and inhibited by miR-199a-3p *(***Figure 16*B*)**.

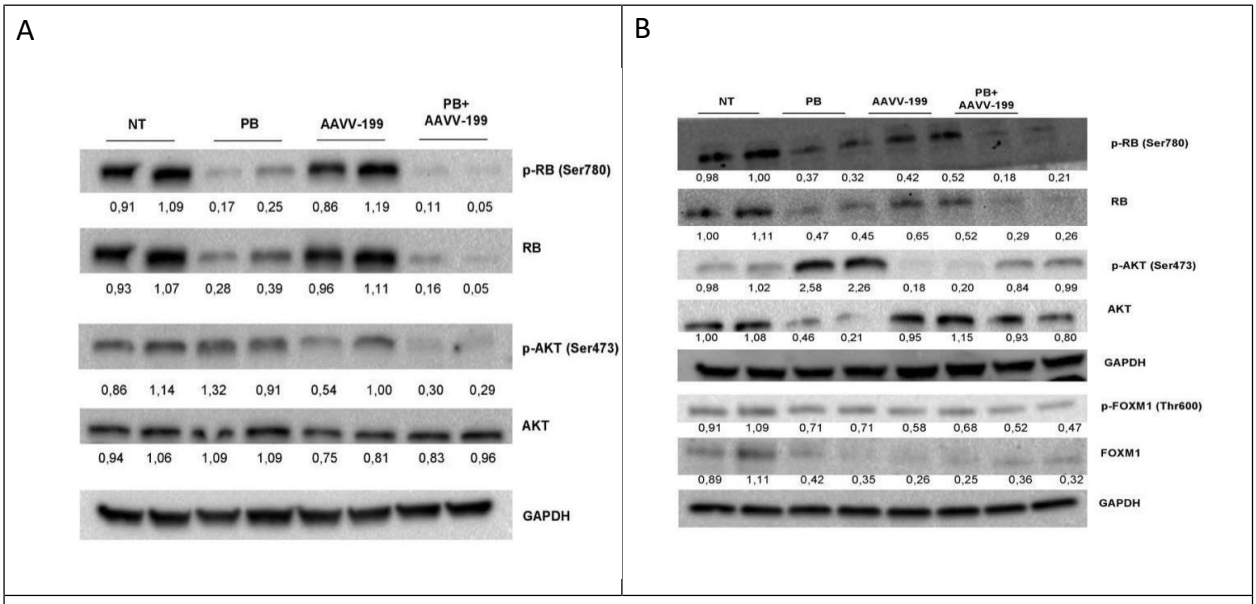


Figure 16. Molecular effects of Palbociclib and miR-199a-3p on HepG2 and Hep3B cells.

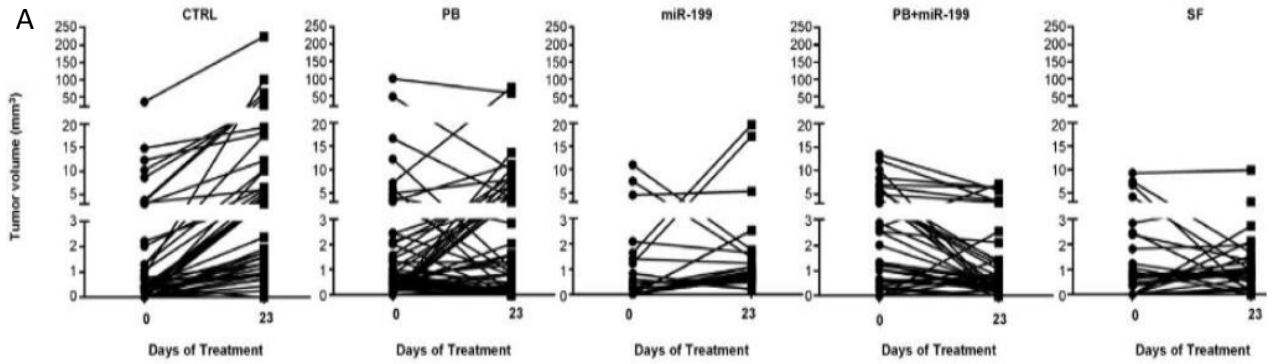
(A) RB, AKT protein expressions in HepG2 cells; (B). RB, AKT, FOXM1 protein expressions in Hep3B cells. The values are normalized on the GAPDH protein and compared to the average levels detected in the untreated cells.

Combination therapy of Palbociclib and miR-199a-3p in *in vivo* HCC models

The in vivo study of this therapy was very similar to the previous one. Once the tumors were 2-3mm^3 and the mice were 6 months old, they were separated into the treatment groups 1. CTRL (vehicle), 2. PB (100mg/kg), 3. miR-199a-3p mimics (5mg/kg), 4. PB + miR-199a-3p (100 mg/kg + 5mg/kg), and 5. Sorafenib treatment. Palbociclib and Sorafenib were administered through oral route for 3 weeks, every day at their respective doses. miRNA mimics were administered thrice a week intraperitoneally for 3

weeks. Tumor volumes were measured using ultrasonography at the beginning and at the end of the treatments. The animal group in the combination treatment showed a higher regression of tumor size than the individual treatments Palbociclib or miR-199 *(***Figure 17*A)***. The Sorafenib-treated group exhibited effects on tumors that were pretty much like the combination-treated group.

A



TABLES

Table 2. In vivo anti-cancer activity of Palbociclib and miR-199a-3p

CTRL = untreated; PB= palbociclib; SF= sorafenib

	Number of mice	Increase of Tumor Volume between post and pre-treatment (Mean ± SD, mm³)
CTRL	9	11,30 ± 30,90
PB	11	0,69 ± 12,50
PB+miR199	11	-1,62 ± 2,90
SF	9	-0,43 ± 2,13
miR199	9	1,29 ± 5,45

B

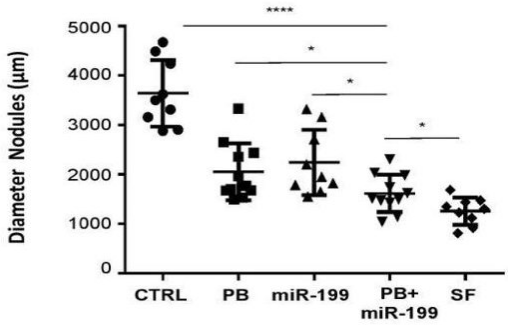


Figure 17. *miR-199a-3p increased anti-tumoral Palbociclib efficacy in vivo*

(A) Tumor nodules volumes in DEN-treated TG221 mice of the following groups: (1) scramble oligonucleotides (CTRL) (n=9); (2) Palbociclib (PB) (n=11); (3) miR-199a-3p mimics (miR-199) (n=9); (4) Palbociclib + miR-199a-3p mimics (PB+miR-199) (n=11); (5) sorafenib (SF) (n=9). Experimental therapies started at 6 months, when all mice presented one or more tumor nodules in their livers. Single tumor nodules were monitored by ultrasound at the beginning and end of treatment and mean tumor size for the different treated groups was reported in the Table; **(B)** Tumor size measurement in each experimental group. *: p value ≤ 0.05; ****: p value ≤ 0.0001

Once the treatment period ended, tumors from each treated mice group were compared and the lesion size was measured *(***Figure 17*A*)*. The effects of the combination of miR-199a-3p plus Palbociclib on the development and size of tumors were confirmed by the reduction in the size of tumor lesions. The weight data suggests that the combination treatment did not result in severe weight loss as compared to the individual treatments *(***Figure 18***)*. indicating that toxicity was tolerable and largely caused by Palbociclib, with minimal contribution of miR-199a-3p.

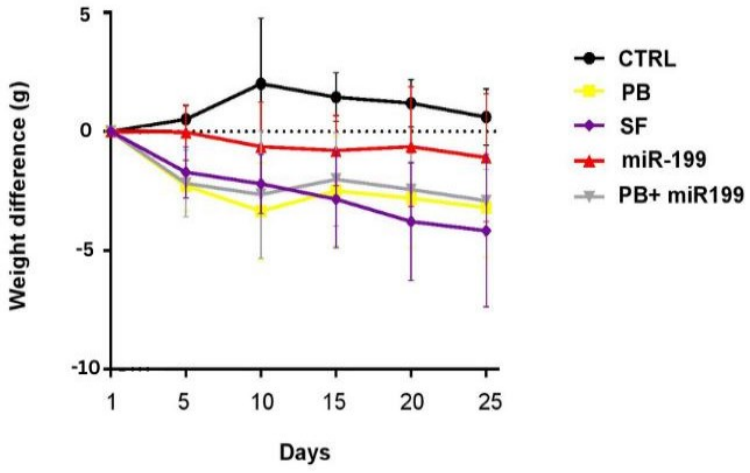


Figure 18. Weight measurements of the animal throughout the experiment.

Data are represented as mean +/- SD

The tumors collected from each experimental group went through immunohistochemical analysis for the apoptotic marker cleaved caspase-3 (Persad et al. 2004) and the tumor proliferation marker Ki67 (Wu et al. 2020). An increase in apoptosis and a decrease in cell proliferation were detected in mice treated with miR-199a-3p and Palbociclib combination in comparison with single agents or untreated controls. *(***Figure 19***).*

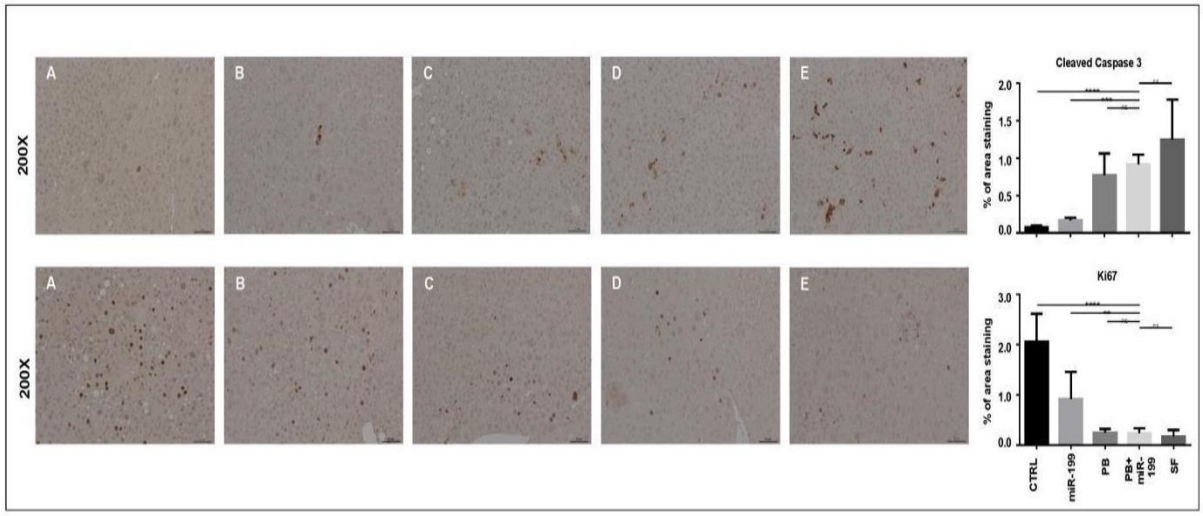


Figure 19. Immunohistochemical analysis for testing apoptosis and cell proliferation in TG221-derived HCC

(A) CTRL, (B) Palbociclib, (C) miR-199a-3p, (D) Palbociclib+ miR199a-3p, and (E) Sorafenib; The graphs indicate the percentage of areas stained for Caspase3 (upper panel) and Ki67 (lower panel). Magnification 200X, scale bar = 50µm. Data are represented as mean +SD. *: p value ≤ 0.05; **: p value ≤ 0.01; ***: p value ≤ 0.001; ****: p value ≤ 0.0001.

To assess the molecular effects of treatments in tumors, protein expressions were tested from the tumor samples obtained from all the treatment groups. The protein extracted from the HCC samples obtained from the *in vivo* models was used in pairs as biological replicates. The combination treatment resulted in a decreased phosphorylated RB1 and AKT proteins.

A key target of miR-199a-3b was also downregulated (PAK4) and was the confirmation of the miRNA mimics in action (**Figure 20*A***). miR-199 levels were evaluated in HCC samples to confirm miRNA expression (**Figure 20*B***).

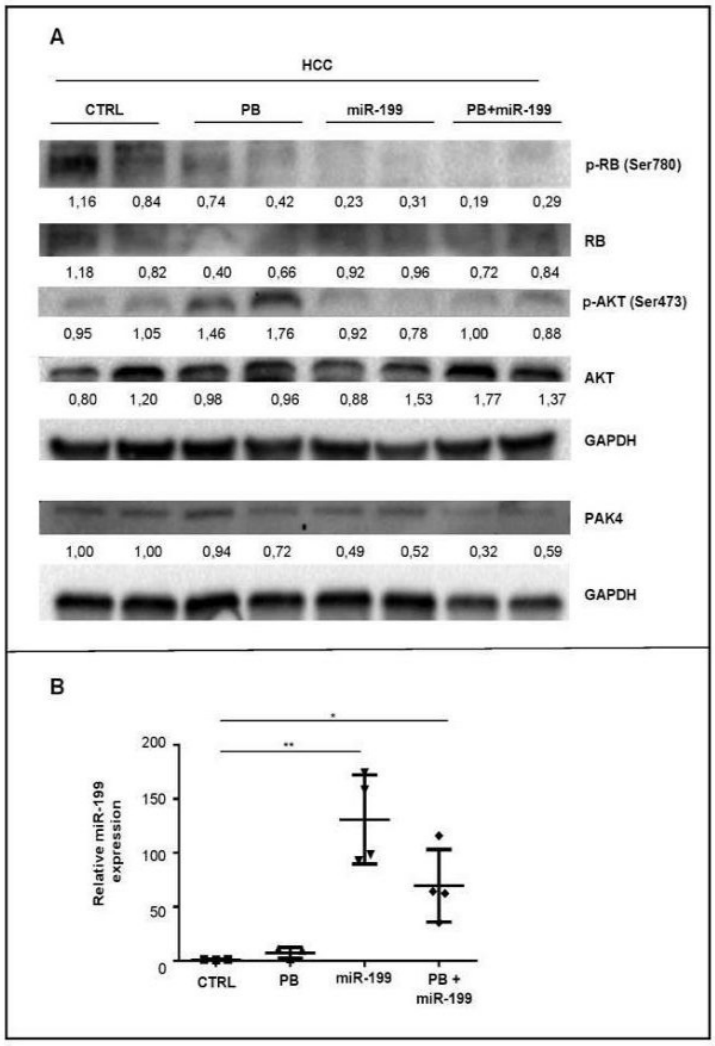


Figure 20. Molecular target expressions of miR-199a-3p and Palbociclib

(A) Expression of the molecular targets of the Palbociclib, miR-199a-3b, and the combination in in vivo model. The values are normalized on the GAPDH protein and compared to the average levels detected in the untreated cells**; (B)** Graphical representation of miR-199 expression in the experimental groups in in vivo models., *: p value ≤ 0.05; **: p value ≤ 0.01.

Overall, the reported findings support the notion that the combined treatment of Palbociclib and miR-199a-3p can exert a biological and molecular synergistic effect against HCC with tolerable toxic effects. Our investigation shows that miR-199a-3p can increase the efficacy of Palbociclib in the treatment of HCC and represent a promising therapeutic option for HCC, not only because it can enhance the antitumor activity of Palbociclib but also because it does not add significant toxicity.

DISCUSSION

The present study focuses on the combination of AKT inhibitors with Palbociclib in *in vivo* and *in vitro* models of liver cancer (HCC). In this study, we hypothesized that miRNA-based therapies could improve the efficacy of drugs already in clinical use either to increase their efficacy reduce their toxic effects or counteract the emergence of resistance phenomena. To this end, we investigated miR-199a-3p in combination with Palbociclib in experimental *in vitro* and *in vivo* models of HCC.

Palbociclib is a cyclin-dependent kinase 4/6 (CDK4/6) inhibitor that works by inhibiting the activity of CDK4 and CDK6, enzymes that are involved in cell cycle progression. By inhibiting CDK4/6 activity, Palbociclib slows down the cell cycle and prevents the growth and proliferation of cancer cells. More specifically, CDK4/6 are enzymes that play a critical role in the G1 phase of the cell cycle. In this stage, the cell prepares for DNA synthesis and cell division. CDK4/6, together with other proteins, phosphorylate and inhibit retinoblastoma protein (Rb), which in turn releases the transcription factor E2F; E2F then promotes the expression of genes required for the cell to progress through the cell cycle. Palbociclib acts by selectively inhibiting CDK4/6 activity. This causes cancer cells to remain in the G1 phase, preventing them from progressing through the cell cycle and dividing. Additionally, Palbociclib may also induce senescence, a state in which cells no longer divide but remain metabolically active.

Palbociclib is currently being tested in patients who have failed or are intolerant of standard first-line sorafenib therapy (NCT01356628) (Littman, Brus, and Burkart 2015). The use of Palbociclib in combination with other drugs has also been tested in models of HCC: additive effects have been demonstrated by the combination of Palbociclib and regorafenib (Digiacomo et al. 2020) or Palbociclib with sorafenib in xenograft models of human HCC (Bollard et al. 2017).

MiRNAs have emerged as promising molecules or targets for cancer therapy due to their role in the regulation of gene expression and their involvement in various cancer-related processes. MiRNAs that act as tumor suppressors can be reverted to suppress

tumor cell growth, while oncogenic miRNAs can be inhibited to reduce tumor cell proliferation and survival (Gramantieri et al. 2008) (Fornari et al. 2019) (Xu et al. 2018).

miR-199a-3p is a miRNA molecule that regulates gene expression by targeting messenger RNA (mRNA) molecules for degradation or translational inhibition. miR-199a-3p is downregulated in various cancer types, including hepatocellular carcinoma, where it has been found downregulated in virtually all liver cancers (Hou et al. 2011).

The function of miR-199a-3b varies depending on specific target genes and cellular context. miR-199a-3p can regulate two oncoproteins, MTOR and PAK4, which are crucial components of the PI3K-AKT-PTEN pathway involved in cell survival and proliferation (Engelman 2009). miR-199a-3b can also target the expression of cyclin D1 and CDK6, two key proteins involved in the cell cycle G1-S phase transition (Shen et al. 2015). By inhibiting the expression of these proteins, miR-199a-3b can slow or stop cell proliferation. miR-199a-3b also promotes apoptosis (programmed cell death) in cancer cells by targeting anti-apoptotic genes, such as BCL-2 and MCL-1 (Duan et al. 2011). It also targets the expression of matrix metalloproteinase-2 (MMP-2), a protein involved in the degradation of extracellular matrix components and in the promotion of tumor cell invasion and migration (Shen et al. 2015).

It has been previously demonstrated that *in vivo* administration of miR-199a-3p shows antitumor activity in TG221 mice and is well tolerated by mice (Callegari et al. 2018) (Callegari et al. 2019). The antitumor activity of miR-199a- 3p has also been demonstrated in various models of liver cancer, including subcutaneous and orthotopic models (W. Zhang et al. 2019) (Hou et al. 2011) (Ghosh et al. 2017), as well as models using patient-derived liver cells tumors (Shao et al. 2020).

Combining miRNA-based therapies with existing drugs is not new, and some studies have shown that this type of combination therapy can lead to better outcomes than using single agents alone (Cappuyns and Llovet 2022) (Ray 2020) (T. Zhang et al. 2021). Combining miRNA-based therapies with drugs already in clinical use might increase the treatment's efficacy by reducing its toxic/side effects and preventing the development of drug resistant phenomenon.

Here, we tested miR-199a-3p for its ability to enhance the antitumor activity of Palbociclib in the TG221 mouse model. The rationale for the use of Palbociclib in combination with miR-199a-3p is that Palbociclib, while suppressing CDK4/6 kinase activity, simultaneously increases AKT activity. Thus, we hypothesize that inhibition of the AKT pathway may synergize with Palbociclib to enhance antitumor activity. Indeed, we confirmed the suitability of the hypothesis by detecting a strong synergistic effect between Palbociclib and the pan-AKT inhibitor, MK-2206. However, the strong toxicity prevented the possibility of using this combination *in vivo*. Conversely, the combination of miR-199a-3p with Palbociclib demonstrated good therapeutic efficacy and excellent tolerability, providing preclinical proof-of-principle of the value of the combination of miR-199a-3p and Palbociclib in anti-HCC therapy. These results suggest that inhibition of AKT activity represents a general approach to enhance the antitumor efficacy of Palbociclib.

The ability of miR-199a-3p to enhance the antitumor activity of Palbociclib was investigated in the TG221 mouse model of liver cancer (HCC). The TG221 mouse strain is a transgenic mouse model that overexpresses the miR-221 miRNA in the liver (Callegari et al. 2012). This overexpression leads to the development of liver cancer, making it a valuable model for studying the disease. We have previously demonstrated that this miR-221 transgenic mouse model represents a fairly accurate liver cancer model for performing preclinical investigations aimed at testing miRNA-based therapies. Indeed, in addition to the upregulation of miR-221, other miRNAs known to play a key role in human HCC, such as miR-199a-3p, or miR-122 or miR-21 were deregulated in tumors arising from the TG221 model in a manner like the human HCC (Callegari et al. 2012). These disruptions in miRNA levels make the TG221 model an ideal preclinical model for testing miRNA-based therapies in liver cancer (Callegari et al. 2012).

The basis for using CDK4/6 inhibitors, such as Palbociclib, as an anticancer agent is the presence of cells with functional RB1 since RB1 protein is one of the major targets phosphorylated by activated CDK4/6.

The RB1 gene (also known as the retinoblastoma gene) plays a crucial role in cell cycle regulation and is frequently mutated in various cancer types (Mehyar et al. 2020). As

mentioned earlier, CDK4/6, along with other proteins, phosphorylate and inhibit retinoblastoma protein (Rb), which in turn releases the transcription factor E2F, which promotes the expression of genes necessary for the cell to progress through G1-S phases of the cell cycle. The regulation of RB1 protein levels and its phosphorylation status is a complex process that is not yet fully understood. The results of various studies, including ours, are consistent with the observation that CDK4/6 inhibitors, such as Palbociclib, are responsible not only for a decrease in RB1 phosphorylation, but can also cause a decrease in protein levels. The downregulation of RB1 protein observed in response to Palbociclib treatment is a puzzling phenomenon since RB1 is a well-known tumor suppressor protein. Several hypotheses have been proposed to explain the decrease in RB1 protein levels in response to CDK4/6 inhibition (Digiacomo et al. 2020) (Bollard et al. 2017) (Rubio et al. 2019).

Furthermore, our results challenge the idea that inhibition of RB1 phosphorylation is the only mechanism associated with the antitumor action of Palbociclib, considering that its growth-inhibiting effect is also observed in Hep3B cells, which carry a STOP codon in the RB1 gene and have very low intrinsic expression of RB1 protein. The presence of a stop codon in the RB1 gene in HEP3B cells results in a truncated, non-functional protein that is unable to perform its normal function. This type of mutation can lead to a loss of cell cycle regulation and contribute to the development of cancer.

Instead, this study highlights the importance of FOXM1, an oncogenic transcription factor crucial in cell cycle regulation, DNA damage repair, and cell survival (Laoukili et al. 2005). FOXM1 is aberrantly upregulated in several types of human cancers, including HCC (Yu et al. 2016) (Laoukili et al. 2005), and correlates with more aggressive tumor phenotypes, higher resistance to chemotherapy and radiotherapy, and worse prognosis. The mechanism of action of FOXM1 involves the regulation of the expression of many target genes involved in a broad range of cellular processes. These target genes include genes involved in cell cycle regulation (e.g., cyclin B1), DNA damage response (e.g., Rad51), and apoptosis (e.g., Bcl-2). FOXM1 also plays a critical role in regulating cell migration and invasion by controlling the expression of genes involved in epithelial-mesenchymal transition (EMT) and angiogenesis.

FOXM1 is known as a substrate of CDK4/6 (Anders et al. 2011), by which it is activated. Its lack of phosphorylation by CDK4/6 favors its degradation and the inhibition of its oncogenic activity (Anders et al. 2011).

Thus, the anticancer effects of Palbociclib may not only be due to the inhibition of RB1 phosphorylation but may also involve the inhibition of the oncogenic transcription factor FOXM1. This inhibition may also occur through a potential role of miR-199a-3p, as FOXM1 is also activated via the AKT pathway (Yao, Fan, and Lam 2018) (Chesnokov et al. 2021).

CONCLUSIONS

In general, this study suggests that miRNA-based therapy could find a useful application through combination with drugs already in clinical use to improve their efficacy without increasing toxicity. The combination of Palbociclib and miR-199a-3p could overcome some toxic effects when compared with sorafenib, a standard therapy in advanced HCC. Sorafenib has reported of toxic effects over time in patients with advanced HCC (Zhou and Fountzilas 2019) (Li, Gao, and Qu 2015) (Cabral, Tiribelli and Sukowati 2020), which makes the use of sorafenib limited by its toxic side effects during long-term treatments.

In summary, this study proposes the use of miRNA-based therapy in combination with existing drugs to enhance their efficacy while not increasing toxicity. The combination of Palbociclib with miR-199a-3p was indeed effective without increasing toxicity in *in vivo* models and may represent a potentially valuable alternative to sorafenib in advanced HCC. Furthermore, although not tested in this study, the combination of Palbociclib with miR-199a-3p could not only be effective in sorafenib-naïve conditions, but, considering the different mechanisms of action, it could find potential application in overcoming sorafenib resistance mechanisms. While a few clinical trials aimed at evaluating safety and efficacy of using miRNA modulation as a cancer treatment strategy are currently underway (ClinicalTrials.gov Identifier: NCT01829971; NCT02369198; NCT02580552; NCT03713320) (Hong et al. 2020) (van Zandwijk et al. 2017) (Witten and Slack 2020), the present study provides further progress in this direction.

BIBLIOGRAPHY

- Abou-Alfa, Ghassan K., Tim Meyer, Ann-Lii Cheng, Anthony B. El-Khoueiry, Lorenza Rimassa, Baek-Yeol Ryoo, Irfan Cicin, et al. 2018. "Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma." *The New England Journal of Medicine* 379 (1): 54–63. https://doi.org/10.1056/NEJMoa1717002.
- Anders, Lars, Nan Ke, Per Hydbring, Yoon J. Choi, Hans R. Widlund, Joel M. Chick, Huili Zhai, et al. 2011. "A Systematic Screen for CDK4/6 Substrates Links FOXM1 Phosphorylation to Senescence Suppression in Cancer Cells." *Cancer Cell* 20 (5): 620–34. https://doi.org/10.1016/j.ccr.2011.10.001.
- Beaver, Julia A., Laleh Amiri-Kordestani, Rosane Charlab, Wei Chen, Todd Palmby, Amy Tilley, Jeanne Fourie Zirkelbach, et al. 2015. "FDA Approval: Palbociclib for the Treatment of Postmenopausal Patients with Estrogen Receptor-Positive, HER2-Negative Metastatic Breast Cancer." *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 21 (21): 4760–66. https://doi.org/10.1158/1078-0432.CCR-15-1185.
- Befeler, Alex S., and Adrian M. Di Bisceglie. 2002. "Hepatocellular Carcinoma: Diagnosis and Treatment." *Gastroenterology* 122 (6): 1609–19. https://doi.org/10.1053/gast.2002.33411.
- Bollard, Julien, Verónica Miguela, Marina Ruiz de Galarreta, Anu Venkatesh, C. Billie Bian, Mark P. Roberto, Victoria Tovar, et al. 2017. "Palbociclib (PD-0332991), a Selective CDK4/6 Inhibitor, Restricts Tumour Growth in Preclinical Models of Hepatocellular Carcinoma." *Gut* 66 (7): 1286–96. https://doi.org/10.1136/gutjnl-2016-312268.
- Bonelli, Mara A., Graziana Digiacomo, Claudia Fumarola, Roberta Alfieri, Federico Quaini, Angela Falco, Denise Madeddu, et al. 2017. "Combined Inhibition of CDK4/6 and PI3K/AKT/mTOR Pathways Induces a Synergistic Anti-Tumor Effect in Malignant Pleural Mesothelioma Cells." *Neoplasia (New York, N.Y.)* 19 (8): 637–48. https://doi.org/10.1016/j.neo.2017.05.003.
- Bonelli, Mara, Rita Terenziani, Silvia Zoppi, Claudia Fumarola, Silvia La Monica, Daniele Cretella, Roberta Alfieri, et al. 2020. "Dual Inhibition of CDK4/6 and PI3K/AKT/mTOR Signaling Impairs Energy Metabolism in MPM Cancer Cells." *International Journal of Molecular Sciences* 21 (14): 5165. https://doi.org/10.3390/ijms21145165.
- Bressac, B., M. Kew, J. Wands, and M. Ozturk. 1991. "Selective G to T Mutations of P53 Gene in Hepatocellular Carcinoma from Southern Africa." *Nature* 350 (6317): 429–31. https://doi.org/10.1038/350429a0.
- Bruix, Jordi, Shukui Qin, Philippe Merle, Alessandro Granito, Yi-Hsiang Huang, György Bodoky, Marc Pracht, et al. 2017. "Regorafenib for Patients with Hepatocellular Carcinoma Who Progressed on Sorafenib Treatment (RESORCE): A Randomised, Double-Blind, Placebo-Controlled, Phase 3 Trial." *Lancet (London, England)* 389 (10064): 56–66. https://doi.org/10.1016/S0140-6736(16)32453-9.

- Cabral, Loraine Kay D., Claudio Tiribelli, and Caecilia H. C. Sukowati. 2020. “Sorafenib Resistance in Hepatocellular Carcinoma: The Relevance of Genetic Heterogeneity.” *Cancers* 12 (6): 1576. https://doi.org/10.3390/cancers12061576.
- Callegari, Elisa, Lucilla D’Abundo, Paola Guerriero, Carolina Simioni, Bahaeldin K. Elamin, Marta Russo, Alice Cani, et al. 2018. “miR-199a-3p Modulates MTOR and PAK4 Pathways and Inhibits Tumor Growth in a Hepatocellular Carcinoma Transgenic Mouse Model.” *Molecular Therapy. Nucleic Acids* 11 (June): 485–93. https://doi.org/10.1016/j.omtn.2018.04.002.
- Callegari, Elisa, Marco Domenicali, Ram Charan Shankaraiah, Lucilla D’Abundo, Paola Guerriero, Ferdinando Giannone, Maurizio Baldassarre, et al. 2019. “MicroRNA-Based Prophylaxis in a Mouse Model of Cirrhosis and Liver Cancer.” *Molecular Therapy. Nucleic Acids* 14 (March): 239–50. https://doi.org/10.1016/j.omtn.2018.11.018.
- Callegari, Elisa, Bahaeldin K. Elamin, Lucilla D’Abundo, Simonetta Falzoni, Giovanna Donvito, Farzaneh Moshiri, Maddalena Milazzo, et al. 2013. “Anti-Tumor Activity of a miR-199-Dependent Oncolytic Adenovirus.” *PloS One* 8 (9): e73964. https://doi.org/10.1371/journal.pone.0073964.
- Callegari, Elisa, Bahaeldin K. Elamin, Ferdinando Giannone, Maddalena Milazzo, Giuseppe Altavilla, Francesca Fornari, Luciano Giacomelli, et al. 2012. “Liver Tumorigenicity Promoted by microRNA-221 in a Mouse Transgenic Model.” *Hepatology (Baltimore, Md.)* 56 (3): 1025–33. https://doi.org/10.1002/hep.25747.
- Cappuyns, Sarah, and Josep M. Llovet. 2022. “Combination Therapies for Advanced Hepatocellular Carcinoma: Biomarkers and Unmet Needs.” *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 28 (16): 3405–7. https://doi.org/10.1158/1078-0432.CCR-22-1213.
- Chesnokov, Mikhail S., Marianna Halasi, Soheila Borhani, Zarema Arbieva, Binal N. Shah, Rick Oerlemans, Irum Khan, Carlos J. Camacho, and Andrei L. Gartel. 2021. “Novel FOXM1 Inhibitor Identified via Gene Network Analysis Induces Autophagic FOXM1 Degradation to Overcome Chemoresistance of Human Cancer Cells.” *Cell Death & Disease* 12 (7): 704. https://doi.org/10.1038/s41419-021-03978-0.
- Cretella, Daniele, Andrea Ravelli, Claudia Fumarola, Silvia La Monica, Graziana Digiacomo, Andrea Cavazzoni, Roberta Alfieri, et al. 2018. “The Anti-Tumor Efficacy of CDK4/6 Inhibition Is Enhanced by the Combination with PI3K/AKT/mTOR Inhibitors through Impairment of Glucose Metabolism in TNBC Cells.” *Journal of Experimental & Clinical Cancer Research: CR* 37 (1): 72. https://doi.org/10.1186/s13046-018-0741-3.
- Cristofanilli, Massimo, Hope S. Rugo, Seock-Ah Im, Dennis J. Slamon, Nadia Harbeck, Igor Bondarenko, Norikazu Masuda, et al. 2022. “Overall Survival with Palbociclib and Fulvestrant in Women with HR+/HER2- ABC: Updated Exploratory Analyses of PALOMA-3, a Double-Blind, Phase III Randomized Study.” *Clinical Cancer Research: An Official Journal of the American*

Association for Cancer Research 28 (16): 3433–42. https://doi.org/10.1158/1078-0432.CCR-22-0305.

- D'Amico, Gennaro, Alberto Morabito, Mario D'Amico, Linda Pasta, Giuseppe Malizia, Paola Rebora, and Maria Grazia Valsecchi. 2018. "Clinical States of Cirrhosis and Competing Risks." *Journal of Hepatology* 68 (3): 563–76. https://doi.org/10.1016/j.jhep.2017.10.020.
- Di Martino, Maria Teresa, Mariamena Arbitrio, Daniele Caracciolo, Alessia Cordua, Onofrio Cuomo, Katia Grillone, Caterina Riillo, et al. 2022. "miR-221/222 as Biomarkers and Targets for Therapeutic Intervention on Cancer and Other Diseases: A Systematic Review." *Molecular Therapy. Nucleic Acids* 27 (March): 1191–1224. https://doi.org/10.1016/j.omtn.2022.02.005.
- Digiacomo, Graziana, Claudia Fumarola, Silvia La Monica, Mara A. Bonelli, Daniele Cretella, Roberta Alfieri, Andrea Cavazzoni, et al. 2020. "Simultaneous Combination of the CDK4/6 Inhibitor Palbociclib With Regorafenib Induces Enhanced Anti-Tumor Effects in Hepatocarcinoma Cell Lines." *Frontiers in Oncology* 10: 563249. https://doi.org/10.3389/fonc.2020.563249.
- Duan, Zhenfeng, Edwin Choy, David Harmon, Xianzhe Liu, Michiro Susa, Henry Mankin, and Francis Hornicek. 2011. "MicroRNA-199a-3p Is Downregulated in Human Osteosarcoma and Regulates Cell Proliferation and Migration." *Molecular Cancer Therapeutics* 10 (8): 1337–45. https://doi.org/10.1158/1535-7163.MCT-11-0096.
- Engelman, Jeffrey A. 2009. "Targeting PI3K Signalling in Cancer: Opportunities, Challenges and Limitations." *Nature Reviews. Cancer* 9 (8): 550–62. https://doi.org/10.1038/nrc2664.
- Fornari, Francesca, Laura Gramantieri, Elisa Callegari, Ram C. Shankaraiah, Fabio Piscaglia, Massimo Negrini, and Catia Giovannini. 2019. "MicroRNAs in Animal Models of HCC." *Cancers* 11 (12): 1906. https://doi.org/10.3390/cancers11121906.
- Fornari, Francesca, Maddalena Milazzo, Pasquale Chieco, Massimo Negrini, George Adrian Calin, Gian Luca Grazi, Daniela Pollutri, Carlo Maria Croce, Luigi Bolondi, and Laura Gramantieri. 2010. "MiR-199a-3p Regulates mTOR and c-Met to Influence the Doxorubicin Sensitivity of Human Hepatocarcinoma Cells." *Cancer Research* 70 (12): 5184–93. https://doi.org/10.1158/0008-5472.CAN-10-0145.
- Fracanzani, A. L., D. Conte, M. Fraquelli, E. Taioli, M. Mattioli, A. Losco, and S. Fargion. 2001. "Increased Cancer Risk in a Cohort of 230 Patients with Hereditary Hemochromatosis in Comparison to Matched Control Patients with Non-Iron-Related Chronic Liver Disease." *Hepatology (Baltimore, Md.)* 33 (3): 647–51. https://doi.org/10.1053/jhep.2001.22506.
- Freemantle, Nick, Patrick Mollon, Tim Meyer, Ann-Lii Cheng, Anthony B. El-Khoueiry, Robin K. Kelley, Ari D. Baron, Fawzi Benzaghou, Milan Mangeshkar, and Ghassan K. Abou-Alfa. 2022. "Quality of Life Assessment of Cabozantinib in Patients with Advanced Hepatocellular Carcinoma in the CELESTIAL Trial."

European Journal of Cancer (Oxford, England: 1990) 168 (June): 91–98. https://doi.org/10.1016/j.ejca.2022.03.021.

- Friemel, Juliane, Markus Rechsteiner, Marion Bawohl, Lukas Frick, Beat Müllhaupt, Mickaël Lesurtel, and Achim Weber. 2016. “Liver Cancer with Concomitant TP53 and CTNNB1 Mutations: A Case Report.” *BMC Clinical Pathology* 16: 7. https://doi.org/10.1186/s12907-016-0029-5.
- Fry, David W., Patricia J. Harvey, Paul R. Keller, William L. Elliott, Maryanne Meade, Erin Trachet, Mudher Albassam, et al. 2004. “Specific Inhibition of Cyclin-Dependent Kinase 4/6 by PD 0332991 and Associated Antitumor Activity in Human Tumor Xenografts.” *Molecular Cancer Therapeutics* 3 (11): 1427–38.
- Ghosh, Alip, Debanjali Dasgupta, Amit Ghosh, Shrabasti Roychoudhury, Dhiraj Kumar, Mahadeo Gorain, Ramesh Butti, et al. 2017. “MiRNA199a-3p Suppresses Tumor Growth, Migration, Invasion and Angiogenesis in Hepatocellular Carcinoma by Targeting VEGFA, VEGFR1, VEGFR2, HGF and MMP2.” *Cell Death & Disease* 8 (3): e2706. https://doi.org/10.1038/cddis.2017.123.
- Giovannini, Catia, Francesca Fornari, Rossella Dallo, Martina Gagliardi, Elisa Nipoti, Francesco Vasuri, Camelia Alexandra Coadă, Matteo Ravaioli, Luigi Bolondi, and Laura Gramantieri. 2018. “MiR-199-3p Replacement Affects E-Cadherin Expression through Notch1 Targeting in Hepatocellular Carcinoma.” *Acta Histochemica* 120 (2): 95–102. https://doi.org/10.1016/j.acthis.2017.12.004.
- Global Burden of Disease Liver Cancer Collaboration, Tomi Akinyemiju, Semaw Abera, Muktar Ahmed, Noore Alam, Mulubirhan Assefa Alemayohu, Christine Allen, et al. 2017. “The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level: Results From the Global Burden of Disease Study 2015.” *JAMA Oncology* 3 (12): 1683. https://doi.org/10.1001/jamaoncol.2017.3055.
- Gramantieri, Laura, Francesca Fornari, Elisa Callegari, Silvia Sabbioni, Giovanni Lanza, Carlo M. Croce, Luigi Bolondi, and Massimo Negrini. 2008. “MicroRNA Involvement in Hepatocellular Carcinoma.” *Journal of Cellular and Molecular Medicine* 12 (6A): 2189–2204. https://doi.org/10.1111/j.1582-4934.2008.00533.x.
- Grieco, A., M. Pompili, G. Caminiti, L. Miele, M. Covino, B. Alfei, G. L. Rapaccini, and G. Gasbarrini. 2005. “Prognostic Factors for Survival in Patients with Early-Intermediate Hepatocellular Carcinoma Undergoing Non-Surgical Therapy: Comparison of Okuda, CLIP, and BCLC Staging Systems in a Single Italian Centre.” *Gut* 54 (3): 411–18. https://doi.org/10.1136/gut.2004.048124.
- Guichard, Cécile, Giuliana Amaddeo, Sandrine Imbeaud, Yannick Ladeiro, Laura Pelletier, Ichrafe Ben Maad, Julien Calderaro, et al. 2012. “Integrated Analysis of Somatic Mutations and Focal Copy-Number Changes Identifies Key Genes and Pathways in Hepatocellular Carcinoma.” *Nature Genetics* 44 (6): 694–98. https://doi.org/10.1038/ng.2256.
- Hirai, Hiroshi, Hiroshi Sootome, Yoko Nakatsuru, Katsuyoshi Miyama, Shunsuke Taguchi, Kyoko Tsujioka, Yoko Ueno, et al. 2010. “MK-2206, an Allosteric Akt

Inhibitor, Enhances Antitumor Efficacy by Standard Chemotherapeutic Agents or Molecular Targeted Drugs in Vitro and in Vivo." *Molecular Cancer Therapeutics* 9 (7): 1956–67. https://doi.org/10.1158/1535-7163.MCT-09-1012.

- Holczbauer, Ágnes, Kirk J. Wangensteen, and Soona Shin. 2022. "Cellular Origins of Regenerating Liver and Hepatocellular Carcinoma." *JHEP Reports: Innovation in Hepatology* 4 (4): 100416. https://doi.org/10.1016/j.jhepr.2021.100416.
- Hou, Jin, Li Lin, Weiping Zhou, Zhengxin Wang, Guoshan Ding, Qiongzhu Dong, Lunxiu Qin, et al. 2011. "Identification of miRNomes in Human Liver and Hepatocellular Carcinoma Reveals miR-199a/b-3p as Therapeutic Target for Hepatocellular Carcinoma." *Cancer Cell* 19 (2): 232–43. https://doi.org/10.1016/j.ccr.2011.01.001.
- Kaplan, David E., and K. Rajender Reddy. 2003. "Rising Incidence of Hepatocellular Carcinoma: The Role of Hepatitis B and C; the Impact on Transplantation and Outcomes." *Clinics in Liver Disease* 7 (3): 683–714. https://doi.org/10.1016/s1089-3261(03)00060-6.
- Kelley, Robin K., Patrick Mollon, Jean-Frédéric Blanc, Bruno Daniele, Thomas Yau, Ann-Lii Cheng, Velichka Valcheva, Florence Marteau, Ines Guerra, and Ghassan K. Abou-Alfa. 2020. "Comparative Efficacy of Cabozantinib and Regorafenib for Advanced Hepatocellular Carcinoma." *Advances in Therapy* 37 (6): 2678–95. https://doi.org/10.1007/s12325-020-01378-y.
- Kobayashi, Masaki, Kenjiro Sawada, Koji Nakamura, Akihiko Yoshimura, Mayuko Miyamoto, Aasa Shimizu, Kyoso Ishida, et al. 2018. "Exosomal miR-1290 Is a Potential Biomarker of High-Grade Serous Ovarian Carcinoma and Can Discriminate Patients from Those with Malignancies of Other Histological Types." *Journal of Ovarian Research* 11 (1): 81. https://doi.org/10.1186/s13048-018-0458-0.
- Koj, A., A. Guzdek, J. Potempa, E. Korzus, and J. Travis. 1994. "Origin of Circulating Acute Phase Cytokines: Modified Proteins May Trigger IL-6 Production by Macrophages. Preliminary Report." *Journal of Physiology and Pharmacology: An Official Journal of the Polish Physiological Society* 45 (1): 69–80.
- Konstantinou, Dimitris, and Melanie Deutsch. 2015. "The Spectrum of HBV/HCV Coinfection: Epidemiology, Clinical Characteristics, Viralinteractions and Management." *Annals of Gastroenterology* 28 (2): 221–28.
- Kota, Janaiah, Raghu R. Chivukula, Kathryn A. O'Donnell, Erik A. Wentzel, Chrystal L. Montgomery, Hun-Way Hwang, Tsung-Cheng Chang, et al. 2009. "Therapeutic microRNA Delivery Suppresses Tumorigenesis in a Murine Liver Cancer Model." *Cell* 137 (6): 1005–17. https://doi.org/10.1016/j.cell.2009.04.021.
- Laoukili, Jamila, Matthijs R. H. Kooistra, Alexandra Brás, Jos Kauw, Ron M. Kerkhoven, Ashby Morrison, Hans Clevers, and René H. Medema. 2005. "FoxM1 Is Required for Execution of the Mitotic Programme and Chromosome Stability." *Nature Cell Biology* 7 (2): 126–36. https://doi.org/10.1038/ncb1217.

- Lee, Y. I., S. Lee, G. C. Das, U. S. Park, S. M. Park, and Y. I. Lee. 2000. “Activation of the Insulin-like Growth Factor II Transcription by Aflatoxin B1 Induced P53 Mutant 249 Is Caused by Activation of Transcription Complexes; Implications for a Gain-of-Function during the Formation of Hepatocellular Carcinoma.” *Oncogene* 19 (33): 3717–26. https://doi.org/10.1038/sj.onc.1203694.
- Lencioni, Riccardo A., Hans-Peter Allgaier, Dania Cioni, Manfred Olschewski, Peter Deibert, Laura Crocetti, Holger Frings, et al. 2003. “Small Hepatocellular Carcinoma in Cirrhosis: Randomized Comparison of Radio-Frequency Thermal Ablation versus Percutaneous Ethanol Injection.” *Radiology* 228 (1): 235–40. https://doi.org/10.1148/radiol.2281020718.
- Li, Ye, Zu-Hua Gao, and Xian-Jun Qu. 2015. “The Adverse Effects of Sorafenib in Patients with Advanced Cancers.” *Basic & Clinical Pharmacology & Toxicology* 116 (3): 216–21. https://doi.org/10.1111/bcpt.12365.
- Lingiah, Vivek A., Mumtaz Niazi, Raquel Olivo, Flavio Paterno, James V. Guarrera, and Nikolaos T. Pyrsopoulos. 2020. “Liver Transplantation Beyond Milan Criteria.” *Journal of Clinical and Translational Hepatology* 8 (1): 69–75. https://doi.org/10.14218/JCTH.2019.00050.
- Littman, Susan Joy, Christina Brus, and Ashlie Burkart. 2015. “A Phase II Study of Palbociclib (PD-0332991) in Adult Patients with Advanced Hepatocellular Carcinoma.” *Journal of Clinical Oncology* 33 (3_suppl): 277–277. https://doi.org/10.1200/jco.2015.33.3_suppl.277.
- Liu, Ming, Lingxi Jiang, and Xin-Yuan Guan. 2014. “The Genetic and Epigenetic Alterations in Human Hepatocellular Carcinoma: A Recent Update.” *Protein & Cell* 5 (9): 673–91. https://doi.org/10.1007/s13238-014-0065-9.
- Llovet, J. M., C. Brú, and J. Bruix. 1999. “Prognosis of Hepatocellular Carcinoma: The BCLC Staging Classification.” *Seminars in Liver Disease* 19 (3): 329–38. https://doi.org/10.1055/s-2007-1007122.
- Llovet, Josep M., Jessica Zucman-Rossi, Eli Pikarsky, Bruno Sangro, Myron Schwartz, Morris Sherman, and Gregory Gores. 2016. “Hepatocellular Carcinoma.” *Nature Reviews. Disease Primers* 2 (April): 16018. https://doi.org/10.1038/nrdp.2016.18.
- Lorentzen, Cathrine Lund, John B Haanen, Özcan Met, and Inge Marie Svane. 2022. “Clinical Advances and Ongoing Trials of mRNA Vaccines for Cancer Treatment.” *The Lancet Oncology* 23 (10): e450–58. https://doi.org/10.1016/S1470-2045(22)00372-2.
- Loudianos, G., and J. D. Gitlin. 2000. “Wilson’s Disease.” *Seminars in Liver Disease* 20 (3): 353–64. https://doi.org/10.1055/s-2000-9389.
- Magnussen, Arvin, and Mansour A. Parsi. 2013. “Aflatoxins, Hepatocellular Carcinoma and Public Health.” *World Journal of Gastroenterology* 19 (10): 1508–12. https://doi.org/10.3748/wjg.v19.i10.1508.

- Mahadeva, R., W. S. Chang, T. R. Dafforn, D. J. Oakley, R. C. Foreman, J. Calvin, D. G. Wight, and D. A. Lomas. 1999. “Heteropolymerization of S, I, and Z Alpha1-Antitrypsin and Liver Cirrhosis.” *The Journal of Clinical Investigation* 103 (7): 999–1006. https://doi.org/10.1172/JCI4874.
- Marrero, Jorge A., Laura M. Kulik, Claude B. Sirlin, Andrew X. Zhu, Richard S. Finn, Michael M. Abecassis, Lewis R. Roberts, and Julie K. Heimbach. 2018. “Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases.” *Hepatology (Baltimore, Md.)* 68 (2): 723–50. https://doi.org/10.1002/hep.29913.
- Martín-Navarro, Carmen M., Atteneri López-Arencibia, Ines Sifaoui, María Reyes-Batlle, Alfonso M. Cabello-Vílchez, Sutherland Maciver, Basilio Valladares, Jose E. Piñero, and Jacob Lorenzo-Morales. 2014. “PrestoBlue® and AlamarBlue® Are Equally Useful as Agents to Determine the Viability of Acanthamoeba Trophozoites.” *Experimental Parasitology* 145 Suppl (November): S69-72. https://doi.org/10.1016/j.exppara.2014.03.024.
- Masuzaki, Ryota, Seth J. Karp, and Masao Omata. 2012. “New Serum Markers of Hepatocellular Carcinoma.” *Seminars in Oncology* 39 (4): 434–39. https://doi.org/10.1053/j.seminoncol.2012.05.009.
- Mehyar, Mustafa, Mohammad Mosallam, Abdelghani Tbakhi, Ala Saab, Iyad Sultan, Rasha Deebajah, Imad Jaradat, et al. 2020. “Impact of RB1 Gene Mutation Type in Retinoblastoma Patients on Clinical Presentation and Management Outcome.” *Hematology/Oncology and Stem Cell Therapy* 13 (3): 152–59. https://doi.org/10.1016/j.hemonc.2020.02.006.
- Molife, L. Rhoda, Li Yan, Joanna Vitfell-Rasmussen, Adriane M. Zernhelt, Daniel M. Sullivan, Philippe A. Cassier, Eric Chen, et al. 2014. “Phase 1 Trial of the Oral AKT Inhibitor MK-2206 plus Carboplatin/Paclitaxel, Docetaxel, or Erlotinib in Patients with Advanced Solid Tumors.” *Journal of Hematology & Oncology* 7 (January): 1. https://doi.org/10.1186/1756-8722-7-1.
- Mu, Xueru, Regina Español-Suñer, Ingmar Mederacke, Silvia Affò, Rita Manco, Christine Sempoux, Frédéric P. Lemaigre, et al. 2015. “Hepatocellular Carcinoma Originates from Hepatocytes and Not from the Progenitor/Biliary Compartment.” *The Journal of Clinical Investigation* 125 (10): 3891–3903. https://doi.org/10.1172/JCI77995.
- Nadarevic, Tin, Agostino Colli, Vanja Giljaca, Mirella Fraquelli, Giovanni Casazza, Cristina Manzotti, Davor Štimac, and Damir Miletic. 2022. “Magnetic Resonance Imaging for the Diagnosis of Hepatocellular Carcinoma in Adults with Chronic Liver Disease.” *The Cochrane Database of Systematic Reviews* 5 (5): CD014798. https://doi.org/10.1002/14651858.CD014798.pub2.
- Niu, Zhao-Shan, Xiao-Jun Niu, and Wen-Hong Wang. 2016. “Genetic Alterations in Hepatocellular Carcinoma: An Update.” *World Journal of Gastroenterology* 22 (41): 9069–95. https://doi.org/10.3748/wjg.v22.i41.9069.

- Parmar, J. S., and D. A. Lomas. 2000. “Alpha-1-Antitrypsin Deficiency, the Serpinopathies and Conformational Disease.” *Journal of the Royal College of Physicians of London* 34 (3): 295–300.
- Persad, Rajendra, Chen Liu, Tsung-Teh Wu, Patrick S. Houlihan, Stanley R. Hamilton, Anna M. Diehl, and Asif Rashid. 2004. “Overexpression of Caspase-3 in Hepatocellular Carcinomas.” *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc* 17 (7): 861–67. https://doi.org/10.1038/modpathol.3800146.
- Pillai-Kastoori, Lakshmi, Amy R. Schutz-Geschwender, and Jeff A. Harford. 2020. “A Systematic Approach to Quantitative Western Blot Analysis.” *Analytical Biochemistry* 593 (March): 113608. https://doi.org/10.1016/j.ab.2020.113608.
- Pons, Fernando, Maria Varela, and Josep M. Llovet. 2005. “Staging Systems in Hepatocellular Carcinoma.” *HPB: The Official Journal of the International Hepato Pancreato Biliary Association* 7 (1): 35–41. https://doi.org/10.1080/13651820410024058.
- Ray, Katrina. 2020. “A Combination Therapy to Keep Hepatocellular Carcinoma in Check.” *Nature Reviews Gastroenterology & Hepatology* 17 (8): 452–452. https://doi.org/10.1038/s41575-020-0337-1.
- Reyes, Cesar V. 2008. “Hepatocellular Carcinoma in Wilson Disease-Related Liver Cirrhosis.” *Gastroenterology & Hepatology* 4 (6): 435–37.
- Rimassa, Lorenza, and Armando Santoro. 2009. “Sorafenib Therapy in Advanced Hepatocellular Carcinoma: The SHARP Trial.” *Expert Review of Anticancer Therapy* 9 (6): 739–45. https://doi.org/10.1586/era.09.41.
- Roayaie, Sasan, Ghalib Jibara, Bachir Taouli, and Myron Schwartz. 2013. “Resection of Hepatocellular Carcinoma with Macroscopic Vascular Invasion.” *Annals of Surgical Oncology* 20 (12): 3754–60. https://doi.org/10.1245/s10434-013-3074-7.
- Rong, Minhua, Gang Chen, and Yiwu Dang. 2013. “Increased miR-221 Expression in Hepatocellular Carcinoma Tissues and Its Role in Enhancing Cell Growth and Inhibiting Apoptosis in Vitro.” *BMC Cancer* 13 (January): 21. https://doi.org/10.1186/1471-2407-13-21.
- Rubio, Carolina, Mónica Martínez-Fernández, Cristina Segovia, Iris Lodewijk, Cristian Suarez-Cabrera, Carmen Segrelles, Fernando López-Calderón, et al. 2019. “CDK4/6 Inhibitor as a Novel Therapeutic Approach for Advanced Bladder Cancer Independently of RB1 Status.” *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 25 (1): 390–402. https://doi.org/10.1158/1078-0432.CCR-18-0685.
- Schneller, Doris, and Peter Angel. 2019. “Cellular Origin of Hepatocellular Carcinoma.” In *Hepatocellular Carcinoma*, edited by Janina E. E. Tirnitz-Parker. Brisbane (AU): Codon Publications. http://www.ncbi.nlm.nih.gov/books/NBK549196/.

- Schulze, Kornelius, Sandrine Imbeaud, Eric Letouzé, Ludmil B. Alexandrov, Julien Calderaro, Sandra Rebouissou, Gabrielle Couchy, et al. 2015. “Exome Sequencing of Hepatocellular Carcinomas Identifies New Mutational Signatures and Potential Therapeutic Targets.” *Nature Genetics* 47 (5): 505–11. https://doi.org/10.1038/ng.3252.
- Sebaugh, J. L. 2011. “Guidelines for Accurate EC50/IC50 Estimation.” *Pharmaceutical Statistics* 10 (2): 128–34. https://doi.org/10.1002/pst.426.
- Shao, Shiyi, Qida Hu, Wangteng Wu, Meng Wang, Junming Huang, Xinyu Zhao, Guping Tang, and Tingbo Liang. 2020. “Tumor-Triggered Personalized microRNA Cocktail Therapy for Hepatocellular Carcinoma.” *Biomaterials Science* 8 (23): 6579–91. https://doi.org/10.1039/D0BM00794C.
- Shen, Liang, Chunming Sun, Yanyan Li, Xuetao Li, Ting Sun, Chuanjin Liu, Youxin Zhou, and Ziwei Du. 2015. “MicroRNA-199a-3p Suppresses Glioma Cell Proliferation by Regulating the AKT/mTOR Signaling Pathway.” *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine* 36 (9): 6929–38. https://doi.org/10.1007/s13277-015-3409-z.
- Sia, Daniela, Augusto Villanueva, Scott L. Friedman, and Josep M. Llovet. 2017. “Liver Cancer Cell of Origin, Molecular Class, and Effects on Patient Prognosis.” *Gastroenterology* 152 (4): 745–61. https://doi.org/10.1053/j.gastro.2016.11.048.
- Sun, Virginia Chih-Yi, and Linda Sarna. 2008. “Symptom Management in Hepatocellular Carcinoma.” *Clinical Journal of Oncology Nursing* 12 (5): 759–66. https://doi.org/10.1188/08.CJON.759-766.
- Sutter, Olivier, Joyce Calvo, Gisèle N’Kontchou, Jean-Charles Nault, Raffik Ourabia, Pierre Nahon, Nathalie Ganne-Carrié, et al. 2017. “Safety and Efficacy of Irreversible Electroporation for the Treatment of Hepatocellular Carcinoma Not Amenable to Thermal Ablation Techniques: A Retrospective Single-Center Case Series.” *Radiology* 284 (3): 877–86. https://doi.org/10.1148/radiol.2017161413.
- Tarao, Kazuo, Yasushi Rino, Shinichi Ohkawa, Setsuo Tamai, Kaoru Miyakawa, Hideki Takakura, Osamu Endo, Michiyasu Yoshitsugu, Norihito Watanabe, and Shohei Matsuzaki. 2002. “Close Association between High Serum Alanine Aminotransferase Levels and Multicentric Hepatocarcinogenesis in Patients with Hepatitis C Virus-Associated Cirrhosis.” *Cancer* 94 (6): 1787–95. https://doi.org/10.1002/cncr.10391.
- Tornesello, Maria Lina, Luigi Buonaguro, Fabiana Tatangelo, Gerardo Botti, Francesco Izzo, and Franco M. Buonaguro. 2013. “Mutations in TP53, CTNNB1 and PIK3CA Genes in Hepatocellular Carcinoma Associated with Hepatitis B and Hepatitis C Virus Infections.” *Genomics* 102 (2): 74–83. https://doi.org/10.1016/j.ygeno.2013.04.001.
- Willson, J. S. B., T. D. Godwin, G. a. R. Wiggins, P. J. Guilford, and J. L. McCall. 2013. “Primary Hepatocellular Neoplasms in a MODY3 Family with a Novel HNF1A Germline Mutation.” *Journal of Hepatology* 59 (4): 904–7. https://doi.org/10.1016/j.jhep.2013.05.024.

- Witten, Lisa, and Frank J. Slack. 2020. “miR-155 as a Novel Clinical Target for Hematological Malignancies.” *Carcinogenesis* 41 (1): 2–7. https://doi.org/10.1093/carcin/bgz183.
- Wong, Kristen, Francesca Di Cristofano, Michela Ranieri, Daniela De Martino, and Antonio Di Cristofano. 2019. “PI3K/mTOR Inhibition Potentiates and Extends Palbociclib Activity in Anaplastic Thyroid Cancer.” *Endocrine-Related Cancer* 26 (4): 425–36. https://doi.org/10.1530/ERC-19-0011.
- Wu, Hongzhen, Xiaorui Han, Zihua Wang, Lei Mo, Weifeng Liu, Yuan Guo, Xinhua Wei, and Xinqing Jiang. 2020. “Prediction of the Ki-67 Marker Index in Hepatocellular Carcinoma Based on CT Radiomics Features.” *Physics in Medicine & Biology* 65 (23): 235048. https://doi.org/10.1088/1361-6560/abac9c.
- Wu, Xiong-Zhi, and Dan Chen. 2006. “Origin of Hepatocellular Carcinoma: Role of Stem Cells.” *Journal of Gastroenterology and Hepatology* 21 (7): 1093–98. https://doi.org/10.1111/j.1440-1746.2006.04485.x.
- Xing, Yan, Nancy U. Lin, Matthew A. Maurer, Huiqin Chen, Armeen Mahvash, Aysegul Sahin, Argun Akcakanat, et al. 2019. “Phase II Trial of AKT Inhibitor MK-2206 in Patients with Advanced Breast Cancer Who Have Tumors with PIK3CA or AKT Mutations, and/or PTEN Loss/PTEN Mutation.” *Breast Cancer Research: BCR* 21 (1): 78. https://doi.org/10.1186/s13058-019-1154-8.
- Xu, Ruliang, Moueen Bu-Ghanim, M. Isabel Fiel, Thomas Schiano, Emil Cohen, and Swan N. Thung. 2007. “Hepatocellular Carcinoma Associated with an Atypical Presentation of Wilson’s Disease.” *Seminars in Liver Disease* 27 (1): 122–27. https://doi.org/10.1055/s-2007-967203.
- Xu, Xin, Yuquan Tao, Liang Shan, Rui Chen, Hongyuan Jiang, Zijun Qian, Feng Cai, Lifang Ma, and Yongchun Yu. 2018. “The Role of MicroRNAs in Hepatocellular Carcinoma.” *Journal of Cancer* 9 (19): 3557–69. https://doi.org/10.7150/jca.26350.
- Yang, Fan, Qing-jun Li, Zhen-bin Gong, Liang Zhou, Nan You, Su Wang, Xiao-lei Li, et al. 2014. “MicroRNA-34a Targets Bcl-2 and Sensitizes Human Hepatocellular Carcinoma Cells to Sorafenib Treatment.” *Technology in Cancer Research & Treatment* 13 (1): 77–86. https://doi.org/10.7785/tcrt.2012.500364.
- Yao, Shang, Lavender Yuen-Nam Fan, and Eric Wing-Fai Lam. 2018. “The FOXO3-FOXM1 Axis: A Key Cancer Drug Target and a Modulator of Cancer Drug Resistance.” *Seminars in Cancer Biology* 50 (June): 77–89. https://doi.org/10.1016/j.semcancer.2017.11.018.
- Yin, Jian, Peng Hou, Zhiqiang Wu, Tao Wang, and Yanxiao Nie. 2015. “Circulating miR-375 and miR-199a-3p as Potential Biomarkers for the Diagnosis of Hepatocellular Carcinoma.” *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine* 36 (6): 4501–7. https://doi.org/10.1007/s13277-015-3092-0.
- Yip, Terry Cheuk-Fung, Vincent Wai-Sun Wong, Henry Lik-Yuen Chan, Yee-Kit Tse, Grace Chung-Yan Lui, and Grace Lai-Hung Wong. 2020. “Tenofovir Is Associated With Lower Risk of Hepatocellular Carcinoma Than Entecavir in

Patients With Chronic HBV Infection in China." *Gastroenterology* 158 (1): 215 225.e6. https://doi.org/10.1053/j.gastro.2019.09.025.

- Yu, Jie, and Ping Liang. 2017. "Status and Advancement of Microwave Ablation in China." *International Journal of Hyperthermia: The Official Journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group* 33 (3): 278–87. https://doi.org/10.1080/02656736.2016.1243261.
- Yu, Min, Zheng Tang, Fandi Meng, Minghui Tai, Jingyao Zhang, Ruitao Wang, Chang Liu, and Qifei Wu. 2016. "Elevated Expression of FoxM1 Promotes the Tumor Cell Proliferation in Hepatocellular Carcinoma." *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine* 37 (1): 1289–97. https://doi.org/10.1007/s13277-015-3436-9.
- Zampino, Rosa, Maria A. Pisaturo, Grazia Cirillo, Aldo Marrone, Margherita Macera, Luca Rinaldi, Maria Stanzione, et al. 2015. "Hepatocellular Carcinoma in Chronic HBV-HCV Co-Infection Is Correlated to Fibrosis and Disease Duration." *Annals of Hepatology* 14 (1): 75–82.
- Zandwijk, Nico van, Nick Pavlakis, Steven C. Kao, Anthony Linton, Michael J. Boyer, Stephen Clarke, Yennie Huynh, et al. 2017. "Safety and Activity of microRNA-Loaded Minicells in Patients with Recurrent Malignant Pleural Mesothelioma: A First-in-Man, Phase 1, Open-Label, Dose-Escalation Study." *The Lancet. Oncology* 18 (10): 1386–96. https://doi.org/10.1016/S1470-2045(17)30621-6.
- Zein, N. N., J. J. Poterucha, J. B. Gross, R. H. Wiesner, T. M. Therneau, A. A. Gossard, N. K. Wendt, P. S. Mitchell, J. J. Germer, and D. H. Persing. 1996. "Increased Risk of Hepatocellular Carcinoma in Patients Infected with Hepatitis C Genotype 1b." *The American Journal of Gastroenterology* 91 (12): 2560–62.
- Zhang, Shu, Yuming Liu, Jing Chen, Hong Shu, Siyun Shen, Yin Li, Xinyuan Lu, et al. 2020. "Autoantibody Signature in Hepatocellular Carcinoma Using Seromics." *Journal of Hematology & Oncology* 13 (1): 85. https://doi.org/10.1186/s13045-020-00918-x.
- Zhang, Ti, Philippe Merle, Huaqi Wang, Haitao Zhao, and Masatoshi Kudo. 2021. "Combination Therapy for Advanced Hepatocellular Carcinoma: Do We See the Light at the End of the Tunnel?" *Hepatobiliary Surgery and Nutrition* 10 (2): 180–92. https://doi.org/10.21037/hbsn-2021-7.
- Zhou, Kehua, and Christos Fountzilas. 2019. "Outcomes and Quality of Life of Systemic Therapy in Advanced Hepatocellular Carcinoma." *Cancers* 11 (6): 861. https://doi.org/10.3390/cancers11060861.
- Zhu, Andrew X., Yoon-Koo Kang, Chia-Jui Yen, Richard S. Finn, Peter R. Galle, Josep M. Llovet, Eric Assenat, et al. 2019. "Ramucirumab after Sorafenib in Patients with Advanced Hepatocellular Carcinoma and Increased α-Fetoprotein Concentrations (REACH-2): A Randomised, Double-Blind, Placebo-Controlled, Phase 3 Trial." *The Lancet. Oncology* 20 (2): 282–96. https://doi.org/10.1016/S1470-2045(18)30937-9.

ANALYTICAL INDEX