Published in final edited form as:

Target Oncol. 2017 August ; 12(4): 449–462. doi:10.1007/s11523-017-0501-x.

Synthesis and Characterization of Novel BMI1 Inhibitors Targeting Cellular Self-Renewal in Hepatocellular Carcinoma

Monica Bartucci¹, Mohamed S. Hussein^{1,2}, Eric Huselid^{1,3}, Kathleen Flaherty¹, Michele Patrizii^{1,3}, Saurabh V. Laddha^{1,4}, Cindy Kui^{5,6}, Rachel A. Bigos^{5,6}, John A. Gilleran^{5,6}, Mervat M. S. El Ansary⁷, Mona A. M. Awad², S. David Kimball^{1,5,6}, David J. Augeri^{1,5,6}, Hatem E. Sabaawy^{1,3,8}

¹Rutgers Cancer Institute of New Jersey, Rutgers University, New Brunswick, NJ 08901, USA

²Clinical and Chemical Pathology, National Research Centre, Cairo, Egypt

³Graduate Program in Cellular and Molecular Pharmacology, Graduate School of Biomedical Sciences, Rutgers University, New Brunswick, NJ 08901, USA

⁴Graduate Program in Quantitative Biomedicine, Institute for Quantitative Biomedicine at Rutgers University, New Brunswick, NJ 08901, USA

⁵Molecular Design and Synthesis Laboratory, Rutgers Translational Sciences, Rutgers University, Piscataway, NJ 08854, USA

⁶Department of Medicinal Chemistry, EMSOP, Rutgers University, Piscataway, NJ 08854, USA

⁷Department of Clinical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt

⁸Department of Medicine, RBHS-Robert Wood Johnson Medical School, Rutgers University, New Brunswick, NJ 08901, USA

Abstract

Background—Hepatocellular carcinoma (HCC) represents one of the most lethal cancers worldwide due to therapy resistance and disease recurrence. Tumor relapse following treatment could be driven by the persistence of liver cancer stem-like cells (CSCs). The protein BMI1 is a member of the polycomb epigenetic factors governing cellular self-renewal, proliferation, and stemness maintenance. BMI1 expression also correlates with poor patient survival in various cancer types.

Objective—We aimed to elucidate the extent to which BMI1 can be used as a potential therapeutic target for CSC eradication in HCC.

Methods—We have recently participated in characterizing the first known pharmacological small molecule inhibitor of BMI1. Here, we synthesized a panel of novel BMI1 inhibitors and examined

Hatem E. Sabaawy, sabaawhe@cinj.rutgers.edu.
Monica Bartucci and Mohamed S. Hussein contributed equally to this work

Conflict of Interest The authors declare no conflict of interest.

Electronic supplementary material The online version of this article (doi:10.1007/s11523-017-0501-x) contains supplementary material, which is available to authorized users.

their ability to alter cellular growth and eliminate cancer progenitor/stem-like cells in HCC with different p53 backgrounds.

Results—Among various molecules examined, RU-A1 particularly downregulated BMI1 expression, impaired cell viability, reduced cell migration, and sensitized HCC cells to 5-fluorouracil (5-FU) in vitro. Notably, long-term analysis of HCC survival showed that, unlike chemotherapy, RU-A1 effectively reduced CSC content, even as monotherapy. BMI1 inhibition with RU-A1 diminished the number of stem-like cells in vitro more efficiently than the model compound C-209, as demonstrated by clonogenic assays and impairment of CSC marker expression. Furthermore, xenograft assays in zebrafish showed that RU-A1 abrogated tumor growth in vivo.

Conclusions—This study demonstrates the ability to identify agents with the propensity for targeting CSCs in HCC that could be explored as novel treatments in the clinical setting.

1 Introduction

Hepatocellular carcinoma (HCC) represents one of the most frequent cancers in developing countries. Owing to its aggressiveness, it is the third most common cause of cancer-related deaths worldwide with a 5-year overall survival rate of 17% [1]. Unfortunately, at the time of diagnosis most symptomatic HCC cases are in advanced stages and surgical resection is no longer an option. For this group of patients, due to high relapse rates after chemotherapy and radiation, the prognosis after any kind of therapy remains bleak [2].

Highly therapy-resistant cancer stem-like cells (CSCs), also termed tumor-initiating cells (TICs), bear both cancer and stem cell-like properties [3] and have critical roles in the genesis, progression, and recurrence of HCC [4]. Hence, molecular pathways and effectors promoting CSC survival and maintenance should be prioritized for therapeutic targeting [5].

Among other factors, BMI1 (B cell-specific Moloney murine leukemia virus integration site 1), the integral component of the epigenetic Polycomb Repressive Complex 1 (PRC1), plays a fundamental role in regulating the transcription of master genes controlling cell fate decisions in the functioning of tissue stem cells and CSCs [6-8]. In HCC, BMI1 acts as a key regulator during tumor initiation and progression by multiple mechanisms, including epigenetic gene regulation [9]. Consequently, BMI1 expression positively correlates with poor patient survival [10] and has been suggested as an attractive and plausible therapeutic target to achieve CSC eradication [7]. Indeed, we and others have identified BMI1 as an essential factor in the tumor-seeding abilities of various cancer-initiating cells [11-16]. Subsequently, targeting of the BMI1 RNA and/or its post-transcriptional regulatory mechanisms with our small-molecule inhibitor caused TICs loss, ultimately impairing cancer progression and growth [11, 13]. Nevertheless, in-depth investigation of targeting BMI1 and its role in HCC development and progression remain to be further clarified.

Based on the RNA three-dimensional (3D) structure of BMI1, we have developed a series of inhibitors and examined their ability to function as antineoplastic agents, alone or when combined with standard therapy. Furthermore, and more critically, we evaluated their abilities to eliminate cancer progenitor/stem-like cells in HCC.

We found that, among different small molecules, one compound in particular, called RU-A1, reduced BMI1 expression in HCC cells, regardless of their p53 status. BMI1 inhibition prevented cell proliferation, most likely through an irreversible cell cycle arrest, impaired migration in vitro and sensitized HCC cells to 5-fluorouracil (5-FU) treatment. More importantly, exposure to RU-A1 decreased the number of CSCs in culture and in an in vivo zebrafish xenograft model of human HCC. Notably, CSC impairment was not observed with chemotherapy alone. Altogether, our data indicate that BMI1 may function as an important driver of liver cancer onset and progression and support large-scale preclinical studies that have the potential to identify promising new therapeutic approaches for HCC.

2 Material and Methods

2.1 Cell Culture

Human HCC cell lines HepG2 (HB-8065) and PLC/PRF/5 (CRL-8024) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Minimum Essential Medium (MEM) or Eagle's Minimum Essential Medium (EMEM), respectively. Huh1 cells [17] (a kind gift of Dr. Zhaohui Feng, Rutgers University) and HEK 293 were both cultured in Dulbecco Modified Eagle's Medium (DMEM). All media were supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA), 100 U/mL penicillin (Sigma-Aldrich, St Louis, MO, USA) and 100 mg/mL streptomycin (Sigma-Aldrich).

2.2 Immunohistochemistry

A tissue microarray (TMA) of 110 liver carcinomas across different clinical stages and pathology grades, and 10 normal tissues (BC03119a), was obtained from US Biomax (Rockville, MD). Pathological diagnosis and detailed HCC patient's specifications are included in Supplementary Table 2. The TMA slide was deparaffinized and antigen retrieval was performed using CC1 (Cell Conditioning Solution, Ventana Medical Systems, Tucson, AZ, Cat# 950-124). The primary antibodies anti-BMI1 (Cell Signaling, Danvers, MA, #6964) and anti-CD45 (Leica, Buffalo Grove, IL, Cat# PA0042) were applied in 1:50 dilution for 1 h at 37 °C, followed by universal secondary antibody (Ventana Medical Systems, #760-4205) staining and the chromogenic detection kit DABMap (Ventana Medical Systems). The slide was counterstained with hematoxylin, sections were observed through a Zeiss Axiostar plus (Zeiss, Jena, Germany) and images were taken using capture DC software or scanned at the Center for Biomedical Imaging and Informatics at Rutgers Cancer Institute of New Jersey (RCINJ). An automated computer-based scoring of the TMA was done.

2.3 Gene Expression Analysis

An Oncomine search was conducted for the terms "BMI1" and "Liver Cancer" to find microarray datasets with matching normal and tumor samples. We selected public datasets from Oncomine plus TCGA Liver cancer. BMI1 expression values for respective samples (tumor and normal) were downloaded from NCBI GEO for these datasets. The dataset information is presented in Supplementary Table 3 including identification, platform, number of samples, *p*-value and median expression values for normal and tumor samples.

2.4 Lentiviral Transduction

The lentiviral vectors CS-H1-shRNA-EF-1 α -EGFP – expressing short hairpin RNA (shRNA) of human BMI1 (target sequence: sh-BMI1#1, 5'-CAGATGAAGATAAGAGAAT-3') and luciferase (sh-Luc target sequence: 5' ACGCTGAGTACTTCGAAAT 3') (kind gifts from Dr. Atsushi Iwama, Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, Japan) were used. Lentiviral supernatants were produced following transduction of HEK 293 cells and one cycle of infection with lentiviral supernatants was carried out on HCC cultures. Enhanced green fluorescent protein (EGFP) positive cells were isolated using BD Influx High-speed Cell Sorter (BD, San Jose, CA) at RCINJ, expanded in short-term cultures and then used in subsequent experiments. HepG2 and HepG2 Sh-BMI1 cells were transiently transfected with 2 μ g of pCMV3-BMI1-Flag (SinoBiologicals, Beijing, P.R. China) using Lipofectamine 2000 (Invitrogen, ThermoFisher Scientific, Waltham, MA), according to the manufacturer's instructions. Cells were collected 24 h after transfection for further analyses.

2.5 Compounds for HCC Cells Treatment

BMI1 inhibitors were developed by the Molecular Design and Synthesis laboratory, Rutgers Translational Sciences at Rutgers University. The scheme of chemical synthesis and structures of the compounds are described in the supplemental methods. C209 (Generic name is PTC-209) was characterized in our laboratory among a screen for post-transcriptional inhibitors of BMI1 [11, 13]. The chemotherapeutic agent 5-fluorouracil (5-FU) was obtained from Sigma-Aldrich. Compounds were used at a fixed 0.5% final DMSO concentration in all treatment and control group experiments.

2.6 Cell Viability Assays

HCC cells (3×10^5 /well) were seeded into 96-well tissue culture plates and treated with different BMI1 inhibitors (10 nM or 10 μ M) in appropriate media. Dose response curves were generated by plating HepG2, PLC/PRF/5 and Huh1 cells (5×10^3 /well) into 96-well plates and exposing them to different concentrations of RU-A1 and C-209 (0.010–10 μ M) for 72 h. Cells were counted using a Beckman Coulter Vi-CELL Cell Viability Analyzer (Beckman Coulter, Brea CA), and cell viability was confirmed by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to standard protocols and analyzed with a Victor 2 plate reader (Wallac, Turku, Finland). For rescue experiments, 2×10^5 untransfected, sh-Luc, sh-BMI1 and BMI1-transfected (FLAG-BMI1 and Sh + FLAG-BMI1) HepG2 cells were plated in a 6-well plate and treated with 10 μ M RU-A1 for 48 h. For chemoresistance comparison, after treating HCC cells for 72 h with RU-A1 and C-209 at their respective IC₅₀, cells were exposed to 10 and 50 μ g/ml of 5-FU for 48 h. Cell viability was then determined as described above.

2.7 Western Blotting

For western blot analysis, 20 μ g of whole cell lysates were used. Nitrocellulose membranes were incubated with anti-BMI1 (Cell Signaling, #6964), anti-p21 (Cell Signaling, #2947), and anti- β -actin (Santa Cruz, Dallas, TX, #69879).

2.8 Colony Formation Assay

Colony formation assays were carried out for HCC cells treated with RU-A1, C-209, and 5-FU either alone or in combination. Cells were washed and 500 single cells were plated in six-well dishes coated with 1% agar (SeaPlaque Agarose, Cambrex, NJ) as described previously [13]. Cultures were incubated at 37 °C for 2–3 weeks. Colonies were stained with crystal violet (0.01% in 10% methanol), visualized and counted under a microscope, and photographed.

2.9 Limiting Dilution Assay

For limiting dilution analysis, different numbers of untreated control, RU-A1- and C209-treated cells (1 cell, 10 cells, 100 cells and 1000 cells) were plated in a 96-well plate and incubated for about 7–10 days before colonies were counted. Calculations to assess the stem cell potential were done using extreme limiting dilution analysis (ELDA) software [18]. Results are given as confidence interval for 1/ (stem cell frequency).

2.10 Migration Assay

Cell migration was assessed in 24-well transwell Boyden chambers (Costar Scientific Corporation, Cambridge, MA). HepG2 and PLC/PRF/5 cells (2×10^4) untreated or treated with RU-A1 and C-209 at their respective IC₅₀ for 24 h were subsequently washed, suspended in fresh growth media, and placed into the upper chamber (2×10^4 /chamber). After incubation for 24 h, migrated cells were stained with Coomassie Brilliant Blue and counted under a microscope.

2.11 PI/Annexin V Staining

Control, RU-A1-, C-209-, and camptothecin-treated HCC cells (1×10^6) were washed and stained with Annexin VFITC Early Apoptosis Detection kit (Cell Signaling, #6592) according to the manufacturer's instructions before analysis on a Beckman Coulter FC500 Analyzer.

2.12 Cell Cycle Analysis

Control, RU-A1- and C-209-treated HCC cells (1×10^6) were fixed by adding cold 75% ethanol drop by drop with constant agitation. Samples were stored in ethanol overnight at –20 °C. The following day, the alcohol was washed off with PBS and 500 µl of PI/RNase staining buffer (BD Biosciences, #550825) was added to each sample before analysis through a Beckman Coulter FC500 Analyzer.

2.13 Quantitative Real-Time PCR and RT-PCR

Treated and untreated HCC cells were lysed, total RNA was extracted, and quantitative PCRs were performed using SYBR green master mix and specific primers (Supplementary Table 4).

2.14 Transplantation of HCC Cells in Zebrafish

Luc-EGFP HepG2 cells were treated with 10 µM RU-A1 for 72 h. For zebrafish xenotransplantation, casper zebrafish 48-h post fertilization (hpf) larvae were anesthetized

(0.5× tricaine methanesulfonate, MS-222; Sigma Aldrich) and treated or untreated Luc-EGFP transduced HepG2 cells were injected into the perivitelline space (above the yolk) using a microinjector (Harvard Apparatus, Holliston, MA) as previously described [19]. After transplantation, embryos were incubated for 2 days at 34 °C and then maintained in a humidified incubator. HepG2 cells were monitored under fluorescent microscopy (Zeiss) for homing and tissue repopulation. EGFP fluorescent bulk cells were quantified using Image J software (<http://rsb.info.nih.gov/ij/>).

2.15 Statistical Analysis

Statistical significance for BMI1 expression on the TMA was assessed by Mantel-Haenszel chi-squared test. Statistical significance for BMI1 gene expression between normal and HCC samples was evaluated by student's t-test. Other statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., www.graphpad.com). Data are presented as mean ± standard deviation (SD). Statistical significance was determined by student's t-test and ANOVA, with Dunnett's post-test, where appropriate. A *p*-value <0.05 is represented by a single asterisk, a *p*-value <0.01 is represented by a double asterisk, three asterisks indicate a *p* value <0.001, while four asterisks indicate *p*-value <0.0001.

3 Results

3.1 BMI1 Expression in HCC

High BMI1 levels have been linked to poor prognosis in HCC patients [10]. Thus, to determine whether up-regulation of BMI1 is correlated with clinicopathological features in HCC, we initially assessed BMI1 expression by immunohistochemistry on a TMA containing both normal and liver carcinoma tissues (Fig. 1a and Supplementary Tables 1 and 2). To rule out BMI1 expression in infiltrating non-tumoral blood cells, co-staining with CD45 was also assessed (Supplementary Fig. 1). Examining a total of 110 core tumor biopsies of liver cancer patients and 10 cores of normal liver tissues, 25 tumor samples (22.7%) exhibited high BMI1 expression (BMI1 2+ or 3+), 52 (47.3%) displayed moderate BMI1 expression (BMI1 1+ or +/-) and 33 (30%) showed negative staining for BMI1. In contrast, only one core of normal liver tissue (10%) presented weak BMI1 expression, while 9 (90%) showed negative staining. In summary, BMI1 is statistically overexpressed (*p* < 0.0001) in this cohort of malignant tissues compared to normal liver tissues (Fig. 1a and Supplementary Table 1). To further confirm these initial findings on a larger scale, we analyzed BMI1 gene expression in a total of 653 HCC vs 301 normal liver tissues from multiple databases by using the cancer microarray platform Oncomine (<https://www.oncomine.org/>). We found BMI1 to be consistently and significantly expressed at higher levels in tumor vs normal tissues in every dataset examined. (Fig. 1b and Supplementary Table 3).

3.2 Development of Pharmacological BMI1 Inhibitors

To investigate whether pharmacological targeting of BMI1 would provide a potential therapy to target tumorigenic cells within an HCC tumor mass, we have previously screened a small molecule library for BMI1 inhibitors exploiting both luciferase- and GFP-reporters encompassing the 5' and 3' UTRs [11, 13]. Among these small molecules, C-209, in

particular, appeared to bind to the BMI1 mRNA, and modulate its post-transcriptional expression [13].

In silico docking experiments of C-209 and a commercially available library of analogs on the BMI1 mRNA structure were done with 3D structural models of BMI1 built by homology modeling using Modeler 9v8 for structure activity relationship (SAR). Coordinates for the analogs were extracted from their co-crystal structure. This virtual screening approach identified potentially more potent BMI1 inhibitors, named RU-compounds (Fig. 2a, left diagram). The Glide (Grid-Based Ligand Docking With Energetics) algorithm approximates a systematic search of positions, orientations, and conformations of the ligand in the binding site using a series of hierarchical filters [20]. The virtual screening approach established that the synthesized compounds had more negative Glide score values - simulating a binding free energy - which represents tighter binders than the prototype inhibitor [21]. The docking rank (Glide score) for RU-A1 was -10.07 , whereas it was only -7.62 for compound C-209 (Fig. 2b). We deduced that changes in steric ring positions alter the activity of these molecules significantly, and identified critical structural features that are important for the efficacy of BMI1 modulators (Figs. 2a and b). These molecules were synthesized at Rutgers University Molecular Design and Synthesis laboratory, Rutgers Translational Sciences, based on the SAR studies utilizing the BMI1 mRNA 3D structure (Supplementary methods). To show that the compounds had no adverse effects in vivo, we performed toxicological assays in zebrafish embryos and investigated whether the compounds would interfere with embryonic development (data not shown). Ranging from 5 nM to 50 μ M concentrations, out of the twelve different analogs tested, three showed a promising toxicology profile. These compounds were next tested on different HCC cell lines.

3.3 BMI1 Inhibition Effects on HCC

HCC arises from the accumulation of multiple genetic alterations. Among these, TP53 mutations are the most critical [22]. Hence, we initially evaluated cell proliferation in HCC HepG2 cells (p53 wild-type) and PLC/PRF/5 cells (p53 mutant) upon exposure to the RU-A1, RU-A5 and RU-A12 compounds. First, cells were treated with low (10 nM) and high (10 μ M) concentrations of all three molecules for 72 h (Supplementary Fig. 2a). Among these, the RU-A1 analog appeared to be the most effective in reducing HCC cell viability in both HCC cell lines, particularly in p53 mutated PLC/PRF/5 cells. Overall, RU-A1 was even more efficient than the reference substance C-209 (Supplementary Fig. 2a). Consequently, we selected the RU-A1 compound for further studies. To examine the antitumor activity of RU-A1, we next determined IC_{50} values in three different HCC cell lines (HepG2, PLC/PRF/5, and Huh1 (p53 wild-type) (Fig. 2c and Table 1). Interestingly, cell proliferation impairment could be directly correlated with BMI1 expression in all three different cell lines, with an IC_{50} of 300 nM in BMI1 low expressing PLC/PRF/5 cells vs an IC_{50} of 10 μ M in the BMI1 highly expressing HepG2 cells (Table 1 and Fig. 2c and d). Also, the HCC Huh1 cell line, with relatively moderate BMI1 levels, displayed an IC_{50} ranging between 4 and 5 μ M for the RU-A1 compound vs 10 μ M for C-209 (Table 1). Accordingly, Western blot analyses showed that upon RU-A1 treatment, BMI1 downregulation was generally more efficient than observed with C-209 (Fig. 2d), suggesting a higher efficacy of our compound.

3.4 Specificity of the BMI1 Inhibitor RU-A1

To link BMI1 inhibition to the observed anti-proliferative effects, we performed an IC₅₀ dose-response cell viability assay in BMI1-deficient (sh-BMI1) and BMI1-overexpressing (FLAG-BMI1) HepG2 cells, and compared the results to BMI1 rescued (Sh- + FLAG-BMI1) and control transduced Sh-Luc and un-transduced HepG2 cells expressing endogenous levels of the protein (Fig. 3a). To maximize exogenous BMI1 expression (FLAG-BMI1), exposure to RU-A1 analog was reduced to 48 h in these experiments, when the effects of RU-A1 on HepG2 cells were initially notable. Unlike both control cells (HepG2 and Sh-Luc), whose survival after 48 h of exposure to RU-A1 was impaired by ~30%, HepG2 BMI1-deficient cells (with low expression levels of the target) were particularly sensitive with an overall survival of 42.2% (Fig. 3a). Also, BMI1-overexpressing (with excess target) cells showed a lower sensitivity, with their cell viability being reduced by only ~10%. Notably, survival analyses comparison between sh-BMI1 and BMI1 rescued (Sh- + FLAG-BMI1) HepG2 cells revealed a significantly lower response of BMI1 rescued HepG2 cells to RU-A1 with an overall survival of ~65% vs 42.2% (Fig. 3a), therefore providing evidence that RU-A1 effects could be selective towards BMI1 targeting.

BMI1 has been found, among other factors, to regulate fundamental cellular processes, such as cell proliferation, apoptosis, and stem cell maintenance [8, 23, 24]. Following RU-A1 exposure, we observed a reduction in cell viability that was not associated with central indicators of cell apoptosis such as caspase 3 activation or Annexin V positivity (data not shown and Fig. 3b). Thus, since BMI1 plays a crucial role in cell cycle regulation [25], following RU-A1 or C-209 treatment, we investigated the distribution of the cell cycle and observed a significant increase in the number of cells accumulating either in the G2/M phase (p53 wild-type HepG2) or the S-G2 phase (p53 mutant PLC/PRF/5 cells) (Fig. 3c), the latter possibly through an increase in p21 levels [26] (Supplementary Fig. 2b). These data would suggest that inhibiting BMI1 may induce an irreversible cell cycle arrest affecting cell proliferation, possibly through the induction of cellular senescence, as we have previously demonstrated for the prototype C-209 compound [13].

3.5 Treatment with BMI1 Inhibitors Impairs Self-Renewal and Tumorigenic Capability in HCC

Self-renewal ability is a distinctive property of stem cells [27], for which BMI1 is a crucial regulator. To estimate the frequency of CSCs within a given tumor, especially after treatments, clonogenic assays are routinely used in laboratory practices [28]. Thus, to investigate the long-term impact of BMI1 inhibition, we performed both clonogenic and limiting dilution analyses (LDA) to appraise differences in colony-forming capacities. The collective results showed that, compared to the controls, the number of colonies was significantly reduced in both HepG2 and PLC/PRF/5 cells following BMI1 inhibition (Fig. 4a). This result indicates that BMI1 downregulation significantly diminished the number of HCC cells with colony forming capacity.

By exhibiting a linear relationship, LDA evaluates the ability of CSCs to form tumors in a dose dependent fashion. LDA defines a tumor cell as a TIC when the cell(s) can initiate tumors at a much higher frequency and at lower numbers, as compared to the bulk tumor

fraction or other cell fractions. To estimate the number of CSCs/TICs existing in the mixed population of our HCC cell lines, LDA was performed on untreated, C-209, and RU-A1-treated cells. A decreasing number of cells were plated (from 1000 to 1 cell/well) and calculations determined by the LDA software highlighted that in the HepG2 and PLC/PRF/5 cells there is about one stem-like cells in 22 (4.5%) and 15 cells (6.6%), respectively. In contrast, in the RU-A1-treated HepG2 and PLC/PRF/5 cells, the frequencies were considerably reduced to about one stem-like cell in 49 cells (2%) and 45 cells (2.2%), respectively (Fig. 4b). This effect was more pronounced for RU-A1 than for C-209, for which the frequency was decreased to about one stem-like cell in 29 cells (3.4%) and 28 cells (3.6%), for HepG2 and PLC/PRF/5 cells, respectively (Fig. 4b). Thus, BMI1 inhibition by RU-A1 effectively, and more powerfully than C-209, decreased the rates of TICs within the total HCC cells.

CSCs can be identified by the expression of specific markers. Several studies have shown that hepatic CSCs could be identified with markers such as CD133, EpCAM (CD326), OCT4, ALDH1, and Sox9 [29]. Thus, to further corroborate our hypothesis, following BMI1 inhibition, we quantitatively assessed the relative expression of stem cell markers in RU-A1- and C-209-treated and untreated HCC cell lines. With both compounds we observed a significant downregulation in the content of stem cell markers (Fig. 4c), thus confirming the impact of BMI1 inhibition on CSC impairment.

3.6 Single BMI1 Inhibitor and Combination Treatment of HCC Cell Lines

The ability to migrate and colonize the same and surrounding organs, as well as to resist various therapies are unique CSC features [30]. BMI1 is emerging as a novel target for reducing the invasive potentials of cancer cells [24]. Following RU-A1 treatment, we did not detect significant changes in cell viability at early time points (12–24 h) (data not shown). Thus, to determine whether RU-A1 could also affect cell motility in vitro, we assessed cell migration on early-treated and untreated HepG2 and PLC/PRF/5 cells. After 24 h, we observed a considerably lower number of cells migrating in the RU-A1-treated group compared to untreated controls in both cell lines (Fig. 5a), suggesting that BMI1 inhibition could be explored to target migrating tumor cells.

Currently, there are no effective therapies to be offered to many patients with advanced HCC. BMI1 overexpression correlates with therapy failure [31–33]. To examine whether BMI1 inhibition sensitizes HCC cells to chemotherapy and targets resistant CSC, we treated HepG2 and PLC/PRF/5 cells with RU-A1 or C-209, and 5-FU, a chemotherapeutic drug used for advanced liver cancer. The use of both inhibitors in combination with chemotherapy significantly reduced HepG2 and PLC/PRF/5 survival in vitro when compared to the control or 5-FU alone, suggesting that BMI1 downregulation could sensitize HCC to antineoplastic agents (Fig. 5b). Next, to examine the long-term impact of treatments, especially in a post-therapy discontinuation setting, cells were washed and replated. Viability and clonogenic assays were used to evaluate differences in cell survival and colony-forming abilities. Both RU-A1 and C-209, as mono- or co-treatments with 5-FU, impaired short-term survival of HCC cells (Fig. 5b). Critically, when we assessed long-term survival, we observed that while chemotherapy-treated cells fully regained their ability to grow, BMI1 inhibitor-treated cells,

alone or 5-FU-combined, were significantly less proliferative (Fig. 5c). Furthermore, HCC cells maintained the ability to form colonies after single treatments with 5-FU, but colony formation became significantly impaired with RU-A1 or C-209 (Fig. 5d), indicating that BMI1 inhibition impairs survival and clonogenic activity of HCC CSCs. The concomitant assessment of the expression of CSC markers further corroborated these outcomes, with RU-A1 being more efficient than C-209 (Fig. 5e).

3.7 BMI1 Inhibition in a HCC Xenograft Zebrafish Model

After examining the effects of RU-A1 treatment on the colony formation capabilities of HCC cells in vitro, we examined these effects in vivo in a human HCC xenograft zebrafish model. HepG2 cells were lentivirally transduced to express enhanced green fluorescent protein (EGFP) and treated with RU-A1 (10 μ M) for 72 h. Subsequently, RU-A1-treated or untreated cells were injected into the perivitelline space of casper zebrafish embryos at 48 h post fertilization (hpf). Cell homing and human tumor cell engraftment as an evidence for tumor initiation were monitored under fluorescent microscopy and EGFP positive cells were quantified using image J (<http://rsb.info.nih.gov/ij/>). Fluorescence intensity significantly increased ($p < 0.01$) over the following two days in the untreated group (BMI1 proficient cells), while it significantly decreased ($p < 0.05$) in the RU-A1 treated group (BMI1 deficient cells), implying that the BMI1 inhibitor RU-A1 diminished the number of CSCs, and reduced the ability for tumor initiation in vivo (Fig. 6a and b). Moreover, we observed a significant prolongation of survival of zebrafish embryos engrafted with RU-A1-treated HCC cells compared to untreated cells (Fig. 6c).

4 Discussion

HCC is the fifth most common cancer worldwide and the third most common cause of cancer-related deaths. Novel therapies targeting the core of HCC development and progression are critically needed. CSCs/TICs in HCC have key roles in relapse, metastasis, and treatment failure [3, 4, 34]. Therefore, targeting the driving factors and (epi)genetic signaling pathways promoting abnormal self-renewal in CSCs should be prioritized for developing effective therapies [5].

Multiple studies have suggested that BMI1, Wnt/ β -catenin, Notch, Hedgehog, FGF, and TGF- β /BMP signaling networks are implicated in the maintenance of tissue homeostasis by regulating self-renewal of normal stem cells as well as proliferation and/or differentiation of CSCs [4, 30, 35]. Our laboratory has recently demonstrated that in CSCs from prostate cancer and glioblastoma patients, BMI1 is overexpressed and associated with stem cell-like traits [13, 36]. Similar findings were revealed in many other human cancers including lymphoma, lung, ovarian, nasopharyngeal carcinoma, breast, medulloblastoma, and HCC [7, 36-41]. As a non-enzymatic protein, targeting BMI1 by traditional methods is challenging. Moreover, a few small molecules are known to specifically target an RNA of interest. To date, the most well-described binding of small molecules to an RNA is the binding of certain antibiotics to the bacterial ribosome. We recently utilized cell-based, zebrafish and mouse xenograft assays to screen a library of small molecule inhibitors, and identified the first known post-transcriptional small molecule inhibitor of BMI1 that targets CSCs in prostate

cancer [11, 13]. Using medicinal chemistry and chemical engineering, we here identified several novel compounds with greater solubility, selectivity and more potent antitumor activity, named RU-A compounds. Employment of these BMI1 inhibitors against HCC cell lines significantly decreased colony formation in vitro and prevented tumor initiation in vivo, thereby indicating a functional effect on CSCs [13]. Furthermore, BMI1 inhibition at the doses utilized, while displaying antitumor activity against human tumor cells in xenografts, did not exert toxic effects on normal host cells in hematopoietic and other tissues, which are likely to have a much less abundant BMI1 expression. These findings suggest that targeting overexpressed BMI1 could have a selective therapeutic window advantage in inhibiting the clonogenic potential of tumor stem-like cells compared to normal cells. From these and other studies, we concluded that BMI1 is a bona fide target for the development of drugs impeding “stemness” and overall tumorigenic cell growth. Thus, we established a program for targeting CSCs with BMI1 inhibitors that supports the development of additional BMI1-targeted therapies for more effective cancer treatments.

BMI1 plays particular role(s) in the self-renewal of adult liver stem cells [42]; it has therefore been proposed as a novel therapeutic target for the eradication of CSCs in HCC [7]. Fan et al. validated the functional significance of BMI1 in regulating hepatic oval cells, the major type of bipotential progenitor cells in the adult liver, as well as the role of BMI1 during hepatocarcinogenesis using BMI1 knockout mice [43]. In HCC patients, BMI1 expression significantly correlates with poor patient survival [10], and it is involved in the maintenance of a so-called tumor-initiating subpopulation [7, 9, 30]. Therefore, targeting BMI1 with small molecules might emerge as a powerful therapeutic approach for eradicating CSCs in HCC [13, 44, 45].

We extensively studied the prototype BMI1 inhibitor C-209 [13] and found it to reduce the tumor initiation capacity of prostate cancer cells, which have lower BMI1 expression than HCC [13]. In the current study, the novel BMI1 inhibitor RU-A1 showed more potency than C-209 in targeting CSCs. Effendi et al. showed variations in BMI1 expression in human HCC cell lines, with higher expression in HepG2 than PLC/PRF/5 [9]. Accordingly, the RU-A1 compound was more efficient in reducing cell viability in the latter.

Proving specificity towards a certain target and/or lack of off-target effects are challenges often faced during the drug discovery process. By performing BMI1 overexpression, silencing and rescue experiments, we provided evidence that RU-A1 effects could be selective towards BMI1 targeting. Moreover, additional studies conducted in our laboratory using microarray analysis showed that RU-A1 and the now well-characterized C-209 BMI1 inhibitor share the modulation of several metabolic and oncogenic pathways. These not only link the effect of these BMI1 inhibitors to the modulation of known pathways that are regulated by BMI1, but also will help in unraveling the key BMI1 target genes and downstream signaling mechanisms that are driving tumor initiation and/or progression and are impacted upon BMI1 inhibition with our compounds (Bartucci M and Sabaawy H., unpublished results).

BMI1 plays an important role in cell cycle regulation, cell immortalization, and cell senescence [46]. BMI1 has been shown to promote proliferation and to protect cells from

apoptosis through inhibition of Ink4a/ARF function [47, 48]. Indeed, BMI1 can potentially regulate the p16-pRb and p53-p21 pathways controlling senescence by down-regulating p16^{INK4a} and p19^{ARF} [49]. RU-A1 showed no notable apoptotic effects in the two cell lines tested. It did, however, induce a significant increase in the number of cells accumulating in the G2 phase in HepG2 cells (wild-type p53) and in cells transitioning from S to G2 phase in PLC/PRF/5 cells (mutant p53), suggesting that the effects of RU-A1 on the cell cycle may vary in relation to p53 activity. Modulating either Ink4a/ARF or p53 pathways could potentially mediate the cell cycle and/or apoptotic effects of targeting BMI1 with the inhibitor RU-A1. BMI1 regulates transcription as a part of polycomb repressor complex-1 (PRC1) recruitment to unmethylated CpG islands to monoubiquitylate histone H2A on lysine 119 [50]. Although the most well-established transcriptional target of BMI1 is the INK4A/ARF locus, containing key cell cycle regulators [23], BMI1 also has direct protein-protein interaction effects, for example, BMI1 also suppresses p21 [51]. We demonstrate that treatment with RU-A1 increases p21 levels in HCC cells with mutant p53 (PLC/PRF/5 cells), which could, at least in part, mediate the arrest of the cell cycle in G2/M phase [26]. These data could also suggest that RU-A1, by targeting BMI1 and tumor initiating cells, could be inducing a cytostatic effect, thus impacting overall HCC cell growth.

Therapy-resistance, self-renewal, and metastatic capacity are specific traits of CSCs [52]. We found that BMI1 inhibition was accompanied by reduced cell motility as well as an increase in sensitivity to 5-FU treatment. More importantly, unlike chemotherapy alone, long-term analyses of treatment with RU-A1 showed that our compound effectively impacts cell survival and reduces CSCs content, as assessed by clonogenic assays and an evaluation of stem cell marker expression, even when used alone. These data are particularly important when considering that the effectiveness of targeted therapy relies not only on achieving tumor size reduction, but also most importantly on preventing relapse and/or secondary clonal lesions. Also, these results suggest that a BMI1 inhibitor monotherapeutic regimen could be effective in the clinic.

Human tumor xenografts in zebrafish have been utilized for multiple cancer types including prostate, breast, pancreas, colon cancers, leukemias, and sarcomas [53]. When comparing the in vitro characteristics of these human cancer cells to their in vivo activities in zebrafish xenografts, their tumorigenic potential in vivo correlated with their advanced or metastatic stage [19, 53]. In our zebrafish xenograft assay, we observed that RU-A1 significantly reduced the tumorigenic potential in vivo, thus validating the in vitro results of treating HCC cells with RU-A1.

Overall, in this study, we demonstrate that the RU-A1 compound downregulates BMI1 levels and interferes with cell viability, migration, chemosensitivity, cell self-renewal and tumor-initiating capability, corroborating the existence of a link between BMI1-expressing self-renewing, chemoresistant TICs and cancer dissemination in HCC. Although future studies are required to delineate the pathways and target genes impacted by BMI1 targeted therapy, we believe that the initial antitumor activities of the RU-A1 compound are promising and worthy of further validation to achieve the full potential of pharmacological targeting of tumor cell self-renewal with potent and selective BMI1 inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Leonard Zon (Harvard University) for the Casper zebrafish. We thank members of Dr. David Augeri's laboratory and core facilities at the Molecular Design and Synthesis laboratory, Rutgers Translational Sciences at Rutgers University for the synthesis, purification and mass spectral analyses of the small molecules utilized in this study.

Funding for the Study This project was supported by the Department of Defense Grant (W81XWH-12-1-0249 to H.S.), National Cancer Institute (P30 CA072720 to R.D.) and New Jersey Health Foundation award (Research grant PC-72-16 to H.S.).

References

1. El-Serag HB. Hepatocellular carcinoma. *N Engl J Med*. 2011;365(12):1118–27. [PubMed: 21992124]
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet*. 2003;362(9399):1907–17. [PubMed: 14667750]
3. Yilmaz G, Akyol G, Cakir A, Ilhan M. Investigation of diagnostic utility and expression profiles of stem cell markers (CD133 and CD90) in hepatocellular carcinoma, small cell dysplasia, and cirrhosis. *Pathol Res Pract*. 2014;210(7):419–25. [PubMed: 24702884]
4. Lee TK, Castilho A, Ma S, Ng IO. Liver cancer stem cells: implications for a new therapeutic target. *Liver Int*. 2009;29(7):955–65. [PubMed: 19490415]
5. Yao Z, Mishra L. Cancer stem cells and hepatocellular carcinoma. *Cancer Biol Ther*. 2009;8(18):1691–8. [PubMed: 19901516]
6. Smith LL, Yeung J, Zeisig BB, Popov N, Huijbers I, Barnes J, et al. Functional crosstalk between Bmi1 and MLL/Hoxa9 axis in establishment of normal hematopoietic and leukemic stem cells. *Cell Stem Cell*. 2011;8(6):649–62. [PubMed: 21624810]
7. Chiba T, Miyagi S, Saraya A, Aoki R, Seki A, Morita Y, et al. The polycomb gene product BMI1 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma. *Cancer Res*. 2008;68(19):7742–9. [PubMed: 18829528]
8. Lukacs RU, Memarzadeh S, Wu H, Witte ON. Bmi-1 is a crucial regulator of prostate stem cell self-renewal and malignant transformation. *Cell Stem Cell*. 2010;7(6):682–93. [PubMed: 21112563]
9. Effendi K, Mori T, Komuta M, Masugi Y, Du W, Sakamoto M. Bmi-1 gene is upregulated in early-stage hepatocellular carcinoma and correlates with ATP-binding cassette transporter B1 expression. *Cancer Sci*. 2010;101(3):666–72. [PubMed: 20085590]
10. Wang H, Pan K, Zhang HK, Weng DS, Zhou J, Li JJ, et al. Increased polycomb-group oncogene Bmi-1 expression correlates with poor prognosis in hepatocellular carcinoma. *J Cancer Res Clin Oncol*. 2008;134(5):535–41. [PubMed: 17917742]
11. Bansal N, Campbell N, Medina D, Dipaola RS, Bertino JR, Sabaawy HE. Targeting Bmi1 in human prostate tumor initiating cells. In: Proceedings of the 101st Annual Meeting of the American Association for Cancer Research 2010, Philadelphia: AACR: Washington, DC. p. Abstract nr 4279.
12. Kreso A, van Galen P, Pedley NM, Lima-Fernandes E, Frelin C, Davis T, et al. Self-renewal as a therapeutic target in human colorectal cancer. *Nat Med*. 2014;20(1):29–36. [PubMed: 24292392]
13. Bansal N, Bartucci M, Yusuff S, Davis S, Flaherty K, Huselid E, et al. BMI-1 targeting interferes with patient-derived tumor-initiating cell survival and tumor growth in prostate cancer. *Clin Cancer Res*. 2016;22(24):6176–91. [PubMed: 27307599]
14. Nishida Y, Maeda A, Chachad D, Ishizawa J, Qiu YH, Kornblau SM, et al. Preclinical activity of the novel B-cell-specific Moloney murine leukemia virus integration site 1 inhibitor PTC-209 in acute myeloid leukemia: implications for leukemia therapy. *Cancer Sci*. 2015;106(12):1705–13. [PubMed: 26450753]

15. Mayr C, Wagner A, Loeffelberger M, Bruckner D, Jakab M, Berr F, et al. The BMI1 inhibitor PTC-209 is a potential compound to halt cellular growth in biliary tract cancer cells. *Oncotarget*. 2016;7(1): 745–58. [PubMed: 26623561]
16. Bolomsky A, Schlangen K, Schreiner W, Zojer N, Ludwig H. Targeting of BMI-1 with PTC-209 shows potent anti-myeloma activity and impairs the tumour microenvironment. *J Hematol Oncol*. 2016;9:17. [PubMed: 26935956]
17. Liu J, Zhang C, Lin M, Zhu W, Liang Y, Hong X, et al. Glutaminase 2 negatively regulates the PI3K/AKT signaling and shows tumor suppression activity in human hepatocellular carcinoma. *Oncotarget*. 2014;5(9):2635–47. [PubMed: 24797434]
18. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods*. 2009;347(1–2):70–8. [PubMed: 19567251]
19. Bansal N, Davis S, Tereshchenko I, Budak-Alpdogan T, Zhong H, Stein MN, et al. Enrichment of human prostate cancer cells with tumor initiating properties in mouse and zebrafish xenografts by differential adhesion. *Prostate*. 2014;74(2):187–200. [PubMed: 24154958]
20. Glide. Available online at http://www.schrodinger.com/docs/2003_1/pdf/firstdiscovery/fd27_technical_notes.pdf. 2003.
21. Perola E, Walters WP, Charifson PS. A detailed comparison of current docking and scoring methods on systems of pharmaceutical relevance. *Proteins*. 2004;56(2):235–49. [PubMed: 15211508]
22. Hussain SP, Schwank J, Staib F, Wang XW, Harris CC. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene*. 2007;26(15):2166–76. [PubMed: 17401425]
23. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature*. 1999;397(6715):164–8. [PubMed: 9923679]
24. Yang MH, Hsu DS, Wang HW, Wang HJ, Lan HY, Yang WH, et al. Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. *Nat Cell Biol*. 2010;12(10):982–92. [PubMed: 20818389]
25. Grinstein E, Mahotka C. Stem cell divisions controlled by the proto-oncogene BMI-1. *J Stem Cells*. 2009;4(3):141–6. [PubMed: 20232599]
26. Cayrol C, Knibiehler M, Ducommun B. p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. *Oncogene*. 1998;16(3):311–20. [PubMed: 9467956]
27. Lajtha LG. Stem cell concepts. *Differentiation*. 1979;14(1–2):23–34. [PubMed: 383561]
28. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc*. 2006;1(5):2315–9. [PubMed: 17406473]
29. Sun JH, Luo Q, Liu LL, Song GB. Liver cancer stem cell markers: progression and therapeutic implications. *World J Gastroenterol*. 2016;22(13):3547–57. [PubMed: 27053846]
30. Oishi N, Wang XW. Novel therapeutic strategies for targeting liver cancer stem cells. *Int J Biol Sci*. 2011;7(5):517–35. [PubMed: 21552419]
31. Mihic-Probst D, Kuster A, Kilgus S, Bode-Lesniewska B, Ingold-Heppner B, Leung C, et al. Consistent expression of the stem cell renewal factor BMI-1 in primary and metastatic melanoma. *Int J Cancer*. 2007;121(8):1764–70. [PubMed: 17597110]
32. Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. *J Clin Invest*. 2005;115(6): 1503–21. [PubMed: 15931389]
33. Hayry V, Tynninen O, Haapasalo HK, Wolfer J, Paulus W, Hasselblatt M, et al. Stem cell protein BMI-1 is an independent marker for poor prognosis in oligodendroglial tumours. *Neuropathol Appl Neurobiol*. 2008;34(5):555–63. [PubMed: 18346113]
34. Martin-Padura I, Marighetti P, Agliano A, Colombo F, Larzabal L, Redrado M, et al. Residual dormant cancer stem-cell foci are responsible for tumor relapse after antiangiogenic metronomic therapy in hepatocellular carcinoma xenografts. *Lab Invest*. 2012;92(7):952–66. [PubMed: 22546866]

35. Katoh M, Katoh M. WNT signaling pathway and stem cell signaling network. *Clin Cancer Res.* 2007;13(14):4042–5. [PubMed: 17634527]
36. Mehta M, Khan A, Danish S, Haffty BG, Sabaawy HE. Radiosensitization of primary human glioblastoma stem-like cells with low-dose AKT inhibition. *Mol Cancer Ther.* 2015;14(5): 1171–80. [PubMed: 25695954]
37. Kim JH, Yoon SY, Jeong SH, Kim SY, Moon SK, Joo JH, et al. Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. *Breast.* 2004;13(5):383–8. [PubMed: 15454193]
38. Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, Saremaslani P, et al. Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. *Nature.* 2004;428(6980):337–41. [PubMed: 15029199]
39. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature.* 2006;444(7120):756–60. [PubMed: 17051156]
40. Berezovska OP, Glinskii AB, Yang Z, Li XM, Hoffman RM, Glinsky GV. Essential role for activation of the Polycomb group (PcG) protein chromatin silencing pathway in metastatic prostate cancer. *Cell Cycle.* 2006;5(16):1886–901. [PubMed: 16963837]
41. Ruan ZP, Xu R, Lv Y, Tian T, Wang WJ, Guo H, et al. Bmi1 knockdown inhibits hepatocarcinogenesis. *Int J Oncol.* 2013;42(1):261–8. [PubMed: 23138990]
42. Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer.* 2006;6(11):846–56. [PubMed: 17060944]
43. Fan L, Xu C, Wang C, Tao J, Ho C, Jiang L, et al. Bmi1 is required for hepatic progenitor cell expansion and liver tumor development. *PLoS One.* 2012;7(9):e46472. [PubMed: 23029524]
44. Cao L, Bombard J, Cintron K, Sheedy J, Weetall ML, Davis TW. BMI1 as a novel target for drug discovery in cancer. *J Cell Biochem.* 2011;112(10):2729–41. [PubMed: 21678481]
45. Siddique HR, Saleem M. Role of BMI1, a stem cell factor, in cancer recurrence and chemoresistance: preclinical and clinical evidences. *Stem Cells.* 2012;30(3):372–8. [PubMed: 22252887]
46. Jiang L, Li J, Song L. Bmi-1, stem cells and cancer. *Acta Biochim Biophys Sin Shanghai.* 2009;41(7):527–34. [PubMed: 19578716]
47. Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev.* 1999;13(20):2678–90. [PubMed: 10541554]
48. Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature.* 2003;425(6961):962–7. [PubMed: 14574365]
49. Dimri GP. What has senescence got to do with cancer? *Cancer Cell.* 2005;7(6):505–12. [PubMed: 15950900]
50. Blackledge NP, Farcas AM, Kondo T, King HW, McGouran JF, Hanssen LL, et al. Variant PRC1 complex-dependent H2A ubiquitylation drives PRC2 recruitment and polycomb domain formation. *Cell.* 2014;157(6):1445–59. [PubMed: 24856970]
51. Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S. shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. *Cell Stem Cell.* 2007;1(1):87–99. [PubMed: 18371338]
52. Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB. Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. *Nat Rev Drug Discov.* 2009;8(10):806–23. [PubMed: 19794444]
53. Teng Y, Xie X, Walker S, White DT, Mumm JS, Cowell JK. Evaluating human cancer cell metastasis in zebrafish. *BMC Cancer.* 2013;13:453. [PubMed: 24089705]

Key Points

BMI1, a transcriptional repressor involved in stem cell maintenance is upregulated in HCC compared to normal liver tissues.

We used molecular and chemical designs to generate novel BMI1 small molecule inhibitors.

RU-A1 reduced tumor growth of HCC cells and zebrafish xenografts, and diminished the number of stem-like cells more effectively than the model compound C-209 (PTC-209).

Fig. 1.

BMI1 expression in HCC. **a)** Left panel: Immunohistochemistry staining for BMI1 in HCC and normal liver tissues. Representative images from normal liver tissue (negative), and HCC tissues with weak (BMI1 1+ or +/-) or strong (BMI1 2+ or 3+) BMI1 expression. Right panel: Quantification of BMI1 expression in cohorts of normal liver tissues and HCC. Staining percentages and statistical analysis are described in Supplementary Table 1. **b)** Oncomine analysis of BMI1 expression in HCC vs normal liver tissues. Four datasets (identified as A-D) are included. Dataset description and patient characteristics are described in Supplementary Table 2

Fig. 2.

Downregulation of BMI1 expression in HCC cells by the BMI1 inhibitor RU-A1. **a**) Side-by-side electrostatic potential plots for RU-A1 and RU-A5. The plots indicate that RU-A5 is more electron-rich than RU-A1. The electrostatic potential was computed from a B3LYP/6-31G* single point calculation using Spartan software. **b**) The Glide (Grid-based ligand docking with energetics) algorithm [21] approximates the position, orientation, and conformation of C-209 and the RU compounds to the BMI1 RNA binding site on precomputed van der Waals and electric grids to generate binding free energy. **c**) Cell viability evaluated in HepG2, PLC/PRF/5, and Huh1 cell lines treated with the BMI1 inhibitors RU-A1 and C-209 (0.01–10 μM) for 72 h. **d**) Left panel: Protein lysates derived from HepG2, PLC/PRF/5, and Huh1 cells treated with either RU-A1 or C-209 at their respective IC₅₀ (Table 1) and probed for BMI1 and β-actin (loading control). Right panel: Quantification of BMI1 expression levels relative to the displayed western blot. All experiments were done three times

Fig. 4.

BMI1 inhibition reduces the self-renewal capacity and CSCs content in vitro. **a)** Left panel: Representative image showing clonogenic capability of untreated, RU-A1-, and C-209-treated HepG2 and PLC/PRF/5 cells. Right panel: Colony quantification for each cell line. Three independent experiments with six replicates for each condition were performed. Data are displayed as mean percentage $\pm$ S.D. **b)** Upper panel: limiting dilution analysis (LDA) plot used to assess the frequency of self-renewing TICs in HCC. Frequencies were calculated using LDA with 99% confidence interval. Correlation coefficient lower, estimate and upper values are displayed. Lower panel: graphic representation of limiting dilution assay in RU-A1- and C-209-treated or untreated HCC cells. **c)** qPCR showing stem cell

marker expression variation in HCC cells following treatments with BMI1 inhibitors. Data are plotted as results of two independent experiments. $*p < 0.05$, $***p < 0.001$ and $****p < 0.0001$. In all experiments, HepG2 cells were treated with both RU-A1 and C-209 at 10 μM , while PLC/PRF/5 cells were exposed to 300 nM RU-A1 and 5 μM C-209, respectively

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5.

BMI1 inhibition negatively impacts CSC features. **a)** Cell motility of HCC cells in vitro. Following short-term exposure (24 h) to RU-A1, both HepG2 and PLC/PRF/5 cells were washed and replated in fresh media to assess cell migration at 24 h. **b)** Viability of HepG2 and PLC/PRF/5 cells treated with 5-FU (10 µg/ml and 50 µg/ml) for 48 h and subsequently treated with RU-A1 and C-209 for an additional 72 h. **c)** Survival of therapy-resistant HCC cells. HepG2 and PLC/PRF/5 cells, treated as described above, were washed and replated in fresh media for an additional 3 days. Experiments in **b** and **c** were done at least 3 times in triplicates. **d)** Cell clonogenicity of therapy-resistant HCC cells. HepG2 and PLC/PRF/5 cells treated as described above, were washed and plated in soft agar. After 2–3 weeks, media were removed, colonies were stained with crystal violet and counted. **e)** Q-PCR analysis of stem cell markers in untreated and treated HCC cells. Results are indicated as

mean $\pm$ S.D. of two independent experiments with 6 replicates for each condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Fig. 6.

Establishment of human tumor xenograft in zebrafish. **a)** Representative images of HepG2 EGFP⁺ cells injected in the perivitelline space of 2dpf embryos and their fate after 2 days. At least 50 embryos were utilized for either control untreated or each treatment with DMSO or RU-A1. Xenograft take rates were 70–80% and were confirmed by fluorescence before initiation of treatment. Untreated cells (upper) and RU-A1 treated (lower). Red circles outline the engrafted tumor areas. **b)** Graphical representation showing area of fluorescence of untreated cells and RU-A1-treated cells when measured at 4dpf. To confirm that fluorescent area measurements correlate with cell counts, matching cell numbers were injected and cell counts representing tumor formation in these zebrafish xenografts between untreated and RU-A1 treated cells on day 0 were not different. **c)** Kaplan–Meier survival analysis of casper zebrafish embryos after injecting untreated and RU-A1 treated HepG2 cells. The survival rate for embryos with untreated cells ($n = 33$) was significantly lower due to continued tumor cell growth when compared to that of embryos with RU-A1 treated cells

($n = 43$) ($p < 0.001$). Survival data are representative for six independent experiments performed each with >50 embryos/treatment. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. All experiments were done at least 3 times

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1Compounds IC₅₀ HCC cell lines

Cell Line	RU-A1	C209	P53 status	BMI1 Expression
HepG2	10μM	10μM	wt	+++
Huh1	4μM	10μM	wt	++
PLC/PRF/5	300nM	5μM	mutated	+