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

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Abbreviation list: ABC, ATP-binding cassette; BrdU, 5-bromo-2’-deoxy-uridine; CDK, cyclin dependent kinase; CTD, C-terminal domain; DEN, diethylnitrosamine; DMSO, dimethylsulfoxide; HCC, hepatocellular carcinoma; IC, inhibitory concentration; MDR,
multiple drug resistance; MTD, maximum tolerated dose; MTT, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHH, primary human hepatocytes; Rb, retinoblastoma; SCID, severe combined immunodeficient; TACE, transarterial chemoembolization; UGT, UDP-glucuronosyl-transferase

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Abstract

Treatment options for hepatocellular carcinoma (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 cyclin dependent kinases (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. Inhibition of those CDKs in human HCC 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 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. 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.
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 p16\textsuperscript{Ink4}, p21\textsuperscript{WAF/CIP1}, p27\textsuperscript{KIP1} and p57\textsuperscript{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 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 through its binding to the ATP-binding site (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.
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 10⁵ 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) (Supplementary Figure S1A) and BP-14 (N2-(4-aminocyclohexyl)-9-cyclopentyl-N6-[[6-(2-furyl)-3-pyridyl]methyl]purine-2,6-diamine) (Supplementary Figure S1B) 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)\textsubscript{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\textsuperscript{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 (Asys HiTech, Salzburg, Austria). MTT assays were repeated 3 times for each drug application. IC\textsubscript{50} values were obtained by log-linear interpolation of data points and are depicted by dose-response curves using the software GraphPad Prism\textsuperscript{®} 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, 1 mM 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 [\textgamma\textsuperscript{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 antibodies (Calbiochem, LaJolla, USA).

**Flow cytometry**

The analysis of cellular DNA content was performed with a multicolor BD LSRFortessa cell analyzer (Becton Dickinson, Franklin Lakes, USA). Prior to the cytofluorometric measurement, about 5 x 10⁵ 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 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 nine month by the determination of IC$_{50}$ values using the MTT assay. HCC cells showing higher IC$_{50}$ 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-S807/811 Rb (Cell Signaling Technology, Beverly, USA), 1:500; anti-Rb (Cell Signaling Technology, Beverly, USA), 1:1000; 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-cleaved caspase-7 (Cell Signaling Technology, Beverly, USA), 1:1.000; anti-caspase-7 (Cell Signaling Technology, Beverly, USA), 1:1.000; 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. The experiments were performed using five 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, 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. The experiments were performed using twenty 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). 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. To detect DNA fragmentation by TUNEL analysis in tumor tissues, an *in situ* cell death detection kit (Roche, Mannheim, Germany) was employed as recommended 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.
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. S2A and S2B). Dose-response curves revealed IC_{50} 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). Notably, roscovitine showed no effect at this concentration which suggests less potency than BA-12 and BP-14 (Fig. 1C). Furthermore, administration of HepG2 and PLC cells with 0.5 µM BA-12 or 0.2 µM BP-14, respectively, caused a significant decrease in CDK2-specific phosphorylation of retinoblastoma (Rb; Fig. 1E and 1F). This was associated with a more moderate lowering of total Rb levels at higher drug concentrations that might be due to apoptosis. In the same line, 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 IC_{50} 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. S3A and S3B). 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. Cleavage of caspase-7 further confirmed data of PARP processing (Fig. 3A and 3B). As BP-14 induced apoptosis at 0.2 µM 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 3D). Accordingly, both BA-12 and BP-14 exhibited IC$_{50}$ 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 IC$_{50}$ values as well as at serial dilutions (1/2 IC$_{50}$, 1/4 IC$_{50}$, 1/8 IC$_{50}$ and 1/16 IC$_{50}$) for up to 9 month. In case HCC cells lower their chemosensitivity by acquiring a resistance mechanism, IC$_{50}$ values rise over time. Most notably, we observed that IC$_{50}$ 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). 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 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 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). Noteworthy, mice treated with BA-12 and BP-14 did not show a decrease in body weight compared to 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 anti-hepatoma 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 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 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 (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. Řezníčková, manuscript in preparation). Besides CDKs, the most sensitive kinases are CK1d and ERK8. In addition, BA-12 also targets CLK2.

Nowadays the standard treatment for unresectable, intermediate stages of HCC is TACE. Several chemotherapeutics are used of which doxorubicin and cisplatin are most commonly used (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 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 (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 glucuronidation 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 IC\textsubscript{50} 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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Table 1. Sustained cytotoxicity in HCC cell lines after long-term exposure to BA-12 and BP-14

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Figure Legends

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. Roscovitine (R) was used at a concentration of 0.03 µM. E and F, reduction of the CDK2-specific phosphorylation of Rb as well as 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 (Pol) 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 and caspase-7 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, volumes of HepG2-(A) and PLC-derived tumors (B) in the absence of compounds (control) and after interference with BA-12 or BP-14. C and D, immunohistochemistry showing tumor sections stained with anti-BrdU antibody. Inserts show BrdU labeling at higher magnification. The panel below shows quantitative analysis of BrdU incorporation. c, control. E and F, TUNEL-positive cells in tumor sections and the respective quantitative analysis. 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). D, effect of drug treatment on mouse body weight. Groups of mice were weighed after the 3 cycles of drug treatment and mean weights are depicted in bars.
Haider et al., Figure 3

A

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Viability (%) vs. concentration (µM)

- BA-12 vs. PHH
- BP-14 vs. PHH

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Molecular Cancer Therapeutics

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

Christine Haider, Markus Grubinger, Eva Reznícková, et al.

*Mol Cancer Ther* Published OnlineFirst August 12, 2013.

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