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„Inhibition of cyclin-dependent kinases in hepatocellular carcinoma“

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1. Abstract

Hepatocellular carcinoma (HCC) is among the most frequent causes of cancer deaths worldwide with a rising incidence in western countries. Through the lack of appropriate screening strategies, most HCC patients are diagnosed with advanced or unresectable tumors. Even with treatment the 5 year survival rate for patients with HCC is only 7%. Most HCC patients develop a resistance against anti-cancer drugs while being treated with chemotherapeutics. Therefore there is an urgent need for novel treatment options. In this study, the therapeutic potential of two novel cyclin-dependent kinase (CDK) inhibitors, designated BA-12 and BP-14, was evaluated for the preclinical treatment of HCC.

Our data revealed strong cytotoxicity of BA-12 and BP-14 on multiple human hepatoma cell lines and efficient inhibition of CDK1, CDK2, CDK5, CDK7 and CDK9. By antagonizing these CDKs in HCC cell lines, reduced clonogenicity by arresting cells in S/G2 and G2/M phase of the cell cycle and induced apoptosis was observed. In contrast, primary human hepatocytes failed to show cytotoxicity and apoptosis. No loss of chemosensitivity was observed in HCC cells after long-term exposure to inhibitors. In vivo, treatment of xenografted human HCC cells with BA-12 or BP-14 effectively repressed tumor formation. Moreover, BA-12 or BP-14 significantly diminished diethylnitrosamine-induced hepatoma development in mice. In conclusion, these data show that BA-12 and BP-14 exhibit strong anti-tumorigenic effects in the absence of chemoresistance, resulting in a superior efficacy compared to currently used chemotherapeutics in HCC.

2. Zusammenfassung

Das hepatozelluläre Karzinom (HCC) gehört weltweit zu den häufigsten Krebs-Todesursachen mit ansteigender Inzidenz in den westlichen Ländern. Durch fehlende Screening Methoden werden die meisten Patienten mit fortgeschrittener oder inoperabler Erkrankung diagnostiziert. Auch die therapeutischen Behandlungen von HCC Patienten führen nur zu einer 5-Jahres Überlebensrate von 7%. Zusätzlich entwickeln die meisten Patienten Resistenzen gegen gängige Chemotherapeutika. Daher gibt es einen dringenden Bedarf nach neuen Therapieoptionen. In dieser Studie wurde das therapeutische Potential von zwei neuen Cyclin-abhängigen Kinase (CDK) Inhibitoren, genannt BA-12 und BP-14, als Therapiemöglichkeit für Leberkrebs evaluiert. Die Untersuchungen zeigten starke zytotoxische Effekte von BA-12 und BP-14 auf HCC Zelllinien und eine effiziente Hemmung von CDK1, CDK2, CDK5, CDK7 und CDK9. Durch diese Inhibition kommt es zu einer verminderten Klonogenität durch Induktion eines Zellzyklusarrestes in S/G2 und G2/M Phasen und durch Induktion von Apoptose in den HCC Zelllinien. Im Gegensatz dazu zeigen primäre Hepatozyten keinen Zellzyklusarrest oder Apoptose. Nach einer Langzeitbehandlung von HCC Zelllinien mit den CDK-Inhibitoren konnte keine verminderte Chemosensitivität festgestellt werden. Weiters wurde beobachtet, dass die Behandlung von humanen xenotransplantierten HCC Zellen das Tumorwachstum mit BA-12 und BP-14 signifikant hemmen. Außerdem konnte gezeigt werden, dass BA-12 und BP-14 das Tumorwachstum in Diethylnitrosamin-induzierten Hepatomen reduziert. Zusammenfassend zeigen diese Resultate eindeutig, dass BA-12 und BP14 starke anti-krebserzeugende Effekte bewirken und dabei keine Chemoresistenz auslösen. BA-12 und BP-14 könnte folglich den derzeitig verwendeten Chemotherapeutika überlegen sein und eine vielversprechende Alternative zu den vorhandenen Therapieoptionen darstellen.

3. Introduction

3.1.Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) derives from hepatocytes and accounts for 90% of all liver cancers (1). It represents the sixth most common diagnosed cancer worldwide and the second most frequent cause of cancer related mortality in men (2). The high mortality rate results from the lack of effective treatment options, especially for patients with advanced stage HCC.

HCC mostly occurs in a background of chronic liver disease and cirrhosis which favors the formation of dysplastic nodules (3). 80% of HCC develop on a cirrhotic or advanced fibrotic background (4). Cirrhosis mostly appears as a result of chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, or metabolic disorders like hemochromatosis (5). More recently, non-alcoholic steatohepatitis (NASH), a manifestation of obesity and metabolic syndrome, is emerging as other factors for cirrhosis and HCC (6, 7) (Table 1).

Table 1. Risk factors for HCC and their association with cirrhosis and advanced fibrosis (8).

Major risk factors for HCC are:	
80%	Cirrhosis or advanced fibrosis, mostly due to - Hepatitis B virus infection - Hepatitis C virus infection - Alcohol - NASH - Congenital disorders such as Hemochromatosis, Wilson's disease, *etc.*
20%	In the absence of Cirrhosis or advanced fibrosis - Hepatitis B virus infection - Aflatoxin (mostly combined with HBV) - Some genetic disorders such as Tyrosinosis - Drug induced (*eg* anabolic steroids)

The heterogeneous distribution of liver cancer incidence throughout the world reflects the huge variation in the main risk factors. Liver cancer incidence is strikingly higher in developing countries with high rates in Eastern and South-Eastern Asia, Middle and West Africa, where the major risk factor is chronic infection with HBV, together with intoxication with Aflatoxin B (AFB1) (3). By contrast, in low rate areas, like North America, Europe and Japan, infection with HCV, alcohol abuse and NASH are the major causes for HCC (9) (Figure 1).

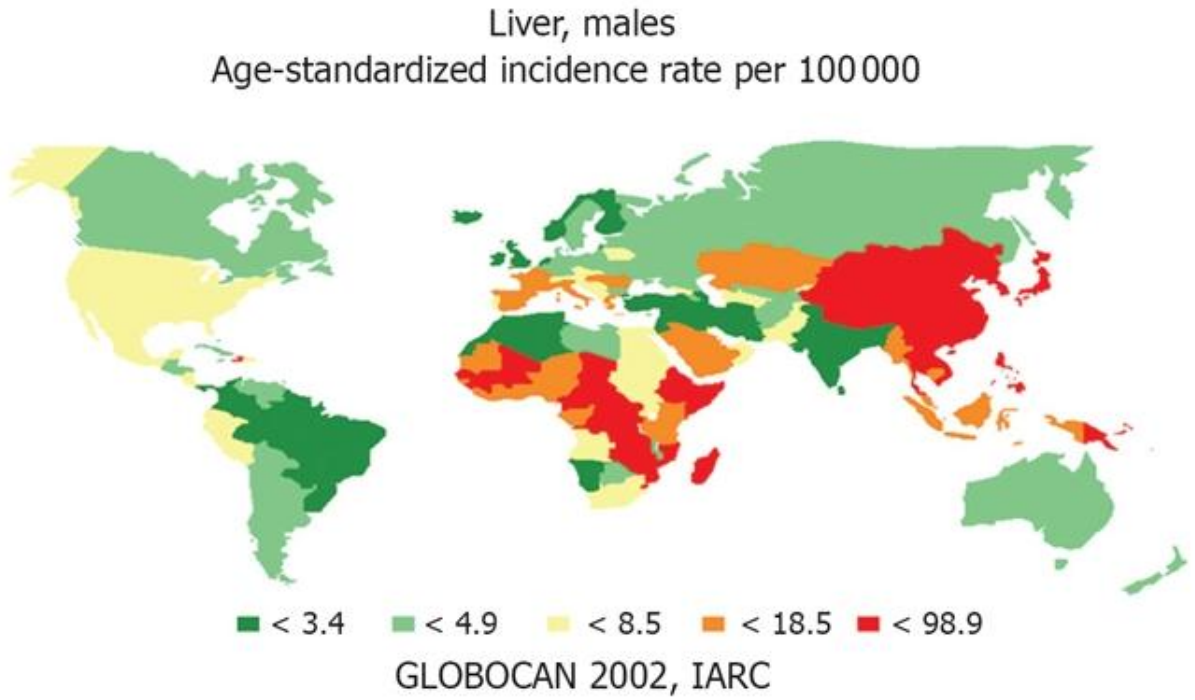

Figure 1. Age-standardized incidence rates of liver cancer in males per 100 000 population (10). Data are estimates for the year 2002 from the Globocan database of the International Agency for Research on Cancer. Geographical distribution is shown via different colors.

In the last few decades, the management of HCC has improved significantly due to ameliorated diagnostic tools, the development of evidence-based staging systems and the availability of improved therapy options (11). Some of the highest risk populations have seen declines in HCC rates, but still the overall global incidence will continue to rise until a probable plateau is reached in 2015-2020 (12). Subsequent decreases in the rates of HCC have been predicted, partly resulting from the expected improvements in the control of HBV and HCV infection (13). However, risk factors such as diabetes and obesity may become increasingly important drivers of future HCC incidence trends.

Through the paucity of effective and well-tolerated treatments and the insufficient surveillance programs many challenges arise with this cancer. There is a need for more detailed and clinically grounded genomic characterization, for a deeper understanding of the mechanisms of genomic instability, host-viral interactions, microenvironmental processes and for the identification of biomarkers to identify early stage disease as well as those at greater risk of developing HCC (14).

3.2.HCC therapy

Treatment of HCC can be divided into curative and palliative (Figure 2). Curative treatments, which are surgical resection, percutaneous ablation and liver transplantation, induce complete responses in a high proportion of patients and are expected to improve survival (15). Palliative treatments, including chemoembolization and the administration of Sorafenib, can obtain good response rates in treating the symptoms and can in some cases even improve survival (5).

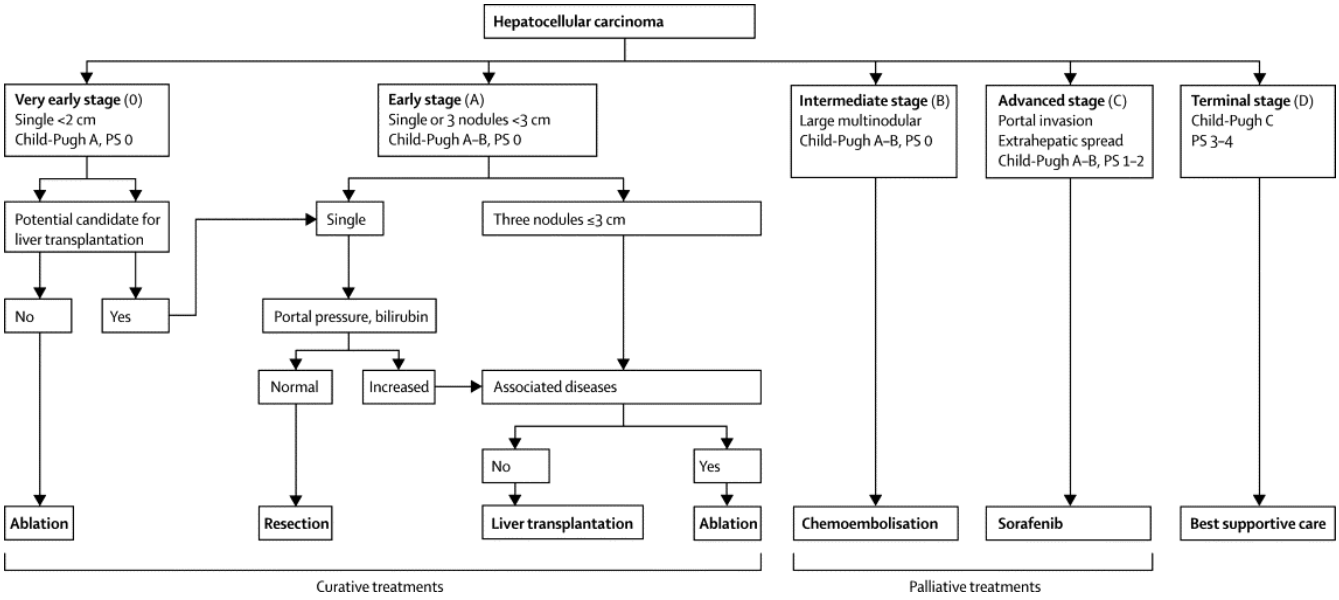

Figure 2. Treatment strategy of HCC in accordance with the BCLC staging system (16). The Barcelona Clinic Liver Cancer (BCLC) staging system links treatment options with the patient status. PS, performance status; Child-Pugh, score to assess the prognosis of chronic liver disease (cirrhosis). HCC patients usually show no symptoms at the early stage. Later they present with pain in the upper right portion of the abdomen and worsening of the liver function, noted by increasing jaundice. Probably the most common sign is an enlarged liver.

Figure 2 shows the BCLC strategy where patients are classified according to outcome and simultaneously links it with treatment indication (16). It establishes treatment recommendations and simultaneously aims to incorporate prognosis estimation (17).

The BCLC system takes several prognostic markers related to tumor (size, number, vascular invasion, N1, M1), liver function (Child-Pugh), and health status (ECOG-Eastern Cooperative Oncology Group Performance Status, PS) into account. The very early stage (BCLC-0, BCLC-A) corresponds to patients with well-preserved liver function (Child-Pugh A) diagnosed with one asymptomatic nodule of less than 2 cm (BCLC 0) or up to 3 nodules smaller than 3 cm (BCLC-A) without vascular invasion or satellites. These patients receive radical therapies such as surgical resection, percutaneous ablation

and liver transplantation (16, 18). For these patients an overall 5 year survival rate of 50-60% can be presumed. Patients with single nodules, who show no sign of cirrhosis or portal hypertension, are treated with resection. Unfortunately, only 10-20% of HCC patients show a resectable tumor. Reasons that exclude surgery are vascular invasion, high risk anatomical location, excessive size or number of lesions, insufficient remnant liver and extrahepatic metastasis (19). The treatment via resection has a 5-year survival rate of 60-70%. However, 70% show either recurrence or a new tumor formation after 5 years (18, 20).

Liver transplantation is used for the treatment of patients with a single nodule less than 5 cm or 3 nodules less than 3 cm, with a proper liver function (Child-Pugh A-B) and a good health status (PS 0). It can cure both, the cancer and the underlying cirrhosis with a recurrence rate of only 10-20%. However, patient selection criteria (Milan criteria) are strict and donor organ availability is limited. Further complications are second neoplasia and infection due to immunosuppression and organ rejection (21). Preventive strategies for recurrence are irradiation with iodine-131-labelled lipiodol and interferon, which showed promising results (22).

If a patient presents with small tumors but with contraindications for surgical therapies, percutaneous treatments are alternative options. Here cancer cells are destroyed by chemical substances, either alcohol or acetic acid, or by radiofrequency, microwave, laser or cryoablation (23). Less than 30-40% of HCC patients are eligible for potentially curative therapies due to advanced stages of disease at the time of diagnosis (24). As a result, patients with intermediate stage disease (BCLC-B) receive systemic chemotherapy. The use of chemotherapy is often combined with transarterial chemoembolization (TACE), where the hepatic artery is obstructed (25). However, chemotherapeutic treatment of HCC most frequently associates with the increased expression of drug resistance genes, resulting in the insensitivity to available chemotherapeutic agents (26). Doxorubicin has shown inefficacy with a response rate of about 0-15% (27, 28), and other chemotherapeutic agents, such as epirubicin, cisplatin, 5-fluorouracil, etoposide and their combinations, demonstrate even lower efficacies. In average this therapy increases median survival by 20-25 months (9).

What should be considered is that if the recommended option according to the BCLC-staging system is not beneficial because of the patient's condition, the treatment approach for the next disease stage should be performed. Accordingly, patients from BCLC stage A may profit from transarterial chemoembolization, BCLC-B patients from sorafenib and those in BCLC-C may enter trials to assess new treatment methods (16). Patients with advanced stage of HCC have a 1-year survival rate of 50% and receive Sorafenib (29). Sorafenib increases the median survival of about three months (30). Patients in terminal stage HCC (BCBC-D) have a median survival time of less than three month and should receive best supportive care (18). Even though there are widespread surveillance programs of at-risk populations, more than half of the patients are diagnosed at a late stage and there treatment options are strongly limited. This strongly indicates the urgent need for novel alternative treatment options.

Metastasis is a major problem in dealing with HCC. The prognosis highly depends on the clinicopathological characteristics regarding cell invasion and dissemination (31). HCC most frequently shows intrahepatic metastasis that are found in 60% of HCC nodules smaller than 5 cm and in 95% of one larger than 5 cm (32), being one of the worst predictors for recurrence after liver transplantation and remaining a major obstacle to further improve the long-term survival after curative HCC resection (33, 34). The prognosis for patients with extrahepatic metastasis to remote organs is still poor, with a 1-year survival rate of 40% (35).

3.3.Mechanisms of HCC development

Carcinogenesis is considered a multistep process (36, 37) where each step represents genetic alterations leading to malignant growth and aberrant differentiation of epithelial tissue (38). According to D. Hanahan and R. Weinberg multiple steps are necessary for this malignant transformation (37):

i) self-sufficiency in growth signals (activation of oncogenes)
ii) insensitivity to growth-inhibitory signals (inactivation of anti-oncogenes or tumor suppressor genes
iii) evasion of apoptosis
iv) limitless replicative potential
v) sustained angiogenesis, tissue invasion and metastasis
vi) reprogramming of energy metabolism and
vii) evasion of immune destruction.

Metastasis itself is again a multistage process in which cells detach from the primary tumor, intravasate into the blood circulation or the lymphatic system, survive in the circulatory system, extravasate and colonize in other tissues (39). Accordingly, hepatocarcinogenesis is a multistep process involving many altered signalling cascades which lead to a heterogeneous molecular profile (40). It is associated and preceded by a number of morphologically distinct lesions, hyperplastic nodules, which then develop to dysplastic nodules and further on to HCC, which can be further classified into well, moderately and poorly differentiated tumors. These have then the capacity to invade the surrounding fibrous stroma and vessels (14). It is believed that the accumulation of genetic alterations in the preneoplastic lesions lead to the development of HCC. These genetic changes may occur independently of etiologic conditions, however, some molecular mechanisms have been frequently related to a specific etiology (Figure 3) (41).

HBV-induced hepatocarcinogenesis involves a number of processes including host-viral interactions followed by sustained cycles of necrosis-inflammation-regeneration, viral-endoplasmic-reticulum (ER) interactions (induction of oxidative stress), viral integration into the host genome (and associated host DNA deletion) and the targeted activation of oncogenic pathways by various viral

proteins. HBV is a non-cytopathic, partially double-stranded hepatotropic DNA virus belonging to the family of hepadnaviridae. Encoded by the genome are a reverse transcriptase/DNA polymerase (pol), the capsid protein (hepatitis B core antigen (HBcAg)), and the L, M and S envelope proteins that associate with the ER membrane during the replication process (42).

Through integration into the genome, host DNA sequences can get lost and cancer relevant genes are modulated. These genes involve telomerase reverse transcriptase (TERT), platelet-derived-growth-factor receptor-β (PDGFR-β), PDGF-β and mitogen activated protein kinase 1 (MAPK1) (43). Also the expression of cancer-related signaling components can be altered, such as Src tyrosine kinases, Ras, Raf, MAPK, ERK, JNK (14). Finally, the tumor suppressor p53 gets frequently inactivated, which increases proliferation, survival and influences DNA-damage checkpoints (44). The host-viral interaction leads to a T-cell response which triggers infection to combat the virus, thus leading to hepatocyte necrosis, inflammation and consequently regeneration (45). This mechanism of regeneration and HBV clearance is an inefficient process and leads to a chronic infection that triggers continuous cycles of necrosis–inflammation–regeneration (46). Such cycles might contribute to the propagation of oncogenic lesions with consequent genomic instability. The viral-ER interaction provokes ER stress and consequently oxidative stress (47), which causes mutations through the generation of free radicals, activates stellate cells, stimulates growth signaling pathways and survival signaling pathways (42). Another proposed mechanism is the escape of the virus from the host's immune response through HBV mutations.

HCV is a non-cytopathic virus belonging to the family of flaviviridae. The RNA genome is positive stranded and encodes non-structural proteins (NS2, NS3, NS4A, NS5A and NS5B), which associate with the ER membrane and viral envelope proteins (E1 and E2) (48). Similar biological processes can be found in HCV-induced hepatocarcinogenesis when compared to HBV-induced HCC, but HCV shows three major differences. It shows a higher tendency (i) of yielding chronic infection, (ii) of promoting liver cirrhosis and (iii) HCV is an RNA virus without a DNA intermediate form and is thus not able to integrate into the host genome (46). Various immune-evasion mechanisms are used by HCV proteins. The HCV core protein and the non-structural protein, NS5A, interact with various factors, such as tumor-necrosis factor-α (TNFα) receptor, and interferon-α (IFNα), to evade from immune-mediated cell killing (49). Like HBV, HCV induces ER stress and thus triggers pro-carcinogenic effects. The HCV core proteins also interact with components of the MAPK signaling pathways (ERK, MEK and Raf) and are thus interfering with cell metabolism and proliferation (50). In addition, it was shown that NS5A interacts with and inactivates p53, thus affecting the p53-regulated pathways that control cell-cycle progression, cell survival, response to hypoxic and genotypic stresses, and tumor angiogenesis (51).

Alcohol-induced hepatocarcinogenesis is associated with the induction of inflammation and consequently, cycles of hepatocyte necrosis and regeneration, oxidative stress and cirrhosis. The chronic uptake of alcohol leads to the production of proinflammatory cytokines through the activation

of monocytes (52), induction of circulating endotoxins, and activation of Kupffer cells. These cells then release chemokines and cytokines (including TNFα, interleukin-1 (IL1), IL6 and prostaglandin E2) which have unfavorable effects on hepatocyte survival. They show increased sensitivity to the cytotoxic effects of TNFα and this leads to chronic hepatocyte destruction, followed by regeneration, stellate cell activation (stellate cells are the main source of collagen in the diseased liver), cirrhosis and finally by HCC (53). Alcohol, as well as HCV and HBV, damages the liver through oxidative-stress mechanisms. Several mechanisms are known. Oxidative stress can either lead to the development of fibrosis and cirrhosis or might also cause the accumulation of oncogenic mutations. Possibly oxidative stress accelerates telomere shortening, what favors the development of liver cirrhosis and chromosomal instability leading to HCC (54). Alcohol-induced oxidative stress might influence HCC-relevant signaling pathways. Recently, it was shown that the tyrosine phosphorylation of signal transducer and activator of transcription 1 (STAT1) is reduced (55).

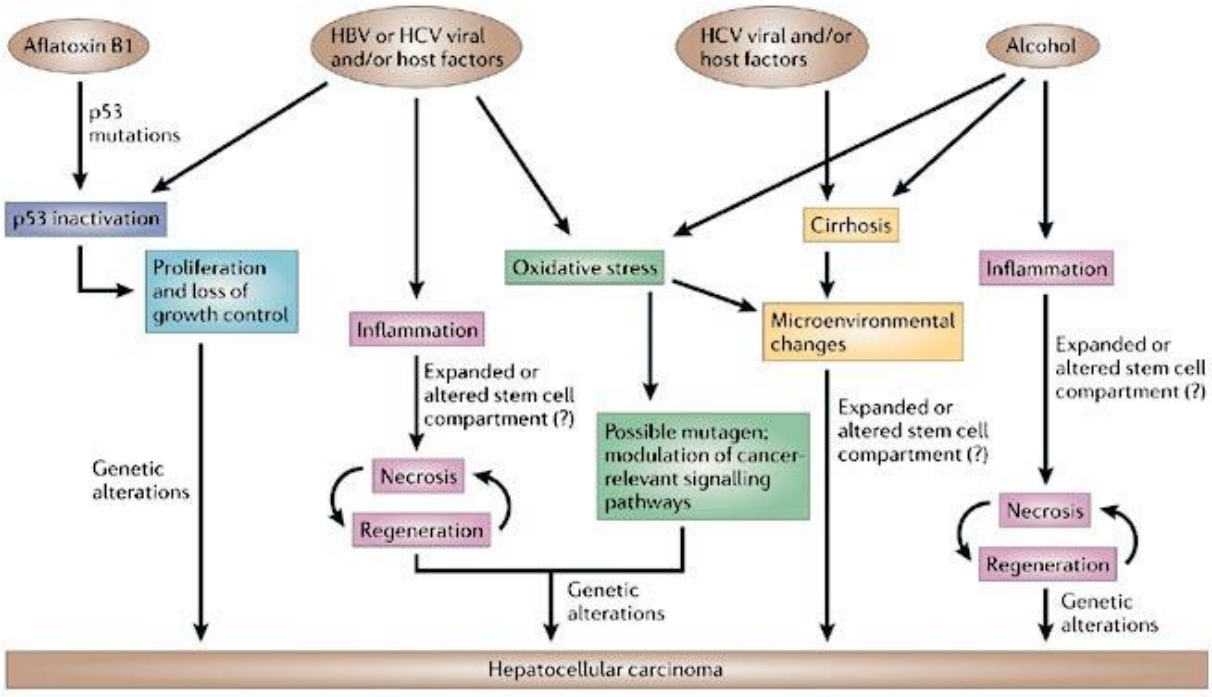

Figure 3. Mechanisms of hepatocarcinogenesis [14]. Summary of mechanisms which are responsible for the development of hepatocellular carcinoma. In addition to these mechanisms, hepatitis B virus (HBV) and Aflatoxin B1 both affect the genome, HBV through the integration into the genome and Aflatoxin B1 through its mutagenic action. The different colors indicate corresponding clusters.

Aflatoxin B1-induced hepatocarcinogenesis is caused by a specific p53 mutation and associates with the mutational activation of oncogenes such as *HRAS* (56). The mutational activation by Aflatoxin B1 is considered the primary driver of HCC development, because no link was found between the toxin and cirrhosis (14).

The molecular analysis of human HCC has shown many genetic and epigenetic alterations that result in the deregulation of key oncogenes and tumor-suppressor genes (14). The main mutations include inactivation of the tumor suppressor p53, mutations in the gene for β-catenin, CTNNB1 (16), overexpression of various ErbB receptor family members including MET and its ligand hepatocyte growth factor (HGF), loss of $p16^{INK4a}$ and E-cadherin and activation of cyclooxygenase 2 (COX2) (14). P53 is found mutated in 25-40% of liver cancer, but until now it is not clear whether p53 mutation contributes to cancer initiation, progression or both. Nowadays it is assumed that this depends on the setting and the aetiology. With Aflatoxin B1, p53 mutation probably drives initiation as with the other aetiologies, p53 mutations are thought to promote tumor progression by facilitating continued proliferation. β-catenin, is a crucial downstream target of the Wnt-signaling pathway and is mutated in about 25%, predominantly in HCV related HCC. The ErbB receptor family consists of four members (ErbB1-4). ErbB1 (EGFR) was found overexpressed in 68% of HCC cases, ErbB3 in 84%, ErbB2 (HER2) in 21% and ErbB4 in 61% (57). EGFR overexpression correlates with a more aggressive tumor, a high proliferation rate, intrahepatic metastasis, de-differentiation and enhanced tumor size (57). MET was shown to be overexpressed in advanced HCC, and plays a crucial role in HCC progression (58).

Genomic instability is a common feature of human HCC. Mechanisms that contribute are telomere erosion, chromosome segregation defects and alterations in the DNA-damage response pathway (14). Telomere shortening correlates with increased chromosomal instability (59). In addition, telomerase is activated in almost 90% of human HCCs (60). Current data suggest that telomere shortening drives chromosomal instability and cancer-promoting lesions during early stages of hepatocarcinogenesis and telomerase re-activation is necessary for malignant progression as it restores chromosomal stability to a level compatible with cancer-cell viability (61). Chromosome segregation defects are a common cytogenic feature of cancer cells, including HCC. Aurora kinase A and its target, hepatoma upregulated protein (HURP), are found overexpressed in HCC (62). Alterations in the DNA-damage response pathway affect mostly p53 and BRCA2. The effect of p53 is already noted above. Loss of heterozygosity (LOH) of the BRCA2 locus is known in HCC, thus probably favoring genomic instability (63).

There are many genomic alterations in HCC. Chromosomal amplifications on 1q, 6p, 8q, 17q and 20q, and deletions on 4q, 8p, 11q, 13q, 16q, and 17p are common and affect important oncogenes and tumor suppressors. These alterations have been surveyed by comparative genomic hybridization (CGH), high-density single nucleotide polymorphism (SNP) arrays or LOH mapping (64). For some loci, the resident cancer gene has been identified and validated, as for example: RB (13q), BRCA2

(13q), FLT2 (13q), TP53 (17p), HBVS2 (hepatitis B virus integration site 2) (4q), hepatoma-derived growth factor (HDGF) (1q), ERBB2 (17q), PIM1 (6p) and MYC (8q).The most frequently affected chromosome arm is 1q with rates of amplification ranging between 58% and 78% in HCC (65). Other high level amplifications have been described in *VEGFA* and cyclinD1, *CCND1*, in 5-10% of patients (65).

Epigenetic alterations are not very well described in HCC but the silencing of tumor suppressors (ie, RASSf1, SOCS1, cyclin D1) and the reactivation of oncogenes (MYC) could be shown (16). Finally, microRNA (miRNA) seems to be able to modulate transcription of key oncogenes. Depending on the genes targeted, miRNAs can act as tumor suppressors or oncogenes (66). Recent evidence shows abnormal expression of miRNAs in HCC, including up-regulation of miR-21, miR-221, miR-151, and down-regulation of Let-7 miRNA, miR-29, and miR-122 (67-72). The expression levels of miRNAs such as miR-26a correlate with poor prognosis in HCC (73).

As a result of these alterations, several signaling cascades related to cell survival and proliferation are activated and respond to targeted treatments in preclinical and early clinical studies. With respect to proliferation cascades, EGFR and Ras signaling is activated in more than 50% of HCC. 40-50% of liver cancers show a disrupted mammalian target of rapamycin (mTOR) pathway, associated with insulin-like growth factor (IGF) pathway activation, PTEN dysregulation and mutations of phosphoinositide-3-kinase (PI3K) (74). IGF-receptor 1 (IGFR1) signaling is active in 20% of early HCC, and overexpression of the HGF and c-MET pathway is a common event (75).

Another important pathway is Wingless (Wnt) signaling, which is activated in a third of hepatocellular carcinomas, often as a result of activating mutations in the transcription factor β-catenin, overexpression of Wnt receptors, or inactivation of E-cadherin. β-catenin was found to be accumulated in the nucleus of 17-40% of human HCCs (76). In addition, HCC is a highly vascularized cancer and angiogenic activity through signaling of VEGFA, ANGPT2 and fibroblast growth factor is a key event.

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3.4. Cyclin-dependent kinases

Protein kinases phosphorylate proteins on serine, threonine and tyrosine residues thus leading to post-translational modifications that are important for regulating protein function in nearly all eukaryotic pathways, such as cell e.g. proliferation and differentiation, cell motility, metabolism and apoptosis.
Cyclin-dependent kinases (CDKs) belong to the family of serine/threonine kinases. Until now, at least 11 variants have been identified that are dependent on cyclin activation (77). Currently, cell cycle roles have been implicated for CDK1-4, 6, 7, 10 and 11. They control cell cycle progression in eukaryotic organisms (78). The cell division cycle allows the reproduction of cells and the transmission of their genes to the next generation. It is based on an orchestrated coordination of enzymatic reactions to diminish all risks of aberrant chromosome segregation and to maintain the stability of genomic DNA (79). Thus progression of the cell through the cell cycle is tightly regulated by cyclin-CDK kinase complexes. These complexes are composed of a catalytic kinase subunit, the CDK, and an activating subunit, the cyclin. The cell division cycle consists of four consecutive but distinct phases, including two gap phases (G1 and G2) that prepare and assess the readiness of cells to enter the S-phase (DNA synthesis phase) and the M-phase (mitotic phase) (80). Cells can reversibly enter a quiescent state referred to as G0 (Figure 4). Progression through the S-phase must be strictly controlled so that cells undergo only a single round of chromosomal DNA replication. M-phase then governs cytokinesis, where the duplicated genetic material is separated into two identical daughter cells (80).
In Schizosaccharomyces pombe and Saccharomyces cerevisiae the cell division cycles are regulated by only a single CDK protein, p34cdc2 and Cdc28 respectively, coupled with several different cyclins that are expressed at different cell cycle phases (81). In contrast, higher organisms use different CDKs that associate with the different cyclins and are important for progression through different cell cycle phases (82). In mammalian cells, mitogenic factors regulate the commitment to cell division. They are active in the G1-phase of the cell cycle to activate CDKs and transverse the restriction point (R), after which the cell is committed to a round of cell division (83). CDK7 forms together with cyclin H and MAT1 a trimeric complex that functions as a CDK-activating kinase (CAK) which phosphorylates and presumably activates all cell cycle CDKs (84). Cyclin C-CDK8 phosphorylates cyclin H to inhibit CAK activity. After initial commitment, CDK4 and CDK6 together with D-type cyclins are activated and are important for the progression during the G1 cell cycle phase (85). The cyclin E-CDK2 complex plays a crucial role during the transition from G1- into the S-phase (8). Cyclin D-CDK4, 6 and cyclin E-CDK2 sequentially phosphorylate the retinoblastoma protein (pRb) and thus relieving the suppression of the activity of members of the E2F family of transcription factors. This allows the transition through the restriction point prior to the G1/S boundary (86). CDK3 might also participate in Rb phosphorylation, maybe in complexion with cyclin C being important in the G0 to G1 transition (87).
During subsequent S- and G2-phase progression, cyclin A-CDK2 and cyclin A-CDK1 play a major role (88, 89). When cells progress to the S-phase, negative regulation of E2F transcriptional function

by cyclin A-CDK2 phosphorylation is necessary (90). CDK10 is thought to be involved in regulating the G2-M phase (91). The progress through the M-phase of the cell cycle is dependent on the cyclin B-CDK1 complex (92) (Figure 4). Also cyclin H-CDK11 likely plays a role in the M-phase, particularly in transcript production and regulation of RNA processing through the interaction with RNA polymerase II (93).

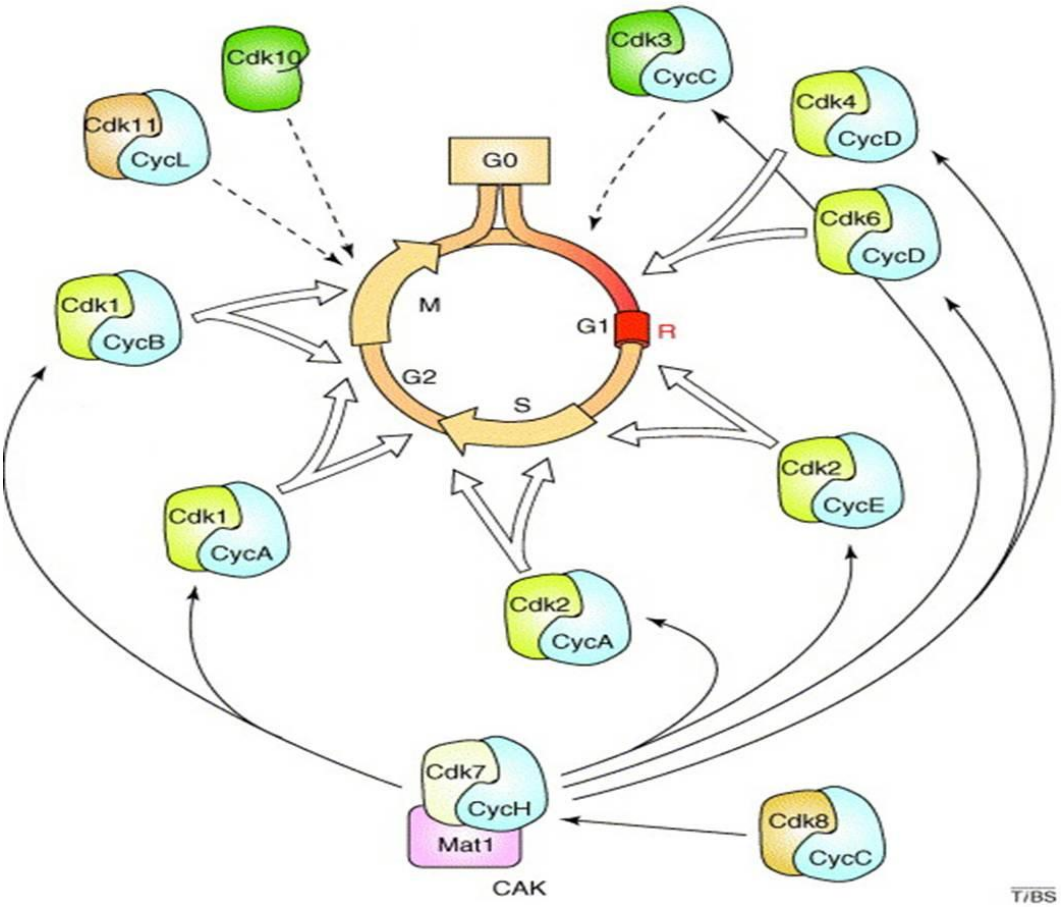

Figure 4. The eukaryotic cell cycle and its cyclin-CDK regulation. The cell cycle consists of a DNA synthesis (S) phase and a mitotic (M) phase, separated by two gap (G1 and G2) phases. In mammalian cells, different cyclin-CDK complexes regulate the progression of cells through the different phases of the cell cycle (77).

Positive or negative regulation of CDKs is modulated by phosphorylation. Two conserved phosphorylation sites are located in the ATP-binding pocket, and when phosphorylated, these sites serve to inhibit protein kinase activity (93). To ensure appropriate cell cycle progression, these kinases are tightly regulated. Therefore, in addition to activating cyclins, CDKs are regulated by the binding of inactivating proteins (cyclin-dependent kinase inhibitory proteins) and activating and inactivating phosphorylation events on critical residues of the CDK subunit. Phosphorylation of the neighboring threonine and tyrosine (Thr14 and Tyr15 in CDK2) in the ATP-binding loop has an inactivating effect on the cyclin-CDK-complex, whereas activation is achieved through the phosphorylation of a specific threonine residue (Thr160/161 in CDK2) in the activation loop (94). In addition to their functions in cell cycle progression, CDK2, 7, 8 and 9 play a role in the regulation of transcription initiation and elongation through phosphorylation of the C-terminal domain of RNA polymerase II (RNAP II) (Figure 5). The CAK complex with CDK7 is also a component of the transcription factor TFIIH and mediates the phosphorylation of serine residue 5 (S5) within the C-terminal domain (CTD) of RNA polymerase II. Cyclin T-CDK9 phosphorylates Serine 2 on the C-terminal domain which results in elongation of the mRNA. Finally, CDK5 is activated by p35 and p39 that are typically expressed in brain. It mostly acts in neural cells and is involved in the regulation of cell survival, transcription, migration and membrane transport (95).

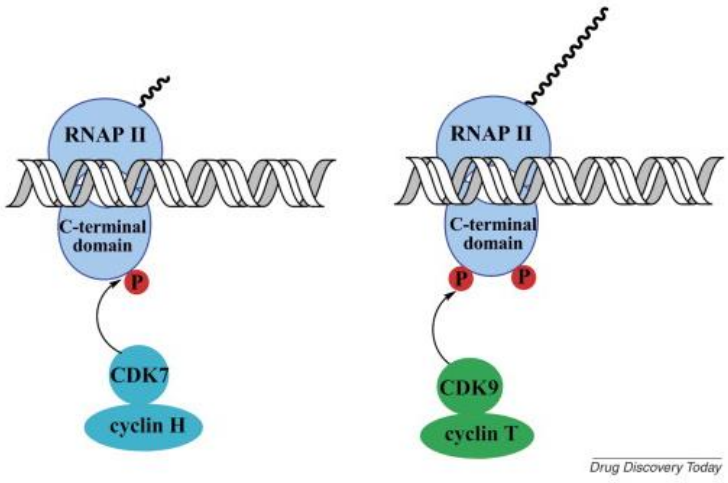

Figure 5. Role of cyclin H-CDK7 and cyclin T-CDK9 in the regulation of transcription. Cyclin H-CDK7 complex (on the left) phosphorylates S5 of the C-terminal domain of the RNA-PolII and is involved in transcription initiation. The phosphorylation of S2 on the C-terminal domain of the RNA-PolII by Cyclin T-CDK9 (shown on the right) results in transcriptional elongation (86).

3.5.In vitro and in vivo hepatoma models

Tumor cell lines are widely used to study the biology of cancer and to examine the factors influencing the response of tumors to therapeutic agents and regimens (96). When selecting *in vivo* or *in vitro* models, several aspects must be considered: (i) the species and strain of animal chosen, (ii) the suitability of the model to human disease, (iii) the extent of animal husbandry required during the in-life pharmacological assessment, (iv) the technical aspects of generating the model and harvesting the tissues for analyses, (v) the cost of research tools in terms of time and money, and (vi) the length of time required for experiments in the model (97). A consideration of translational aspects of the *in vivo* model compared to clinic parameters is also important.

Here, for the evaluation of novel anti-cancer drugs against HCC, several *in vitro* and *in vivo* hepatoma models were used. For *in vitro* experiments, different hepatoma cell lines were used such as PLC/PRF5, HepG2 and Hep3B, and in addition primary human hepatocytes (PHH). In vivo models were employed by human hepatoma xenograft models and DEN (Diethylnitrosamine)-induced hepatocarcinogenesis. These different tumor models are explained in more detail below.

Cell culture models

In general the advantages of using *in vitro* models include handling a homogenous cell population and allowing genetic manipulation. The disadvantages to consider here are that cells growing as a monolayer do not reflect the complexity of cells growing *in vivo*, thus fail to mimic systemic interactions at the molecular and cellular level. Cell culture models lack the heterogeneity that is present in patients with cancer and the study can be easily biased through the choice of cell system.

To study and understand drug metabolism, drug efficacy and toxicity, a reliable research model is of utmost importance. To be considered are the properties of a cell line according to drug-metabolizing enzymes and transporters. These can be divided into three groups: phase I, phase II and phase III. Phase I enzymes catalyze oxidation, reduction, hydrolysis, cyclization, and decyclization reactions (ie, cytochrome P450 (P450)). Phase II enzymes are involved in conjugating ionized groups to the drug, leading to more water soluble metabolites. Phase III enzymes are membrane transporters that pump drugs across cellular barriers (98).

Primary human hepatocytes (PHHs) and hepatoma cell lines such as HepG2 are among the most widely used *in vitro* models in pharmacological and toxicological studies. PHHs remain differentiated and sustain the major drug-metabolizing enzyme activities for a distinct period of time in culture, thus representing a unique *in vitro* system and being the “gold standard” concerning drug metabolism and toxicity (99). However, there are some major drawbacks. PHHs have a high variability in the expression and corresponding activity of drug metabolizing enzymes reflecting the interindividual variability among donors caused by genetic and environmental factors (100). In addition they have short life spans due to a rapid onset of cellular senescence and cell death (98). In contrast, HepG2 hepatoma cells can be cultivated and are widely used for toxicity studies. Despite the lower activity of

cytochrome P450 (CYP) and UDP-glucuronosyl-transferases (101), they show features of normal parenchymal liver cells, such as secretion of lipoproteins, biosynthesis of multiple plasma proteins, and plasma membrane polarity (101). HepG2 cells have been considered a valuable model because of their almost complete set of phase II enzymes and their resulting intact metabolism, proven by several studies (102). Other hepatoma cell lines, such as PLC/PRF5 and Hep3B, have also been used in several drug metabolism and toxicity studies (103).

Mouse models

The cell culture is a useful tool to provide first insight into the metabolism or toxicology but it does not mimic the *in situ* environment of cancer or normal tissue. Therefore, *in vivo* models were established. They have been preclinically used in predicting efficacy and determining toxicity of anti-cancer compounds before entering a clinical trial (104). The laboratory mouse still is one of the best models to study cancer *in vivo* due to the similarity to humans (105).

Subcutaneous xenograft models

Human xenograft models are widely used as *in vivo* models. Here, human HCC cells such as HepG2, PLC/PRF5 and Hep3B are inoculated either subcutaneously (in this work) or in other organs/tissue of the laboratory mouse and are allowed to grow depending on their capability to proliferate (106). Mice used for xenografts are immunocompromised so that they do not reject human cells. Most commonly used are severely compromised immunodeficient (SCID) mice, which are B-cell- and T-cell-deficient, where tumor growth occurs in most of the cases (107). Major challenges in this model are the observation of tumor growth or regression over a period of time and the assessment of treatment efficacy (108). However, with advances in *in vivo* animal imaging, monitoring of response to treatment is now possible, although this evaluation method is still expensive and difficult to establish (109). What has to be considered is that there exist critical differences when comparing tumor xenografts with patient-derived specimens, especially through the crucially altered or lost tumor microenvironment, which includes nearby normal tissue stromal cells, vasculature and lymphatic circulation, and immune cells (110). These differences are possibly relevant in the therapeutic evaluation of compounds, as found out over several years that some new anticancer agents show significant activities in xenografts but are proven to be ineffective in the clinical setting (111). Yet, some studies propose the opposite (112). In conclusion, xenografts should be considered as an intermediate step between cell culture and mouse cancer models, and could be termed “animal culture” (105).

Diethylnitrosamine (DEN)-induced hepatocarcinogenesis

Chemically induced models are favorable for research that requires HCC to develop in a natural background of liver damage. Different hepatotoxic agents have been shown to induce HCC in the mouse liver, of which the genotoxic carcinogen diethylnitrosamine (DEN) is the most efficient one (113). DEN exhibits a strong carcinogenic capacity through its ability to alkylate DNA structures.

Firstly, DEN is hydroxylated to α-hydroxylnitrosamine, which is oxygen- and NADPH-dependent mediated by cytochrome P450 (114). Cytochrome P450 is an enzyme, which has its highest activity in centrilobular hepatocytes. After cleavage of acetaldehyde, an electrophilic ethyldiazonium ion is formed that causes DNA damage by reacting with nucleophiles such as DNA-bases (115). DEN works in a dose-dependent manner (116). It is typically administered to male mice (in this work C57BL/6J) at day 14 by a single intraperitoneal injection (5 µg/g body weight). Other, less efficient possibilities are the injection of a higher dose to older mice or an intraperitoneal DEN-injection 36 hours after a partial hepatectomy (113).

The time needed after a single DEN-injection to develop HCC does not only depend on the administered dose, but also on sex, age and strain of mice (117). The influence of these factors on the development of HCC is a disadvantage of the DEN model. Male mice are much more susceptible to DEN-induced hepatocarcinogenesis, which is due to the inhibitory effect of estrogens and the stimulating effect of androgens on hepatocarcinogenesis (118). HCC occurring faster in younger mice is a result of the higher proliferation rate of the hepatocytes (117). The efficiency of liver tumor formation is strongly dependent on the mouse strain. An explanation for this could be a different methylation status for key genes to develop HCC (119). The above mentioned model mimics the clinical situation best. But there are still inconsistencies between mouse and human carcinogenesis, thus making conclusions from the mouse to the human situation continues to be difficult (120).

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4. Aims

HCC represents the sixth most common cancer worldwide and the second most frequent cause of cancer related mortality in men (1). Most patients are diagnosed at an advanced stage and even with treatment 5 year survival rate of patients with HCC is only 10% (2). Chemotherapy is still very inefficient as most patients develop resistance against anti-cancer drugs while being treated (3). Until now no serious treatment was found to substantially prolong survival, therefore new approaches must be found.

The aim of this work was the preclinical evaluation of the therapeutic potential of two novel cyclin-dependent kinase inhibitors, termed BA-12 and BP14, for the treatment of HCC.

Specific aims include to

(i) evaluate the cytotoxicity of BA-12 and BP-14 in vitro and in vivo models

(ii) identify the main proteins that are regulated by BA-12 and BP-14 with a special focus on CDK1, CDK2, CDK5, CDK7 and CDK9 (because they are known to be targeted by roscovitine)

(iii) determine anti-tumorigenic effects of BA-12 and BP-14 (induction of apoptosis and growth arrest)

(iv) analyze the chemosensitivity of hepatoma cells against both compounds after long- and short-term exposure

(v) assess the efficacy of BA-12 and BP-14 in vivo by employing xenograft mouse models and DEN-induced hepatocarcinogenesis

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6. Manuscript

Novel Inhibitors of Cyclin-Dependent Kinases Combat Hepatocellular Carcinoma without Inducing Chemoresistance

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Running title: Intervention with HCC by Novel CDK Inhibitors

Key words: CDK, roscovitine, drug efficacy, inhibitory concentration, hepatocellular carcinoma, cancer therapy

6.1. Abstract

Purpose: Chemotherapeutic treatment of hepatocellular carcinoma (HCC) is still very ineffective. Most HCC patients escape from treatment with chemotherapeutics by resistance against anti-cancer drugs. We evaluated the therapeutic potential of two novel cyclin-dependent kinase (CDK) inhibitors, designated BA-12 and BP-14, for treatment of HCC.

Experimental Design: Phosphorylations of histone H1 and RNA polymerase II were employed to determine the specificity of CDK inhibition. Cytotoxicity of compounds was analyzed by MTT assay. Clonogenicity as well as cell cycle repression and induction of apoptosis were examined in multiple human hepatoma cell lines. Side effects were monitored in primary human hepatocytes. Diethylnitrosamine (DEN)-induced liver carcinoma and human HCC xenografts were used to assess the efficacy of BA-12 and BP-14 in vivo.

Results: BA-12 and BP-14 displayed strong cytotoxicity on HCC cell lines and efficiently inhibited CDK1, CDK2, CDK5, CDK7 and CDK9. Antagonizing those CDKs in HCC cell lines reduced clonogenicity by arresting cells in S/G2 and G2/M phase of the cell cycle and inducing apoptosis. In contrast, primary human hepatocytes failed to show cytotoxicity and apoptosis. No loss of chemosensitivity was observed in HCC cells after long-term exposure to inhibitors. In vivo, treatment of xenografted human HCC with BA-12 or BP-14 effectively repressed tumor formation. Moreover, BA-12 or BP-14 significantly diminished DEN-induced hepatoma development in mice.

Conclusion: These data show that BA-12 or BP-14 exhibit strong anti-tumorigenic effects in the absence of chemoresistance, resulting in a superior efficacy compared to currently used chemotherapeutics in HCC.

6.2. Translational Relevance

Treatment options for HCC using chemotherapeutics at intermediate and advanced stages of disease are limited as patients most rapidly escape from therapy and succumb to disease progression. Mechanisms of the hepatic xenobiotic metabolism are mostly involved in providing chemoresistance to therapeutic compounds. Given the fact that the aberrant activation of CDKs is frequently observed in HCC, we focused on the efficacy of the novel compounds BA-12 and BP-14 which antagonize CDK1/2/5/7 and CDK9. We show the persistent anti-cancer activity of BA-12 and BP-14 after long-term treatment of various hepatoma models in vitro as well as the regression of human xenografts and chemically induced hepatoma in vivo. Our data provide a preclinical rationale to develop clinical concepts for the use of the CDK inhibitors BA-12 and BP-14 in combating HCC.

6.3. Introduction

Hepatocellular carcinoma (HCC) represents the sixth most common cancer and the third leading cause of cancer deaths worldwide (1). Less than 30-40% of HCC patients are eligible for potentially curative therapies, including surgical resection and orthotopic liver transplantation due to advanced stages of disease at the time of diagnosis (2). As a result, patients with advanced HCC receive systemic chemotherapy. The use of chemotherapy is often combined with transarterial chemoembolization (TACE), where the hepatic artery is obstructed (3). However, chemotherapeutic treatment of HCC most frequently associates with the increased expression of drug resistance genes, resulting in the insensitivity to available chemotherapeutic agents (4). Doxorubicin has shown inefficacy with a response rate of about 0-15% (5, 6), and other chemotherapy agents, such as epirubicin, cisplatin, 5-fluorouracil, etoposide and their combinations, demonstrate even lower efficacies. For doxorubicin, one of the most important causes of chemoresistance is the increased expression of the ATP-binding cassette (ABC) transporters. Overexpression of the ABC member ABCB1 (MDR1) encoding P-glycoprotein (P-gp) is associated with lower accumulation of doxorubicin in HCC cells and with a worse prognosis (7). Sorafenib represents the current treatment standard for advanced HCC by prolonging median survival of patients for about three month (8). These limitations in treatment modalities strongly indicate the urgent need for novel alternative treatment options.

Cyclin-dependent kinases (CDKs) are fundamental for cell cycle control and regulation of apoptosis (9, 10), and are found deregulated in most cancer cells. HCC shows frequent upregulation of CDKs through inactivation of CDK inhibitory proteins including p16Ink4, p21WAF1/CIP1, p27KIP1 and p57KIP2 as well as through increasing levels of cyclins (11, 12). In particular, CDK1 and CDK2 often show an aberrant regulation (13, 14). CDK2 provides S phase entry by binding to cyclin E and allows S phase progression by interacting with cyclin A (15). CDK1/cyclin A activity is essential for the initiation of prophase during the G2/M transition (16). Interestingly, cyclin A was found to be overexpressed in 39% of HCC samples (17). Similarly, CDK1/cyclin B complexes participate and complete mitosis and cyclin B overexpression is frequently observed in HCC (18). Besides the fact that the sustained inhibition of CDK7 and CDK9 induces apoptosis (19), little is known about the deregulation of CDK5, CDK7 and CDK9 in HCC.

Thus, the targeting of CDKs has become an attractive approach in oncology (12). A multitude of small molecule CDK inhibitors has been evaluated as promising anti-proliferative agents for cancer therapy, including (R)-roscovitine (Seliciclib, CYC202). Roscovitine is known to selectively inhibit CDK1, CDK2, CDK5, CDK7 and CDK9 activities (20). Currently, roscovitine is evaluated in a phase 1 clinical trial in combination with Sapacitabine with patients suffering from advanced solid tumors (NCT00999401) and in a phase 2 trial from non-small cell lung cancer patients (21). The effects of roscovitine and its derivatives vary according to cell type, but they are generally able to block the cell cycle at every position (22). The cell cycle arrest is attributed to a direct inhibition of CDK1 and CDK2, while the induction of cell death by roscovitine is considered as a direct consequence of

blocking the CDK7/CDK9-dependent transcription. CDK7 is an integral component of the transcription factor TFIIH (23), which phosphorylates the Ser5 in the C-terminal domain (CTD) of RNA polymerase II (Pol II) to facilitate transcription initiation. CDK9, a portion of the elongation factor P-TEFb (24), performs a complementary function by phosphorylating Ser2 in the CTD of RNA Pol II, which is required for transcription elongation.

In this study we investigated the molecular mechanisms of two novel roscovitine derivatives, designated BA-12 and BP-14, in HCC cells and further determined their anti-cancer activity in xenograft models and chemically induced hepatoma. We show that both compounds inhibit CDK1 and CDK2 on their own, leading to the arrest of HCC cells in S/G2 and G2/M. Moreover, BA-12 and BP-14 reduce the phosphorylation of RNA Pol II at Ser5 and Ser2 and selectively induce apoptosis of HCC cells rather than of primary human hepatocytes. Notably, no chemoresistance against these compounds could be observed after long-term treatment of HCC cells. In vivo, both BA-12 and BP-14 significantly diminished the growth of engrafted human hepatoma and were able to antagonize chemically induced liver cancer formation in mice.

6.4.Material and Methods

Cell culture

The human hepatoma cell lines HepG2, PLC/PRF/5 (PLC), Hep3B and 3sp (formerly described as HCC-1.1) were cultivated as described (25, 26). All cells were kept at 37°C and 5% CO2 and were routinely screened for the absence of mycoplasma. The cell lines HepG2, PLC and Hep3B were obtained from the American Type Culture Collection. The 3sp cells were established from an HCC patient at the Medical University of Vienna (26). All cell lines were verified by short tandem repeat analysis in November 2012.

Primary human hepatocytes (PHHs)

Non-neoplastic tissue samples from liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research, Regensburg, Germany), with the informed patient's consent approved by the local ethical committee of the University of Regensburg. PHHs were isolated using a modified two-step EGTA/collagenase perfusion procedure as described previously (27). Viability of isolated PHHs was determined by trypan blue exclusion and cells with a viability of more than 85% were used for further work. Cells were plated on collagen-coated plates (BD Biosciences, San Jose, USA) at a density of 1.2 x 10^5 cells/cm2. The medium consisted of DMEM with 10% FCS, 2 mM L-glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin and supplements as follows: 125 mU/ml insulin, 7.3 ng/ml glucagon and 0.8 µg/ml hydrocortisone. Cells were incubated at 37°C in a humidified incubator with 5% CO2 and media were changed daily.

Therapeutic agents

BA-12 (2-[[[2-[(4-aminocyclohexyl)amino]-9-cyclopentyl-purin-6-yl]amino]methyl]-4-chloro-phenol) and BP-14 (N2-(4-aminocyclohexyl)-9-cyclopentyl-N6-[[6-(2-furyl)-3-pyridyl]methyl]purine-2,6-diamine) were synthesized by procedures as described (28). Compounds were dissolved in dimethylsulfoxide (DMSO). The stock solution of 100 mM was diluted in assay buffer or in medium to concentrations indicated in the text. The maximum concentration of DMSO in the assays never exceeded 0.1%.

Determination of cell viability and inhibitory concentration (IC)50

Cell viability was determined using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in triplicates at a density of 6 x 10^3 cells per 96-well. After 24 hours, cells were incubated with drug-containing medium for 72 hours. Cells were incubated with MTT solution (5 mg/ml; Sigma, St. Louis, USA) and medium was replaced with DMSO after five hours. The absorbance was measured at 620 nm by employing a microplate reader (AsysHiTech, Salzburg, Austria). MTT assays were repeated 3 times for each drug application. IC50 values were obtained by log-linear interpolation of data points and are depicted by dose-response curves using the software GraphPad Prism® 5.01.

Kinase inhibition assays using cell-free extracts

Whole cell extracts were prepared by lysing Hep3B cells with a buffer containing 20 mM Tris pH 8.0, 100 mM NaCl, 1mM EDTA and 0.5% NP-40. 100 µg of extract was used for immunoprecipitation at 4°C for 4 hours either with 1 µg of the anti-CDK2 antibody M2 (Santa Cruz Biotechnology, Santa Cruz, USA) or with 1 µg of the anti-cyclin B1 antibody GNS1 (Santa Cruz Biotechnology, Santa Cruz, USA). Precipitated protein was resuspended in 20 µl kinase buffer containing 5 µCi [γ-^{32}P]ATP (PerkinElmer, Santa Clara, USA), 1 µg histone 1 (New England Biolabs, Ipswich, USA) and the respective concentration of inhibitor. After incubation for 60 minutes at 30°C, the supernatant was boiled in sample buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and stained with Ponceau S before analysis by autoradiography.

Clonogenic survival assay

500 cells were seeded in a 6-well plate and, either untreated or pretreated with BA-12 or BP-14 for 24 hours, incubated with standard medium for 10 days at 37°C and 5% CO2. Colonies were fixed with methanol/acetic acid (3:1) and stained with 0.25% crystal violet. The crystal violet of fixed cells was solubilized with 1% SDS and the absorbance was photometrically determined at 560 nm.

Cell proliferation analyzed by 5-Bromo-2'-deoxy-uridine incorporation

Cultured cells were grown in medium containing 10 µM 5-bromo-2'-deoxy-uridine (BrdU) for 1 hour. After removing labeling medium, cells were fixed and DNA denatured with a fixing/denaturing solution containing 2 M HCl for 30 minutes at 37°C. To analyze BrdU incorporation in vivo, 200 µl Ringer solution containing 1 mg BrdU was intraperitoneally injected into xenografted mice 2 hours prior to sacrifice. Tumor tissue was fixed in 4% formaldehyde and processed for immunohistochemistry. BrdU incorporation was detected with a monoclonal anti-BrdU antibody (Sigma, St.Louis, USA) and a peroxidase-conjugated secondary antibody (Calbiochem, LaJolla, USA).

Flow cytometry

The analysis of cellular DNA content was performed with a multicolor BD LSR Fortessa cell analyzer (Becton Dickinson, Franklin Lakes, USA). Prior to the cytofluorometric measurement, about 5 x 10^5 cells were washed with phosphate buffered saline (PBS), fixed in 70% ethanol, washed again with PBS and treated with 100 µg RNAse A/50 µg propidium iodide per ml for 10 minutes to stain cellular DNA. The percentage of cells in the various cell cycle positions were calculated using a software package from the same manufacturer.

Determination of long-term chemosensitivity

Hepatoma cells were continuously cultivated in the presence of BA-12 or BP-14 at concentrations lower than the IC50 (1/2 IC50, 1/4 IC50, 1/8 IC50 and 1/16 IC50). The selection of chemoresistant cells was monitored every 6 weeks for nine month by the determination of IC50 values using the MTT assay. HCC cells showing higher IC50 values after treatment with inhibitors as compared to untreated cells are considered as chemoresistant.

Immunoblotting

Immunoblotting was performed as described previously (29). Primary antibodies: anti-phospho-Ser5 RNA Pol II (CDK7; Bethyl Laboratories, Montgomery, USA), 1:1000; anti-phospho-Ser2 RNA Pol II (CDK9; Bethyl Laboratories, Montgomery, USA), 1:1000; anti-RNA Pol II (Santa Cruz Biotechnology, Santa Cruz, USA), 1:1000; anti-PARP (Cell Signaling Technology, Beverly, USA), 1:1000; anti-β-actin (Sigma, St.Louis, USA), 1:2500. Horseradish peroxidase-conjugated secondary antibodies (Calbiochem, LaJolla, USA) were used at dilutions of 1:10000.

Xenografted tumor formation and drug intervention

5x10^6 human hepatoma cells were resuspended in 100 µl Ringer solution and subcutaneously injected into severe combined immunodeficient (SCID) mice (Harlan Laboratories, San Pietro, Italy). Tumor volume was determined as described (29). Pharmacological intervention was performed in tumor-bearing mice for 17 days by daily intraperitoneal injection of either 5 mg/kg BA-12 or 1 mg/kg BP-14 in 100 µl of 0.01% DMSO. Control tumor-bearing mice received DMSO only. All animal experiments were performed according to the Austrian guidelines for animal care and protection.

Diethylnitrosamine-induced liver cancer and drug intervention

To initiate tumor development in the liver, 14-day-old male C57BL/6J mice were intraperitoneally injected with a single dose of diethylnitrosamine (DEN, 25 mg/kg). After 8 month, pharmacological intervention was administrated in DEN-induced mice by 3 cycles of treatment with compounds for 10 days and a release from compounds for 7 days between the cycles. 5 mg/kg BA-12 or 1 mg/kg BP-14 was intraperitoneally injected in 100 µl of 0.01% DMSO. Control mice received

DMSO only. Thereafter, mice were sacrificed and livers were fixed in 4% formaldehyde. Two researchers (C.H. and M.G.) independently scored the diameters of neoplasia that could be monitored at the liver surface. Cancerous nodules with a diameter of up to 1 cm, covering more than 97% of all visible hepatomas, were included into the analysis. All animal experiments were performed according to the Austrian guidelines for animal care and protection.

Immunohistochemistry

Mice were sacrificed and tumors were fixed as described (30). 4 µm thick, paraffin-embedded sections were stained with anti-BrdU (Sigma, St. Louis, USA), 1:200. Biotinylated secondary antibody was used at 1:200. The immunoperoxidase procedure was performed using a Vectastain Elite ABC kit (Vector Laboratories, CA, USA) as described by the manufacturer.

Statistical analysis

Data were expressed as means ± standard deviation (SD) or standard error of the mean (SEM). The statistical significance of differences was evaluated using an unpaired, non-parametric Student's t-test. Significant differences between experimental groups were * $p<0.05$, ** $p<0.01$ or *** $p<0.005$.

6.5.Results

Cytotoxicity and kinase specificity of BA-12 and BP-14

Novel derivatives of roscovitine, designated BA-12 and BP-14, were synthesized based on our knowledge of structure-activity relationships for roscovitine-related compounds (28, 31). Cell viability assays showed strong cytotoxic effects of BA-12 and BP-14 on human HepG2 and PLC hepatoma cells (Fig. 1A and 1B). Analysis of additional established HCC cell lines (Hep3B and 3sp) confirmed the cytotoxicity of BA-12 and BP-14 (Supplementary Fig. S1A and S1B). Dose-response curves revealed IC50 values below 1 µM for both compounds in the various HCC cell lines, reaching as low as 0.02 µM in PLC cells for BP-14 (Supplementary Table S1). Kinase assays using cell-free extracts showed that BA-12 or BP-14 significantly reduced CDK1/CDK2 activity at concentrations of 0.03 µM (Fig. 1C and 1D). Furthermore, treatment of HepG2 and PLC cells with 1 µM and 2 µM BA-12, respectively, resulted in a more than 2.5-fold reduction of RNA polymerase II phosphorylation on serine 5 (CDK7) and serine 2 (CDK9; Fig. 1E and 1F), suggesting inhibition of CDK7/CDK9 activity. Administration of BP-14 below 1 µM led to a strong decrease of CDK7/CDK9 activity in HepG2 and PLC cells. Quantification of CDK inhibition using recombinant CDK substrates displayed IC50 values of BA-12 and BP-14 between 0.01 to 0.05 µM including antagonizing effects on CDK5 (Supplementary Table S2), thus corroborated the data obtained by cell-free extracts. Together, these results suggest that both BA-12 and BP-14 are highly potent cytotoxic compounds on HCC cell lines by the specific inhibition of CDK1/CDK2/CDK5/CDK7 and CDK9.

BA-12 and BP-14 abrogate clonogenicity and repress cell cycle progression

We observed a more than 15-fold reduction of clonogenic growth behavior after treatment of HepG2 and PLC cells with 1 µM BA-12 (Fig. 2A). BP-14-treated cells displayed loss of clonogenicity even at 0.2 µM (Fig. 2B). Analysis of DNA synthesis revealed that treatment of HepG2 or PLC cells with 1 µM of either BA-12 or BP-14 decreased BrdU incorporation more than 2-fold as compared to control (Fig. 2C and 2D), indicating inhibition of DNA synthesis. Proliferation kinetics showed a cytostatic effect of BA-12 at 2 µM and of BP-14 at 0.8 µM in both HepG2 and PLC cells as well as in Hep3B hepatoma cells (Supplementary Fig. S2A and S2B). Accordingly, both compounds induced an accumulation in the G2/M and S/G2 phase of the cell cycle (Fig. 2E and 2F). These data suggest that both BA-12 and BP-14 act anti-proliferative by blocking DNA replication and by arresting HCC cells in the S/G2/M phase of the cell cycle.

BA-12 and BP-14 induce apoptosis in hepatoma cells rather than in primary human hepatocytes (PHHs)

We next examined whether apoptosis is induced by BA-12 or BP-14 in HepG2 cells that harbor wild type p53 and in PLC cells expressing full length but mutated p53 (32). Administration of 1 µM BA-12 induced cleavage of PARP and p53 expression in HepG2 cells (Fig. 3A, left panel). Comparable observations were made after treatment with 2 µM BA-12 in p53-mutated PLC cells (Fig. 3A, right panel). BP-14 was able to trigger PARP cleavage even at a concentration of 0.2 µM (Fig. 3B), thus being more potent to induce apoptosis as compared to BA-12. Yet, both BA-12 and BP-14 failed to induce PARP processing in PHHs, which are the cellular origin of hepatoma (Fig. 3C and 3D). Accordingly, both BA-12 and BP-14 exhibited IC50 values of 26 µM and 20 µM in PHHs, respectively, which was 35-fold (BA-12) or 160-fold (BP-14) higher than observed in HepG2 cells (Supplementary Table S1). From these data we conclude that the novel CDK inhibitors induce apoptosis of HCC cells at low concentration in a p53-independent fashion and fail to execute cytotoxic effects in PHHs.

Long-term cytotoxicity of BA-12 and BP-14 in HCC cells

Most of the chemotherapeutic compounds that are currently available for HCC treatment show low cytotoxic efficacy presumably due to the modification and rapid removal from neoplastic hepatocytes by mechanisms of induced multiple drug resistance (33). This poses one of the major problems in combating liver cancer. Therefore, we analyzed whether BA-12 and BP-14 display changes in cytotoxicity by treating hepatoma cells at concentrations half of their IC50 values as well as at serial dilutions (1/2 IC50, 1/4 IC50, 1/8 IC50 and 1/16 IC50) for up to 9 month. In case HCC cells lower their chemosensitivity by acquiring a resistance mechanism, IC50 values rise over time. Most notably, we observed that IC50 values were maintained in hepatoma cells as compared to control during sustained drug exposure (Table 1). These data provide strong evidence that the cytotoxic effects of BA-12 and BP-14 on HCC cells are maintained upon persistent drug treatment, suggesting that hepatoma cells fail to acquire chemoresistance towards BA-12 and BP-14.

In vivo application of BA-12 and BP-14: Inhibition of xenografts and DEN-induced hepatoma

To examine anti-tumorigenic effects of BA-12 and BP-14 in vivo, we assessed hepatoma xenograft models derived from HepG2 and PLC cells. Tumor-bearing mice were injected either with BA-12 or BP-14 at maximum tolerated doses (MTD). Xenografted mice well tolerated the treatment regimen of BA-12 and BP-14 since 100% of mice survived until the end of drug application. Administration of BA-12 or BP-14 resulted in reduced tumor volumes and tumor stasis of xenografts. Both were effective in the PLC- as well as in the HepG2 model (Fig. 4A and 4B, upper panel). Interestingly, BP-14 exhibited a higher potency than BA-12 in the PLC model resulting in a more pronounced reduction of the tumor volume. Evaluation of S-phase-positive cells in HepG2- and PLC-

derived tumors by BrdU incorporation into DNA revealed a 2-fold decrease after exposure to either BA-12 or BP-14 (Fig. 4A and B, middle and lower panel).

We further analyzed the ability of BA-12 and BP-14 to interfere with endogenous liver cancer development that was chemically induced by the hepatotoxin DEN. Treatment modalities of DEN-induced mice included 3 cycles of treatment at MTDs of BA-12 and BP-14 for 10 days with interim breaks of 7 days (Fig. 5A). Evaluation of tumor nodules that are observed on the surface of cancerous livers revealed that BA-12 causes a 1.4-fold decrease of tumor nodules size as compared to control mice. Intervention with BP-14 showed comparable anti-cancer effects by a 1.3-fold decline of DEN-induced hepatoma (Fig. 5B and 5C). In summary, these data suggest that both BA-12 and BP-14 exhibit strong anti-hepatoma activities in vivo as observed in xenograft models as well as in endogenous liver cancer.

6.6. Discussion

A considerable number of small molecule inhibitors of CDKs have been designed in order to block proliferation of cancer cells (34). Inhibitors of the first generation include roscovitine, a 2,6,9-tri-substituted purine derived from studies evaluating the structure-activity relationship of this compound class (20, 35). It is a selective inhibitor of CDK1/cyclin B, CDK2/cyclin E, CDK5/p35, CDK7/cyclin H and CDK9/cyclin T1. In this study we assessed the anti-cancer activities of the novel roscovitine derivatives BA-12 and BP-14 in hepatoma. These compounds show a much higher potency than roscovitine through considerably lower IC50 values (Supplementary Table S1; Table 1 in (31)) and more efficiently inhibit CDK1, 2, 5, 7 and 9, suggesting an ameliorated selectivity against these kinases.

Nowadays the standard treatment for unresectable, intermediate stages of HCC is TACE. Several chemotherapeutics are used of which doxorubicin and cisplatin are the most commonly used ones (36). Compared to doxorubicin, BA-12 shows a similar IC50 on hepatoma cell lines, whereas BP-14 shows a 10-fold higher efficacy (26). Cisplatin displays an even 10- to 20-fold lower ability of inhibition. The major problem with current chemotherapeutic drugs is that chemoresistance frequently occurs in HCC therapy. Recently Ye et al. described that repeated doses of doxorubicin lead to an overexpression of P-gp and MRP1 and to a subsequent loss of doxorubicin accumulation, thus making cells less susceptible to treatment already after a short period of drug exposure (33). In contrast, our results show that BA-12 and BP-14 fail to trigger resistance mechanisms in various hepatoma cell lines. HCC cells did not exhibit enhanced viability even after long-term treatment. In addition, both compounds were tested using a panel of chemosensitive tumor cell lines and their chemoresistant sublines overexpressing ABCB1. The P-gp overexpressing sublines demonstrated a lower sensitivity against BA-12 and BP-14 by showing IC50 values, which were 2-fold higher than in parental cells (data not shown). Thus, these data propose BA-12 and BP-14 as substrates of P-gp. In agreement, several studies showed that roscovitine represents a high affinity substrate of P-gp as well, yet no concomitant ABCB1-mediated resistance could be observed after treatment with roscovitine (37). Noteworthy, roscovitine was also shown to induce apoptosis in a doxorubicin-resistant human myeloma cell line that overexpresses P-gp (38). Therefore we speculate that despite being P-gp substrates, BA-12 and BP-14 do not induce resistance and are thus being promising novel therapeutic agents with persistent anti-hepatoma activities. BA-12 and BP-14 might be superior to currently available chemotherapeutics, but should be used as first line therapy due to the frequent upregulation of P-gp in drug-treated HCC patients.

Roscovitine is effective in antagonizing CDKs prior to its processing by either glucuronidation or cytochromes P450-mediated metabolism. The cytochromes P450 CYP3A4 and CYP2B6 generate the carboxylate PMF30-128 as major metabolite that lacks inhibitory function on CDKs (39). Glucuronidation takes place via the UDP-glucuronosyl-transferases (UGTs) 1A1, 1A3 and 2B7 (39, 40). Although not tested yet, we assume that BA-12 and BP-14 are metabolized in a similar way.

Currently running experiments focus on the uptake and metabolism of these drugs and investigate whether the compound clearance via 1A1, 1A3 and 2B7 is much more reduced as compared to roscovitine. We further hypothesize that glucoronidation might be the cause for the higher potency of BP-14 as compared to BA-12. BA-12 harbors a hydroxyl group that gets oxidized to glucuronic acid and is thus much more water soluble leading to a more rapid elimination from the body. The faster metabolic clearance of BA-12 might explain the 10-fold difference in IC50 values between BA-12 and BP-14.

HepG2 cells represent a widely used hepatocellular model in pharmacological studies, as these malignant hepatocytes are well differentiated and show features of normal parenchymal liver cells, such as secretion of lipoproteins, biosynthesis of multiple plasma proteins, and plasma membrane polarity (41, 42). However, HepG2 cells show lower levels of CYPs and UDP-glucuronosyl-transferases (43), when compared to primary human hepatocytes (PHHs). Therefore we treated PHHs with compounds and demonstrated via determination of their IC50 values that BA-12 and BP-14 are cytotoxic in PHHs at about 35-fold and 160-fold higher levels as compared to hepatoma cell lines, respectively. Notably, both compounds are not capable to induce apoptosis of PHHs suggesting lack of obvious adverse effects of BA-12 and BP-14 due to the high xenobiotic metabolism under physiological conditions.

BA-12 and BP-14 display further anti-cancer activities as pilot experiments revealed. Both compounds are able to reduce cell invasion and migration of 3-dimensional hepatospheres into the surrounding extracellular matrix (data not shown). In accordance with these findings, CDK7 was found to affect the migration of ovarian, breast, melanoma and prostate cancer cell lines (44). In the same line, CDK9 was shown to be involved in cancer cell invasion, as miR-34a suppresses the assembly of the CDK9-c-Myc-P-TEFb complexes, leading to an inhibition of cell migration and invasion (45). CDK9 further induces tumor necrosis factor-alpha mediated expression of matrix metalloproteinase-9 that facilitates tumor dissemination (46). In addition, inhibition of CDK5 is suggested to be beneficial for anti-cancer therapy as CDK 5 stimulates Rac1-dependent migration of endothelial cells during tumor angiogenesis (47). The putative multiple inhibitory roles of BA-12 and BP-14 in hepatoma growth and migration make them promising new drugs that must be considered for clinical investigation.

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6.9. Figures

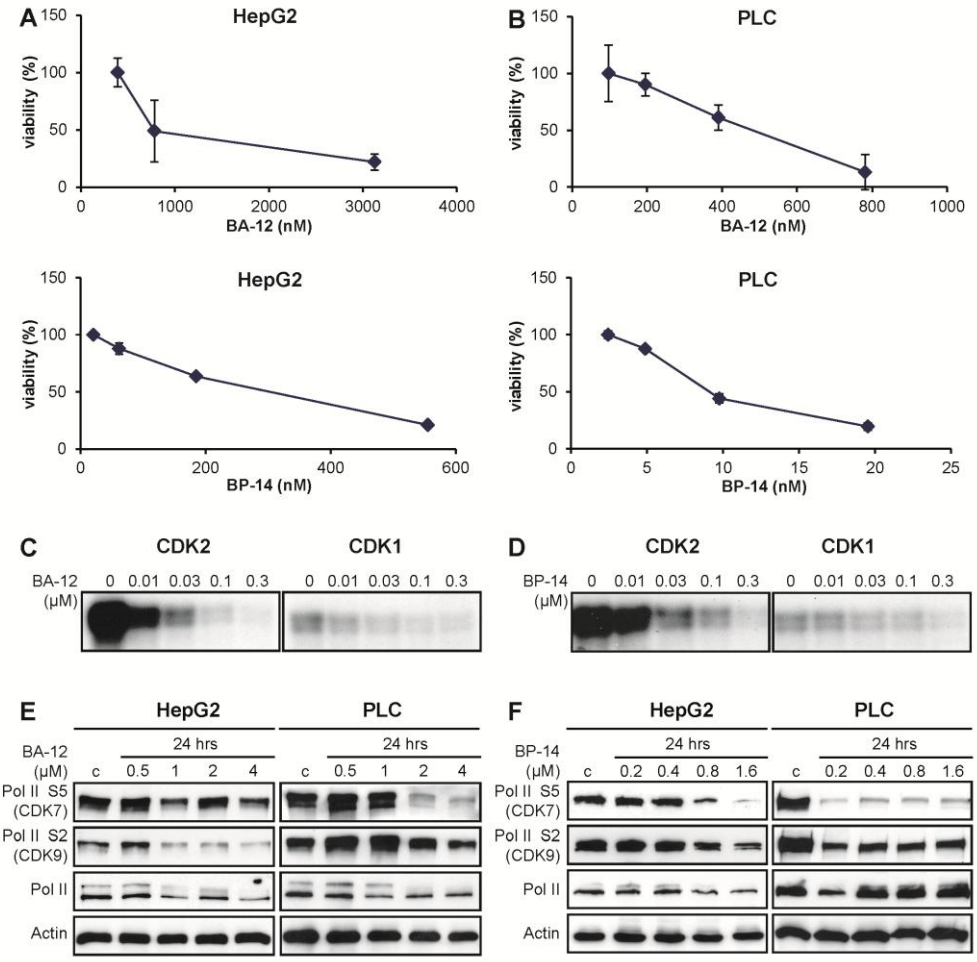


Figure 1. BA-12 and BP-14 diminish cell viability of hepatoma cells and block multiple CDKs.
A and B, dose-dependent effects of BA-12 (A) and BP-14 (B) on the viability of human HepG2 and PLC cells. C and D, inhibition of CDK1 and CDK2 activity by BA-12 (C) and BP-14 (D) in cell-free extracts. E and F, suppression of CDK7 and CDK9 activity after exposure to different concentrations of BA-12 (E) and BP-14 (F) for 24 hours in HepG2 and PLC cells. As detected by immunoblotting, CDK7 and CDK9 activities correspond to serine 5 and serine 2 phosphorylation of RNA polymerase II, respectively. The expression of actin indicates equal loading of protein samples. c, control. Error bars depict SD from at least three individual experiments.

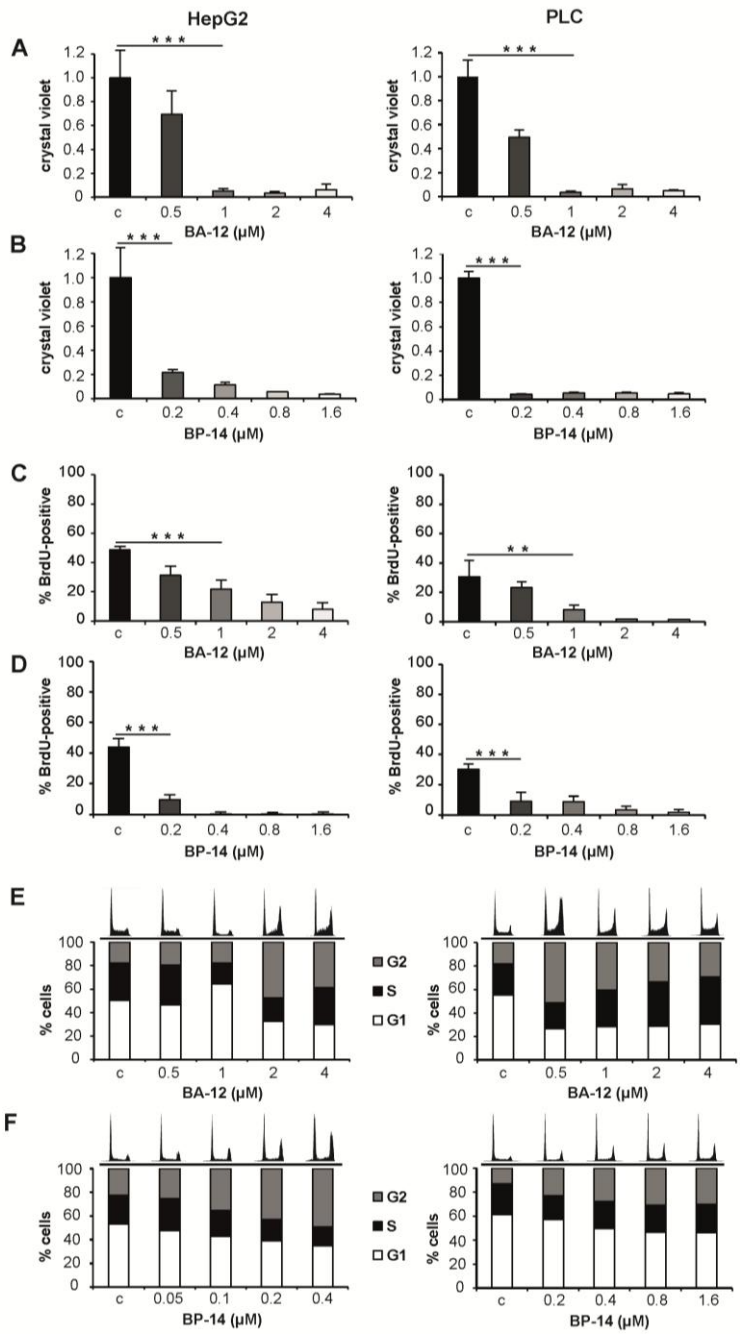


Figure 2. BA-12 and BP-14 interfere with clonogenicity and cell cycle progression of HCC cells. A and B, quantitative evaluation of crystal violet-positive colonies generated from HepG2 (left panel) and PLC cells (right panel). Cells were pretreated with different concentrations of BA-12 (A) or BP-14 (B). C and D, HepG2 (left) and PLC cells (right) were exposed to BA-12 (C) or BP-14 (D) for 24 hours and the DNA synthesis analyzed by BrdU incorporation. E and F, flow cytometry showing the cell cycle distribution of HepG2 (left) and PLC cells (right) after treatment with different concentrations of BA-12 (E) or BP-14 (F) for 24 hours. The cellular DNA content is shown in histograms (upper panel) and the percent of cells in G1, S or G2 phase are depicted in bars after quantification (lower panel). c, control. Error bars depict SD from at least three individual experiments. Statistical significance is indicated with asterisks (** $p<0.01$, *** $p<0.005$).

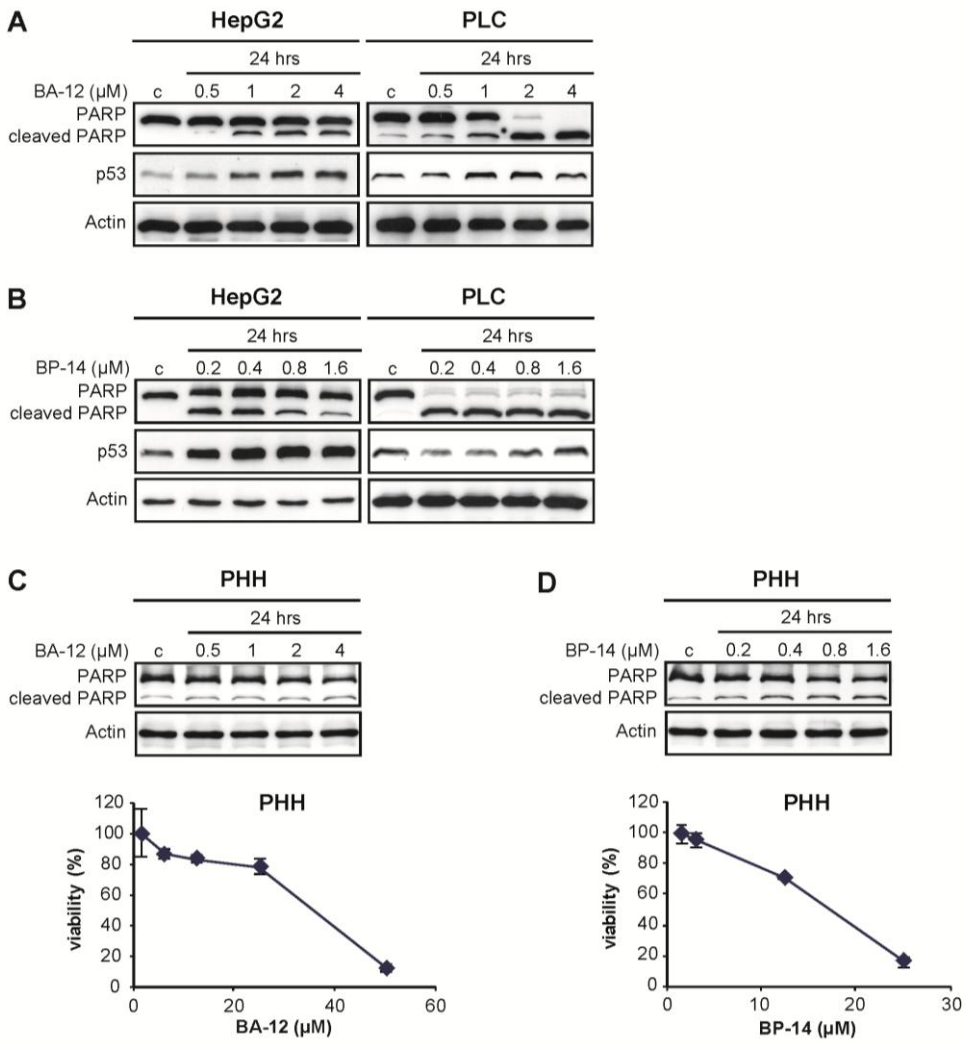


Figure 3. Apoptosis induced by BA-12 and BP-14 in HCC cells but not in primary human hepatocytes (PHHs). A and B, cleavage of PARP after treatment of HepG2 and PLC cells with different concentrations of BA-12 (A) or BP-14 (B) for 24 hours. C, PARP cleavage (upper panel) and determination of dose-dependent effects of BA-12 on the viability (lower panel) of PHHs. D, PARP cleavage and viability of PHHs after treatment with BP-14. Actin is shown as loading control. Error bars depict SD from at least three individual experiments.

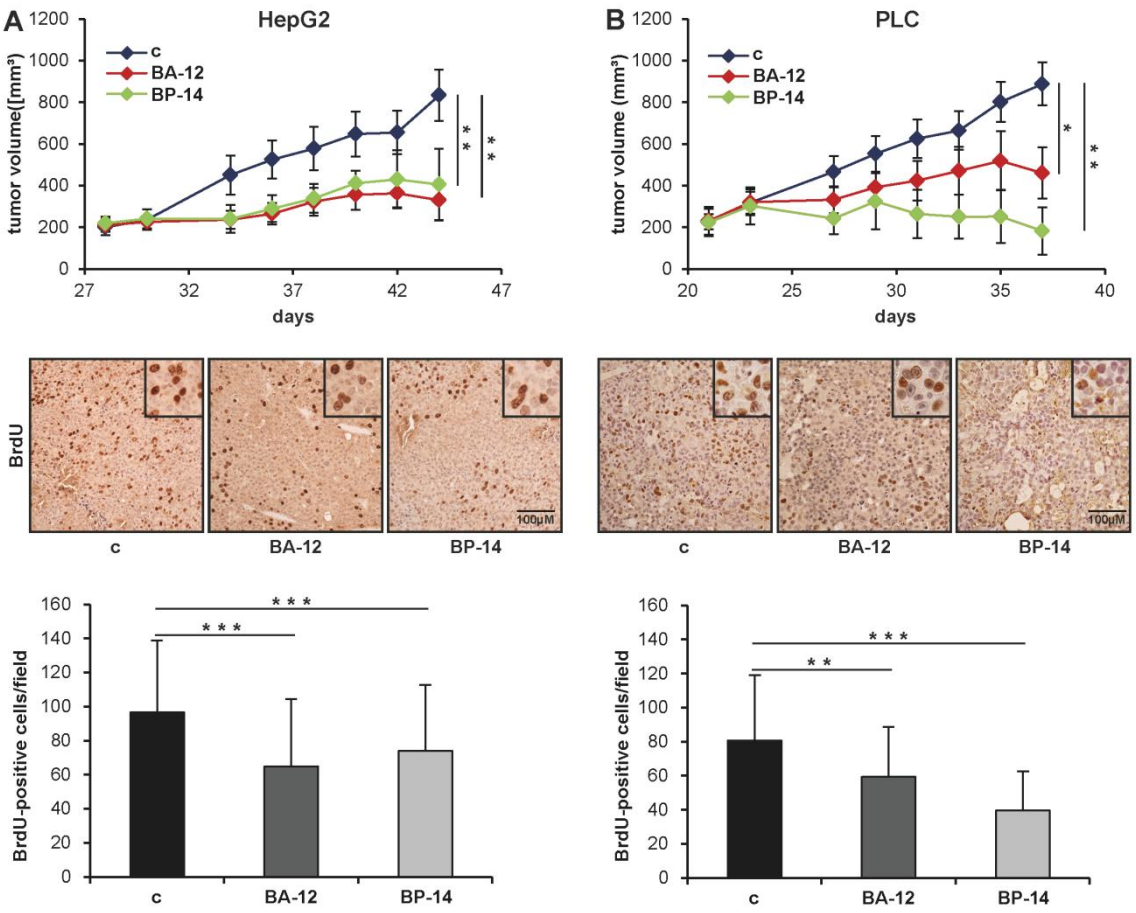


Figure 4. Intervention of xenografted HCC models with BA-12 and BP-14. Tumors were generated by subcutaneous injections of HepG2 and PLC cells into immunodeficient SCID mice. Pharmacological intervention was performed in tumor-bearing mice by daily intraperitoneal injection of either BA-12 or BP-14 for 17 days. A and B (upper panel), volumes of HepG2- (A) and PLC-derived tumors (B) in the absence of compounds (control) and after interference with BA-12 or BP-14. Middle panel, immunohistochemistry showing tumor sections stained with anti-BrdU antibody. Inserts show BrdU labeling at higher magnification. Lower panel shows quantitative analysis of BrdU incorporation. c, control. Error bars depict SD and SEM from three individual experiments that were performed in quadruplicates. Statistical significance is indicated with asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.005$).

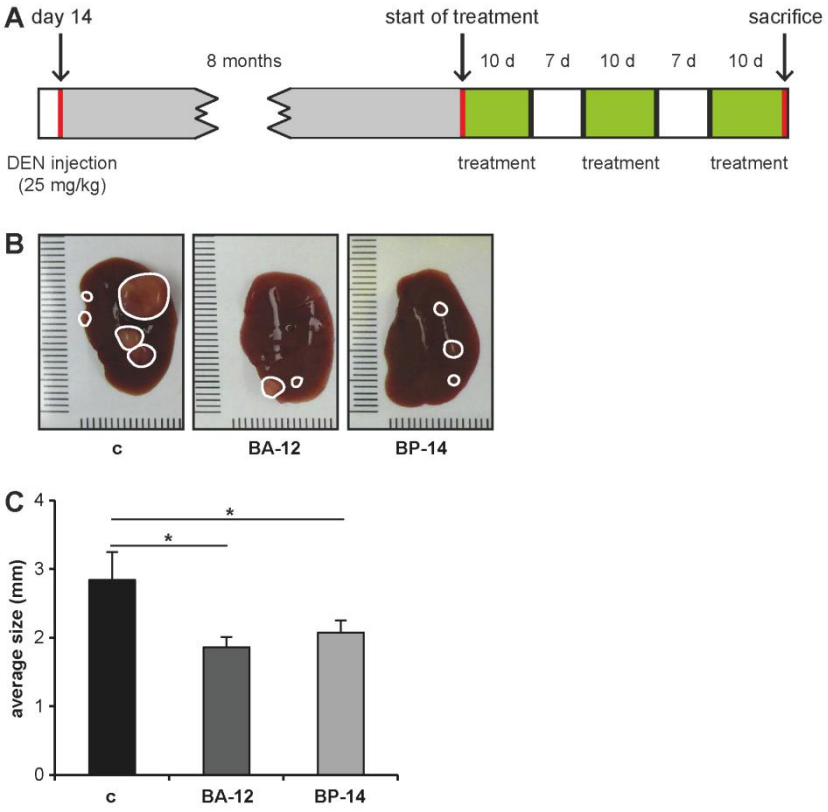


Figure 5. BA-12 or BP-14 reduce DEN-induced hepatoma formation. Endogenous liver cancer was induced by a single DEN injection in 14 days-old male C57BL/6J mice. A, scheme depicting the treatment schedule with BA-12 or BP-14. After 8 month (hatched box), DEN-induced mice were subjected to 3 cycles of drug treatment for 10 days (green boxes) and a release from compound for 7 days between the cycles. B, representative morphologies of DEN-induced hepatoma (control) and those treated with BA-12 and BP-14. White circles indicate cancerous liver nodules in the left lateral liver lobe. C, the diameters of cancerous nodules were scored on the surface of livers and depicted in bars. Statistical significance is indicated with asterisks (* $p<0.05$).

6.10. Supplementary Data

Supplementary Materials and Methods

Kinase inhibition assays using recombinant substrates

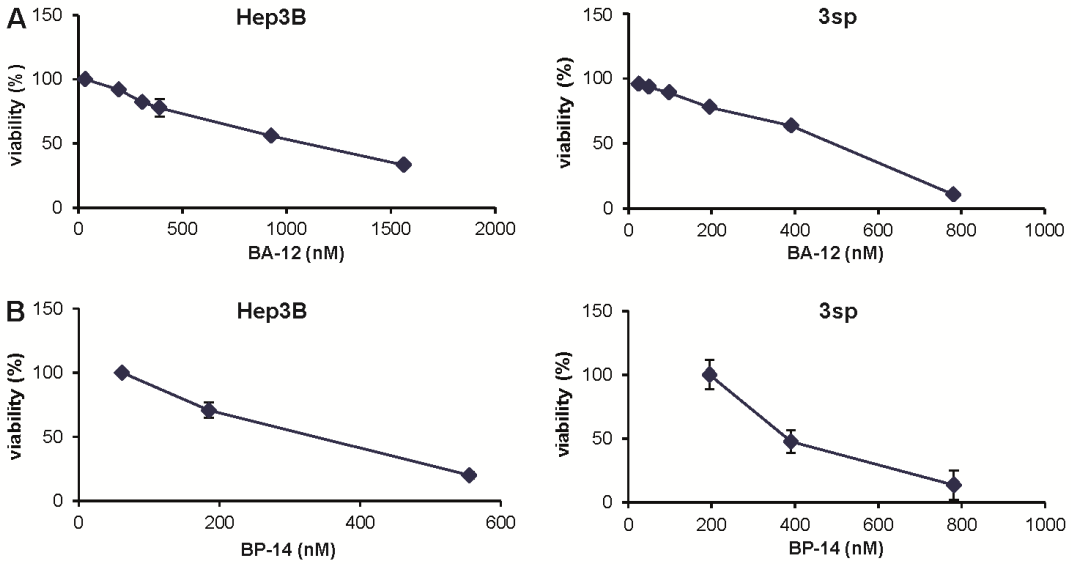
CDK1/cyclin B and CDK2/cyclin E kinases were produced in Sf9 insect cells via baculoviral infection, while CDK5/p35, CDK7/cyclin H/MAT1, and CDK9/cyclin T1 were purchased from ProQinase (Freiburg, Germany) and assayed as described previously (1). The kinase reactions were assayed with 1 mg/ml histone H1 (for CDK2 and CDK5) or (YSPTSPS) 2KK peptide (for CDK7 and CDK9) in the presence of 15/0.15/1.5/1.5 mM ATP (for CDK2/CDK5/CDK7CDK9), 50 µCi [γ-^{33}P]ATP and of the test compound in a final volume of 10 ml. The reaction buffer contained 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 mM Na-orthovanadate, 1.2 mM DTT, 2.5 mg/50 ml PEG20.000. The reactions were stopped by adding 5 ml of 3% H_3PO_4. Aliquots were spotted onto P-81 phosphocellulose (Whatman, GE Healthcare Biosciences, Pittsburgh, USA), washed 3 times with 0.5% H_3PO_4 and finally air-dried. Kinase inhibition was quantified using a FLA-7000 digital image analyzer (Fujifilm, Tokyo, Japan). The concentration of the test compounds required to decrease the CDK activity by 50% was determined from dose-response curves and designated as IC50.

Analysis of proliferation

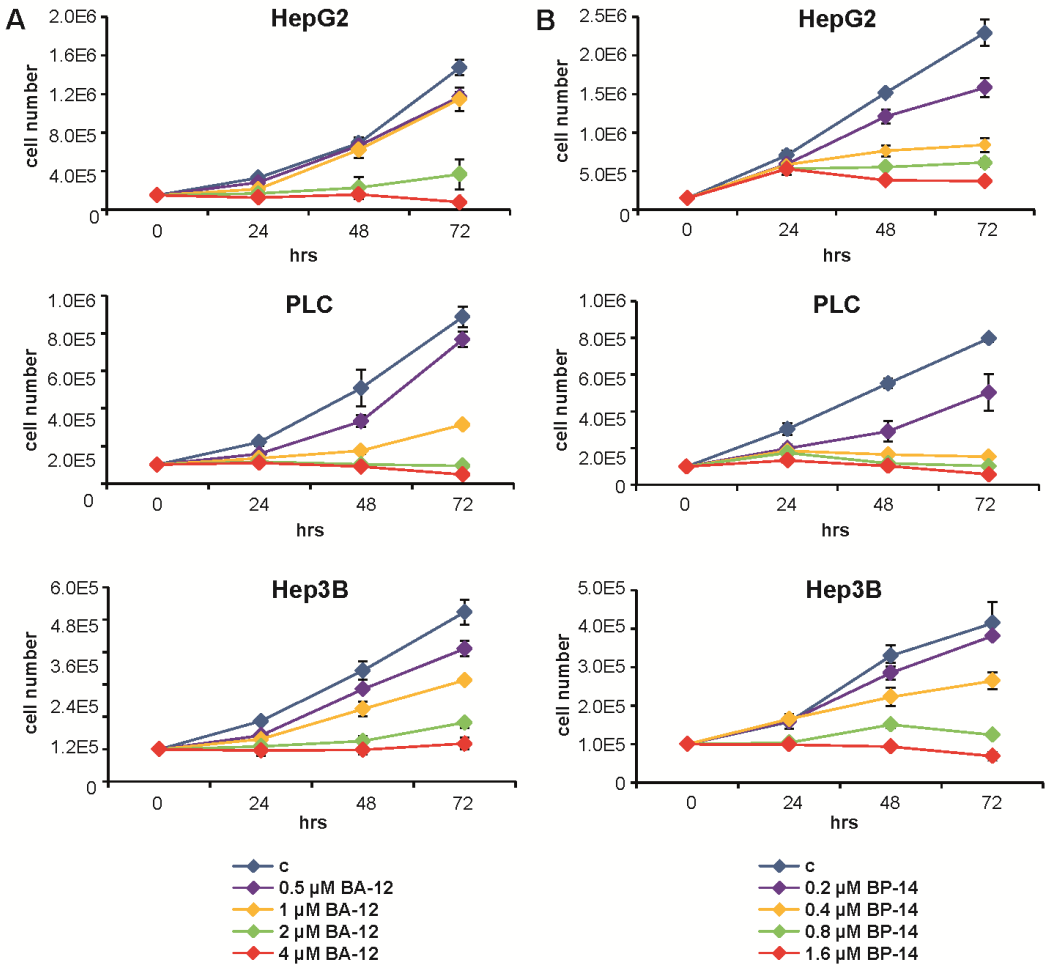
6 x 10^4 cells were seeded on 12-well plates and incubated with inhibitors at different concentrations for 3 days. Cell numbers were determined at various time points after trypsinization using a cell counter (CASY, Schärfe Systems, Reutlingen, Germany). Three independent experiments were performed in triplicates

Supplementary Figures and Tables

Supplementary Figure S1.Cell viability and CDK inhibition of Hep3B and 3sp hepatoma cells. A and B, dose-dependent effects of BA-12 (A) and BP-14 (B) on the viability of human Hep3B and 3sp hepatoma cells. Cell viability was determined by MTT assay. Error bars depict SD from at least three individual experiments.



Supplementary Figure S2. Proliferation of HCC cells after exposure to BA-12 or BP-14. A and B, Proliferation kinetics of HepG2, PLC and Hep3B cells after treatment with different concentrations of BA-12 (A) or BP-14 (B). Error bars depict SD from at least three individual experiments.



Supplementary Table S1. IC50 values of BA-12 and BP-14 in hepatoma cell lines

Cell line	IC_{50} (μM)	
	BA-12	BP-14
HepG2	0.75	0.12
PLC	0.56	0.02
Hep3B	0.63	0.08
3sp	0.77	0.48
PHH	26.07	20.80

Supplementary Table S2. Selectivity of BA-12 and BP-14 on protein kinases using recombinant CDK substrates

Protein kinase	IC_{50} (μM)	
	BA-12	BP-14
CDK1/cyclin B	0.023	0.050
CDK2/cyclin E	0.008	0.010
CDK5/p25NCK	0.021	0.015
CDK7/cyclin H/Mat1	0.048	n.t.
CDK9/cyclin T	0.010	0.007

n.t., not tested.

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8. Curriculum Vitae

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2007	**Internship: quality management** Baxter Bioscience (Quality control)

Publications

Reichl P, Haider C, Grubinger M, Mikulits W. **TGF-beta in epithelial to mesenchymal transition and metastasis of liver carcinoma**. Current pharmaceutical design. 2012;18:4135-47.

Haider C, Grubinger M, Řezníčková E, Weiss T, Rotheneder H, Miklos W, Berger W, Jorda R, Zatloukal M, Gucký T, Strnad M, Kryštof V, Mikulits W (2013) **Novel Inhibitors of Cyclin-Dependent Kinases Combat Hepatocellular Carcinoma without Inducing Chemoresistance**(Clinical Cancer Research, in review)