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

Christine Haider1, Markus Grubinger1, Eva Rezníčková2, Thomas S. Weiss5, Hans Rotheneder2, Walter Miklos1, Walter Berger1, Radek Jorda3, Marek Zatloukal4, Tomáš Gucky4, Miroslav Stmaň3,4, Vladimír Krystof3, and Wolfgang Mikulits1

Abstract

Treatment options for hepatocellular carcinoma 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 cyclin-dependent kinases (CDK) is frequently observed in hepatocellular carcinomas, we focused on the efficacy of the novel compounds BA-12 and BP-14 that antagonize CDK1/2/5/7 and CDK9. Inhibition of those CDKs in human hepatocellular carcinoma cell lines reduced the 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 hepatocellular carcinoma cells after long-term exposure to inhibitors. In vivo, treatment of xenografted human hepatocellular carcinomas with BA-12 or BP-14 effectively repressed tumor formation. Moreover, BA-12 or BP-14 significantly diminished diethylnitrosamine (DEN)-induced hepatoma development in mice. These data show that BA-12 or BP-14 exhibit strong antitumorigenic effects in the absence of chemoresistance, resulting in a superior efficacy compared with currently used chemotherapeutics in hepatocellular carcinomas. Mol Cancer Ther; 12(10): 1947–57. ©2013 AACR.

Introduction

Hepatocellular carcinoma (HCC) represents the sixth most common cancer and the third leading cause of cancer-related deaths worldwide (1). Less than 30% to 40% of patients with hepatocellular carcinomas 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 hepatocellular carcinoma 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 hepatocellular carcinoma 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% to 15% (5, 6), and other chemotherapy agents, such as epirubicin, cisplatin, 5-fluorouracil, etoposide, and their combinations, show 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 hepatocellular carcinoma cells and with a worse prognosis (7). Sorafenib represents the current treatment standard for advanced hepatocellular carcinoma by prolonging median survival of patients for about 3 months (8). These limitations in treatment modalities strongly indicate the urgent need for novel alternative treatment options.

Cyclin-dependent kinases (CDK) are fundamental for cell-cycle control and regulation of apoptosis (9, 10) and are found deregulated in most cancer cells. Hepatocellular carcinoma shows frequent upregulation of CDKs through inactivation of CDK inhibitory proteins including p16INK4a,
p21^{WAF1/CIP1}, p27^{kip1}, and p57^{kip2} 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 hepatocellular carcinoma samples (17). Similarly, CDK1/cyclin B complexes participate and complete mitosis and cyclin B overexpression is frequently observed in hepatocellular carcinoma (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 hepatocellular carcinoma.

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 antiproliferative agents for cancer therapy, including (R)-roscovitine (Seliciclib, CYC202). Roscovitine is known to selectively inhibit CDK1, CDK2, CDK5, CDK7, and CDK9 activities through its binding to the ATP-binding site (20). Currently, roscovitine is evaluated in a phase I clinical trial in combination with sapacitabine with patients suffering from advanced solid tumors (NCT00999401) and in a phase II trial from patients with non–small cell lung cancer (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, whereas 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), conducts 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 hepatocellular carcinoma cells and further determined their anticancer 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 hepatocellular carcinoma 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 hepatocellular carcinoma cells rather than of primary human hepatocytes (PHH). Notably, no chemoresistance against these compounds could be observed after long-term treatment of hepatocellular carcinoma 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.

Materials 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 a patient with hepatocellular carcinoma at the Medical University of Vienna (Vienna, Austria; ref. 26). All cell lines were verified by short-tandem repeat analysis in November 2012.

Primary human hepatocytes

Non-neoplastic tissue samples from liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were conducted 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 trypsin 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) at a density of 1.2 × 10^5 cells/cm2. The medium consisted of Dulbeccos’ modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 mg/mL streptomycin, 100 U/mL penicillin, and supplements as follows: 125 mU/mL insulin, 7.3 mg/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-[[2-[(4-aminocyclohexyl)amino]-9-cyclopen-tyl-purin-6-yl]amino][methyl]-4-chloro-phenol; Supplementary Fig. S1A) and BP-14 (N2-(4-aminocyclohexyl)-9-cyclopentyl-N6-[[6-(2-furyl)-3-pyridyl][methyl]purine-2,6-diamine; Supplementary Fig. S1B) were synthesized by procedures as described (28). Compounds were dissolved in dimethyl sulfoxide (DMSO). The stock solution of 100 mmol/L 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

Cell viability was determined using the MTT assay. Briefly, cells were seeded in triplicates at a density of 6 × 10^3 cells per 96 wells. After 24 hours, cells were incubated with drug-containing medium for 72 hours. Cells were incubated with MTT solution (5 mg/mL; Sigma) and medium was replaced with DMSO after 5 hours. The
absorbance was measured at 620 nm by using a microplate reader (Asys HiTech). MTT assays were repeated three times for each drug application. IC_{50} 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 mmol/L Tris pH 8.0, 100 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% NP-40. Hundred micrograms 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) or with 1 μg of the anti-cyclin B1 antibody GNS1 (Santa Cruz Biotechnology). Precipitated protein was resuspended in 20 μL kinase buffer containing 5 μCi [γ-^32P]ATP (PerkinElmer), 1 μg histone 1 (New England Biolabs), 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**

Five hundred 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/acetate 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 bromodeoxyuridine incorporation**

Cultured cells were grown in medium containing 10 μmol/L bromodeoxyuridine (BrdUrd) for 1 hour. After removing labeling medium, cells were fixed and DNA denatured with a fixing/denaturing solution containing 2 mol/L HCl for 30 minutes at 37°C. To analyze BrdUrd incorporation in vivo, 200 μL Ringer solution containing 1 mg BrdUrd was intraperitoneally injected into xenografted mice 2 hours before sacrifice. Tumor tissue was fixed in 4% formaldehyde and processed for immunohistochemistry. BrdUrd incorporation was detected with a monoclonal anti-BrdUrd antibody (Sigma) and a peroxidase-conjugated secondary antibody (Calbiochem).

**Flow cytometry**

The analysis of cellular DNA content was conducted with a multicolor BD LSRFortessa cell analyzer (Becton Dickinson). Before the cytotoxicometric measurement, about 5 × 10⁶ cells were washed with 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 was 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 IC_{50} (1:2 IC_{50}, 1:4 IC_{50}, 1:8 IC_{50}, and 1:16 IC_{50}). The selection of chemoresistant cells was monitored every 6 weeks for 9 months by the determination of IC_{50} values using the MTT assay. Hepatocellular carcinoma cells showing higher IC_{50} values after treatment with inhibitors than untreated cells are considered as chemoresistant.

**Immunoblotting**

Immunoblotting was conducted as described previously (29). Primary antibodies: anti-S807/811 Rb (Cell Signaling Technology), 1:500; anti-Rb (Cell Signaling Technology), 1:1,000, anti-phospho-Ser5 RNA Pol II (CDK7; Bethyl Laboratories), 1:1,000; anti-phospho-Ser2 RNA Pol II (CDK9; Bethyl Laboratories), 1:1,000; anti-RNA Pol II (Santa Cruz Biotechnology), 1:1,000; anti-S-PARP (Cell Signaling Technology), 1:1,000; anti-cleaved caspase-7 (Cell Signaling Technology), 1:1,000; anti-caspase-7 (Cell Signaling Technology), 1:1,000; anti-β-actin (Sigma), 1:2,500. Horseradish peroxidase-conjugated secondary antibodies (Calbiochem) were used at dilutions of 1:10,000.

**Xenografted tumor formation and drug intervention**

A total of 5 × 10⁶ human hepatoma cells were resuspended in 100 μL Ringer solution and subcutaneously injected into severe combined immunodeficient (SCID) mice (Harlan Laboratories). Tumor volume was determined as described previously (29). Pharmacologic intervention was conducted 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. The experiments were carried out using 5 mice per group and carried out 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, pharmacologic intervention was administered 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. About 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. Haider and M. Gruinger) 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. The experiments were carried out using 20 mice per group and carried out according to the Austrian guidelines for animal care and protection.

**Immunohistochemistry and TUNEL analysis**

Mice were sacrificed and tumors were fixed as described (30). Four-micrometer-thick, paraffin-embedded sections were stained with anti-BrdUrd (Sigma), 1:200. Biotinylated secondary antibody was used at 1:200. The immunoperoxidase procedure was conducted using a Vectastain Elite ABC kit (Vector Laboratories) as described by the manufacturer. To detect DNA fragmentation by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis in tumor tissues, an In Situ Cell Death Detection kit (Roche) was used as recommended by the manufacturer.

**Statistical analysis**

Data were expressed as means ± SD or SEM. The statistical significance of differences was evaluated using an unpaired, nonparametric Student t test. Significant differences between experimental groups were *, \(P < 0.05\); **, \(P < 0.01\); or ***, \(P < 0.005\).

**Results**

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

Novel derivatives of roscovitine, designated BA-12 and BP-14, were synthesized on the basis of 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 B). Analysis of additional established hepatocellular carcinoma cell lines (Hep3B and 3sp) confirmed the cytotoxicity of BA-12 and BP-14 (Supplementary Fig. S2A and S2B). Dose–response curves revealed IC₅₀ values below 1 μmol/L for both compounds in the various hepatocellular carcinoma cell lines, reaching as low as 0.02 μmol/L in PLC cells for
Comparable observations were made after treatment with PARP and p53 expression in HepG2 cells (Fig. 3A, left). In PLC cells expressing full-length but mutated p53 (32), 12 or BP-14 in HepG2 cells that harbor wild-type p53 and rather than in PHHs.

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 μmol/L BA-12 (Fig. 2A). BP-14–treated cells displayed loss of clonogenic even at 0.2 μmol/L (Fig. 2B). Analysis of DNA synthesis revealed that treatment of HepG2 or PLC cells with 1 μmol/L of either BA-12 or BP-14 decreased BrdUrd incorporation more than 2-fold as compared with control (Fig. 2C and D), indicating inhibition of DNA synthesis. Proliferation kinetics showed a cytostatic effect of BA-12 at 2 μmol/L and of BP-14 at 0.8 μmol/L in both HepG2 and PLC cells as well as in Hep3B hepatoma cells (Supplementary Fig. S3A and S3B). Accordingly, both compounds induced an accumulation in the G2–M and S-G2 phase of the cell cycle (Fig. 2E and F). These data suggest that both BA-12 and BP-14 act antiproliferative by blocking DNA replication and by arresting hepatocellular carcinoma cells in the S–G2–M phase of the cell cycle.

BA-12 and BP-14 induce apoptosis in hepatoma cells rather than in 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 μmol/L BA-12 induced cleavage of PARP and p53 expression in HepG2 cells (Fig. 3A, left). Comparable observations were made after treatment with 2 μmol/L BA-12 in p53-mutated PLC cells (Fig. 3A, right). BP-14 was able to trigger PARP cleavage even at a concentration of 0.2 μmol/L (Fig. 3B), thus being more potent to induce apoptosis as compared with BA-12. Cleavage of caspase-7 further confirmed data of PARP processing (Fig. 3A and B). As BA-14 induced apoptosis at 0.2 μmol/L in the absence of CDK7/9 inhibition, these data suggest cytotoxic effects that are also independent of CDK7/9. Yet, both BA-12 and BP-14 failed to induce PARP processing in PHHs, which are the cellular origin of hepatoma (Fig. 3C and D). Accordingly, both BA-12 and BP-14 exhibited IC50 values of 26 and 20 μmol/L 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 hepatocellular carcinoma 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 hepatocellular carcinoma cells

Most of the chemotherapeutic compounds that are currently available for hepatocellular carcinoma 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 months. In case, hepatocellular carcinoma cells lower their chemosensitivity by acquiring a resistance mechanism, IC50 values increase over time. Most notably, we observed that IC50 values were maintained in hepatoma cells as compared with control during sustained drug exposure (Table 1). These data provide strong evidence that the cytotoxic effects of BA-12 and BP-14 on hepatocellular carcinoma cells are maintained upon persistent drug treatment, suggesting that hepatoma cells fail to acquire chemoresistance toward BA-12 and BP-14.

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

To examine antitumorigenic 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 as 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 B). 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 BrdUrd incorporation into...
BA-12 and BP-14 interfere with clonogenicity and cell-cycle progression of hepatocellular carcinoma cells. A and B, quantitative evaluation of crystal violet–positive colonies generated from HepG2 (left) and PLC cells (right). 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 BrdUrd 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 (top) and the percentage of cells in G1, S, or G2 phase are depicted in bars after quantification (bottom). c, control. Error bars depict SD from at least 3 individual experiments. Statistical significance is indicated with asterisks (**, P < 0.01; ***, P < 0.005).
DNA revealed an up to 2-fold decrease after exposure to either BA-12 or BP-14 (Fig. 4C and D). Further analysis showed a 2-fold increase of TUNEL-positive cells after treatment of HepG2-derived tumors with BA-12 or BP-14, which was similar in PLC tumors, indicating that both compounds induce apoptosis in tumor tissues (Fig. 4E and F). Thus, cytostatic effects by reduced DNA synthesis and cytotoxic effects by augmented apoptosis explain the efficacy of these compounds.

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 three 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 with control mice. Intervention with BP-14 showed comparable anticancer treatment of HepG2-derived tumors with BA-12 or BP-14, which was similar in PLC tumors, indicating that both compounds induce apoptosis in tumor tissues (Fig. 4E and F). Thus, cytostatic effects by reduced DNA synthesis and cytotoxic effects by augmented apoptosis explain the efficacy of these compounds.

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 three 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 with control mice. Intervention with BP-14 showed comparable anticancer
effects by a 1.3-fold decline of DEN-induced hepatoma (Fig. 5B and C). Noteworthy, mice treated with BA-12 and BP-14 did not show a decrease in body weight compared with control group (Fig. 5D), suggesting that both compounds do not show obvious side effects. In summary, these data indicate that both BA-12 and BP-14 exhibit strong antihepatoma activities in vivo as observed in xenograft models as well as in endogenous liver cancer.

Discussion

A considerable number of small-molecule inhibitors of CDKs have been designed 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 anticancer activities of the novel roscovitine derivatives BA-12 and BP-14 in hepatoma. These compounds show a much higher potency than roscovitine as indicated by the inhibition of CDK1/2 in cell-free extracts (Fig. 1C) and through considerably lower IC₅₀ values against recombinant CDK1, 2, 5, 7 and 9 (compare Supplementary
Table S2; with Table 1 in ref. 31), suggesting an ameliorated selectivity against these kinases. The kinase selectivity of both BA-12 and BP-14 to CDKs was verified on a panel of 110 human protein kinases (E. Rezníčková, manuscript in preparation). Besides CDKs, the most sensitive kinases are CK1d and ERK8. In addition, BA-12 also targets CLK2.

Nowadays the standard treatment of unresectable, intermediate stages of hepatocellular carcinoma is TACE. Several chemotherapeutics are used of which doxorubicin and cisplatin are most commonly used (36). Compared with doxorubicin, BA-12 shows a similar IC\textsubscript{50} 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 hepatocellular carcinoma therapy. Recently, Ye and colleagues 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. Hepatocellular carcinoma 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 showed a lower sensitivity against BA-12 and BP-14 by showing IC\textsubscript{50} 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 (26). Noteworthy, roscovitine was also shown to induce apoptosis in a doxorubicin-resistant human myeloma cell line that overexpresses P-gp (37). 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 antihepatoma 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 patients with hepatocellular carcinoma.

Roscovitine is effective in antagonizing CDKs before its processing by either glucuronidation or cytochrome P450-mediated metabolism. The cytochromes P450 CYP3A4 and CYP2B6 generate the carboxylate PMF30-128 as major metabolite that lacks inhibitory function on CDKs (38). Glucuronidation takes place via the UDP-glucuronosyltransferases (UGT) 1A1, 1A3, and 2B7 (38, 39). 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 with roscovitine. We further hypothesize that glucuronidation.
might be the cause for the higher potency of BP-14 as compared with 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 pharmacologic 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 (40, 41). However, HepG2 cells show lower levels of CYPs and UDP-glucuronosyl-transferases (42), when compared with PHHs. Therefore, we treated PHHs with compounds and showed via determination of their IC50 values that BA-12 and BP-14 are cytotoxic in PHHs at about 35- and 160-fold higher levels as compared with 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 physiologic conditions.

BA-12 and BP-14 display further anticancer 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 (43). 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 (44). CDK9 further induces TNF-α-mediated expression of MMP-9 that facilitates tumor dissemination (45). In addition, inhibition of CDK5 is suggested to be beneficial for anticancer therapy as CDK5 stimulates Rac1-dependent migration of endothelial cells during tumor angiogenesis (46). 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. Haider, M. Grubinger, M. Strnad, V. Krystof, W. Mikulits
Development of methodology: C. Haider, M. Grubinger
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Haider, E. Reznikova, T.C. Weiss, H. Rotthuener, W. Miklos, W. Berger, R. Jord, E. Reznikova
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Haider, M. Grubinger, W. Miklos, W. Berger, W. Mikulits
Writing, review, and/or revision of the manuscript: C. Haider, M. Grubinger, R. Jord, M. Strnad, V. Krystof, W. Mikulits
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.C. Weiss, M. Zatloukal, T. Gucky
Study supervision: M. Strnad, W. Mikulits

Other: Synthesis of CDK inhibitor BA-12, M. Zatloukal; Synthesis of compounds, T. Gucky

Acknowledgments
The authors thank Heidemarie Huber for excellent technical support and artwork.

Grant Support
This work was supported by the European Union, FP7 Health Research, project number HEALTH-F4-2008-202047 (to W. Mikulits), by the Austrian Science Fund, P2095-B13 (to W. Mikulits), by the Herzerfelder Family Foundation (to H. Rotthuener and W. Mikulits), by the Czech Science Foundation, grants P305/12/0783 and 301/08/1649 (to V. Krystof, M. Zatloukal, and M. Strnad), by the Ministry of Education, Youth and Sports, Czech Republic, grant Mobility 7AMB12A1011 (to V. Krystof and E. Reznikova), and by grant E0007/01/01 of Centre of the Region Hana for Biotechnological and Agricultural Research (to M. Strnad, M. Zatloukal, and T. Gucky).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 5, 2013; revised July 26, 2013; accepted August 2, 2013; published OnlineFirst August 12, 2013.


38. McClue SJ, Stuart I. Metabolism of the trisubstituted purine cyclin-dependent kinase inhibitor seliciclib (R-roscovitine) in vitro and in vivo. Drug Metab Dispos 2008;36:561–70.


40. Altmann B, Giselbrecht S, Weißeahn KF, Welle A, Gottwald E. The three-dimensional cultivation of the carcinoma cell line HepG2 in a perfused chip system leads to a more differentiated phenotype of the cells compared to monolayer culture. Biomed Mater 2008;3:034120.


www.aacrjournals.org
Mol Cancer Ther; 12(10) October 2013

1957
Molecular Cancer Therapeutics

Novel Inhibitors of Cyclin-Dependent Kinases Combat Hepatocellular Carcinoma without Inducing Chemoresistance
Christine Haider, Markus Grubinger, Eva Reznicková, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0263

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2013/08/13/1535-7163.MCT-13-0263.DC1

Cited articles
This article cites 43 articles, 13 of which you can access for free at:
http://mct.aacrjournals.org/content/12/10/1947.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.