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Small Molecule BMH-compounds that Inhibit RNA Polymerase I and Cause Nucleolar Stress

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Abstract

Activation of the p53 pathway has been considered a therapeutic strategy to target cancers. We have previously identified several p53 activating small molecules in a cell-based screen. Two of the compounds activated p53 by causing DNA damage, but this modality was absent in the other four. We recently showed that one of these, BMH-21, inhibits RNA polymerase I (Pol I) transcription, causes the degradation of Pol I catalytic subunit RPA194 and has potent anticancer activity. We show here that three remaining compounds in this screen, BMH-9, BMH-22 and BMH-23, cause reorganization of nucleolar marker proteins consistent with segregation of the nucleolus, a hallmark of Pol I transcription stress. Further, the compounds destabilize RPA194 in a proteasome-dependent manner and inhibit nascent rRNA synthesis and expression of the 45S rRNA precursor. BMH-9 and BMH-22 –mediated nucleolar stress was detected in *ex vivo*-cultured human prostate tissues indicating good tissue bioactivity. Testing of closely related analogs showed that their activities were chemically constrained. Viability screen for BMH-9, BMH-22 and BMH-23 in the NCI60 cancer cell lines showed potent anticancer activity across many tumor types. Finally we show that the Pol I transcription stress by BMH-9, BMH-22 and BMH-23 is independent of p53 function. These results highlight the dominant impact of Pol I transcription stress on p53 pathway activation and bring forward chemically novel lead molecules for Pol I inhibition, and potentially, cancer targeting.

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Introduction

Small molecule chemical library screens have extensively been used to identify chemical entities activating the p53 tumor suppressor protein (1, 2). The rationale has been straightforward – activation of wild type or mutant p53 protein could provide a powerful tool to launch the anti-proliferative, pro-apoptotic and anti-tumor activities of p53 to control tumor growth. Few of these lead molecules have entered clinical trials, such as PRIMA-1 (3, 4) and Nutlin-3 like molecules (5–7). Lead molecules arising from large unbiased screens have a diversity of action mechanisms including inhibition of sirtuins, and regulation of p53 folding and binding (1, 2). We conducted a cell-based high-throughput imaging screen to identify small molecule p53-activators and discovered and validated six lead compounds as activators of p53 based on their ability to stabilize p53, activate p53 reporter and p53 target genes and transcriptional profiling consistent with p53 responses (8). Characterization of their mechanism of p53 activation revealed that two of the compounds caused DNA-damage and mediated p53 stabilization through activation of ATM-signaling pathway. Strikingly, four compounds (BMH-9, BMH-21, BMH-22 and BMH-23) were devoid of DNA-damaging activity as analyzed by several DNA damage markers, and their mechanism of p53 activation remained unresolved. Recently, we showed that one of these compounds, BMH-21, inhibits RNA polymerase I (Pol I) transcription (9).

RNA polymerase I (Pol I) transcription is an emerging tractable process for cancer therapeutics (10–12). The transcription is mediated by a dedicated RNA polymerase holocomplex composed of multisubunit preinitiation and polymerase complexes (13). The transcription is initiated by assembly of the preinitiation complex to the rDNA promoter, followed by stochastic assembly of the Pol I complex (13, 14). Once initiated, Pol I transcription is highly processive, and typically, a single Pol I complex transcribes the entire 13 kb coding region (14). Preinitiation complex assembly is instigated by posttranslational modifications of the preinitiation complex proteins (13). These modifications are effected by cyclin-CDK kinases, providing cell cycle-dependence to the transcription, and by cellular signaling and survival pathways, ERK, Akt/PKB, mTOR, Her2/Neu, and Myc (10, 15).

Pol I transcription is compartmentalized to the nucleolus (16–18). The nucleolus is divided into distinct compartments, namely fibrillar center, dense fibrillar component and granular component, encompassing defined functions that support Pol I transcription, rRNA processing and maturation of the rRNAs and ribosome assembly, respectively (17, 18). These subcompartments are marked by distinct localization of nucleolar proteins that participate in the respective rRNA biogenesis processes (18). Stresses that cause Pol I transcription blocks cause rapid and dynamic reorganization of the nucleolar structures and proteins (18–21). For example, proteins of the fibrillar center and dense fibrillar component relocate to nucleolar cap structures at the nucleolar periphery while granular component proteins typically translocate to the nucleoplasm (18, 22).

The full extent of Pol I deregulation in human cancers is not known. Pol I transcription rates are not being measured, and 18S and 28S mature rRNAs conventionally used for normalization of RNA loading in various experimental approaches have long half lives and poorly reflect changes in the transcription rates. Furthermore, the absence of the multilocus rDNA gene from the human genome assembly obliterates recording of changes in the rRNAs in genome wide approaches. However, it is notable that key oncogenic pathways are ones that increase Pol I transcription rates (10–12). On the other hand, tumor suppressors p53, RB1, ARF, PTEN and GSK3 β have been shown to restrict Pol I and ribosome biogenesis (10, 12). Conversely, Pol I transcription inhibition is a profound signal activating p53 (23–26). Mutations of nucleolar and ribosomal proteins drive tumorigenesis and the nucleolus contains proteins associated with cell cycle regulation, DNA repair and oncogenesis (19, 26, 27). Increased size of the nucleolus and staining of nucleolar organizing regions with silver is a frequent pathognomonic feature of cancer cells (28). These are indications for increased ribosynthetic activity of cancer cells to meet their demands for increased protein synthesis, and provide compelling reasoning to employ targeting of Pol I transcription as a therapeutic strategy.

The present study focused on characterization of the mechanism of action of compounds BMH-9, BMH-22 and BMH-23 arising from our initial high-throughput screen for p53 activators (8). We show here that these compounds function as Pol I inhibitors and similarly to BMH-21, cause proteasome-dependent degradation of RPA194. We further show that the molecules act in a p53-independent manner and that Pol I inhibition occurs upstream of p53 activation. The study defines new lead molecules for targeting Pol I transcription.

Materials and Methods

Cells and compounds

A375 melanoma (CRL-1619), U2OS (HTB96) and SaOS-2 (HTB85) osteosarcoma and HCT116 (CCI-247) cells were from American Type Culture Collection that verifies their identity using genomic fingerprinting. The cells were used at passages < 10 after thawing. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. A375 cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and U2OS and SaOS-2 cells in DMEM supplemented with 15% FBS. Compounds were obtained as follows: BMH-9, BMH-22, BMH-23, BMH-9_A1, BMH-9_A2 and BMH-22_A1 were obtained from ChemDiv and ChemBridge, verified for purity using LC/MS mass spectrometry and ¹H-NMR. Actinomycin D was from Sigma, Nutlin-3 was obtained from Alexis Biochemicals and Sigma, and MG132 from Biomol International LP.

Viability assay

Cells were incubated in the presence of the compounds for the indicated times and viability was determined using WST-1 cell proliferation reagent according to manufacturers instructions (Roche Diagnostics).

Flow cytometry

Cell cycle distribution and cell death were analyzed with flow cytometry. Cells were harvested and fixed in 70% ethanol at -20°C followed by RNaseA treatment and stained with propidium iodide. A total of 10,000 counts were collected (LSR, Becton Dickinson) and cell cycle distribution was analyzed using ModFit LT 3.1 software (Verity Software House). Cells present in sub- G_1 population were analyzed using the acquisition software (CellQuest).

rRNA synthesis assays

rRNA synthesis assays were conducted essentially as in refs. (9) and (29). Cells were labeled with 1 mM 5-FUrd (Sigma) using hypotonic shift and fixed with ice-cold methanol and acetone. Cells were blocked in 3% BSA and FUrd was detected using anti-5-BrdU antibody. DNA was stained with DAPI. Metabolic labeling of rRNA was conducted as in refs. (9) and (29). Cells were labeled with [5, 6- ^3H]-uridine (Perkin Elmer) at 2–3 $\mu\text{Ci}/\text{ml}$. Total RNA was isolated and 3–5 μg of RNA was separated on a 0.8% agarose-formaldehyde gel. RNA was transferred to Hybond- N^+ filter (Amersham), crosslinked, treated with Enhancer (Perkin Elmer) and exposed to film.

Immunofluorescence and image analysis

Immunostaining was performed essentially as in ref. (9). Cells grown on coverslips were fixed in 3.5% paraformaldehyde, permeabilized with 0.5% NP-40 and blocked in 3% BSA. The following primary antibodies were used: UBF (H-300) and RPA194 (C-1) (Santa Cruz Biotechnology), NCL (4E2, Abcam), NPM (FC-61991, Invitrogen), FBL (ab582, Abcam), γH2AX (Upstate), KAP-1 (BD Transduction Laboratories) and p53 (7F5, Cell Signaling Technologies). Secondary Alexa488 and Alexa594-conjugated anti-mouse and anti-rabbit antibodies were from Invitrogen. DNA was stained with DAPI. Images were captured using Axioplan2 fluorescence microscope (Zeiss) equipped with AxioCam HRc CCD-camera and AxioVision 4.5 software using EC Plan-Neofluar objectives (Zeiss). Image analysis was conducted using FrIDA designed for the analysis of RGB color image datasets as in ref. (9). Hue saturation and brightness ranges for green and red fluorescence channel and DNA (blue) were defined for each image set. Image intensities were determined as the fraction of positive cells divided total nuclear area as defined by DNA staining. An average of 100 cells was quantified from two fields for each sample.

Immunoblotting

Cells were lysed in 0.5% NP-40 buffer (25 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, 4 mM NaF, 100 μM Na_3VO_4 , 100 KIU/ml aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin) or RIPA lysis buffer. Proteins were separated on SDS-PAGE, blotted, probed for respective proteins and detected using ECL (Amersham). The primary antibodies used for detection were NCL (4E2), RPA194 (C-1), TIF-IA (Rrn3, C-20, Santa Cruz Biotechnology), TAF₁₁₀ (C-18, Santa Cruz Biotechnology). HRP-conjugated secondary antibodies and were from DAKO or Santa Cruz Biotechnology, HRP-conjugated streptavidin was from DAKO.

Treatment of surgery-derived prostate tissue *ex vivo*

Prostate tissues were isolated from patients undergoing prostatectomy at the Helsinki University Central Hospital with informed written consent and approval by Ethics Committee (#390/E6/06). A cylinder of 8 mm in diameter was cored out of the peripheral region of the prostate, and the tissues were sliced and cultured as detailed in refs. (30) and (31). All experimental treatments of the cultures were performed on days 1–2. The tissues were fixed, embedded in paraffin, and the blocks were sectioned and processed for immunohistochemistry as detailed in refs. (30) and (31) using NPM antibody (Zymed). Secondary anti-mouse conjugated Alexa-488 antibody was from Molecular Probes. The tissues were counterstained with Hoechst 33342 (Molecular Probes). The specimens were imaged using LSM 510 Meta confocal microscope, Plan-Neofluar 40x/1.3 Oil DIC objective and captured with LSM 3.2 software (Zeiss).

Statistical analysis

Statistical analysis was performed by Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

BMH-9, BMH-22 and BMH-23 cause segregation of the nucleolus and proteasome-dependent degradation of RPA194

Segregation of the nucleolus and nucleolar protein translocations are a hallmark of Pol I transcription stress (18, 20). To assess whether BMH-9, BMH-22 and BMH-23 (Fig. 1A) affect the integrity of the nucleolus, we treated A375 melanoma cells with these compounds at 10 μ M concentration for 3 hours after which the cells were fixed and stained for fibrillar center and dense fibrillar component proteins UBF and FBL (Fig. 1B) and granular component proteins NPM and NCL (Fig. 1C). In all above assays we used Actinomycin D (ActD) as control at concentrations at which it inhibits Pol I (50 ng/ml).

BMH-9, BMH-22 and BMH-23 caused segregation of UBF and FBL into nucleolar caps, as did Act D (Fig. 1B). NPM and NCL translocated from the nucleolus into nucleoplasm (Fig. 1C). These phenotypes are consistent with nucleolar stress.

We then analyzed the effect of BMH-9, BMH-22 and BMH-23 on Pol I complex catalytic core subunit RPA194. As analyzed by immunostaining and western blotting, BMH-9, BMH-22 and BMH-23 caused a marked decrease in RPA194 protein, BMH-22 and BMH-23 being most effective in this regard (Fig. 1D and E). ActD caused RPA194 nucleolar cap formation, and did not affect RPA194 protein (Fig. 1D and E). To assess whether the compounds caused RPA194 destabilization, the cells were incubated in the presence of the compounds and cycloheximide, and the turnover was determined. The results showed that BMH-9, BMH-22 and BMH-23 caused RPA194 destabilization in a manner similar to BMH-21 (Fig. 1F and Supplementary Fig. S1). Consistent with our earlier report on BMH-21 (9), the downregulation of RPA194 was dependent on proteasome activity, as its inhibition by MG132 rescued the decrease in RPA194 (Fig. 1D and E). The deregulation of protein stability by BMH-9, BMH-22 and BMH-23 was selective for

RPA194 as Pol I preinitiation complex protein TAF_I110 and a transcription initiation factor TIF-IA were unaffected (Fig. 1E). Nutlin-3, a small molecule MDM2 inhibitor that causes p53 stabilization and activation (5) did not affect RPA194 (Fig. 1E).

BMH-9, BMH-22 and BMH-23 inhibit RNA polymerase I transcription

Segregation of the nucleolus reflects Pol I transcription stress. To assess the effects of BMH-9, BMH-22 and BMH-23 on Pol I activity we first analyzed *de novo* rRNA synthesis using 5-fluorouridine (5-FUrd) incorporation. The BMH-compounds caused potent inhibition of 5-FUrd incorporation (Fig. 2A and B). Further analysis using ³H-uridine metabolic labeling showed that BMH-9, BMH-22 and BMH-23 prominently inhibited the synthesis of the 45S precursor rRNA (Fig. 2C and D). Quantification of two independent experiments showed that the 45S precursor rRNA synthesis was inhibited by over 80% (Fig. 2D). These results demonstrate that BMH-9, BMH-22 and BMH-23 cause robust inhibition of Pol I transcription.

Growth inhibitory activity of BMH-9, BMH-22 and BMH-23 in the NCI60 cancer cell lines

Our initial analysis of BMH-22 in a mouse model of B cell lymphoma showed its marked anti-tumorigenic potential, and that BMH-9, BMH-22 and BMH-23 decreased the viability of several cancer cell lines (8). Furthermore, testing for hematopoietic progenitor colony formation showed that BMH-9 and BMH-22 had negligible toxicity in this regard (8). In order to gain information of the anticancer properties of the compounds in a larger panel of tumor cell lines, we submitted BMH-9, BMH-22 and BMH-23 to the NCI Developmental Therapeutics Program NCI60 screen (32). The compounds demonstrated cytotoxic and cytostatic responses across the NCI60 cell panel with median growth inhibitory concentrations (GI₅₀) of 4.1 μM, 4.3 μM and 2.0 μM for BMH-9, BMH-22 and BMH-23, respectively (Fig. 3A). Comparison of the effects of BMH-9 and BMH-22 in the NCI60 cancer cells to normal cells we analyzed previously (8) indicated better tolerance in the normal cells (Fig. 3B). However, BMH-23 had substantially more toxicity in normal cells indicating its less optimal properties (not shown). However, BMH-23 did not activate the DNA damage response as assessed by Ser139 H2AX and Ser824 KAP1 phosphorylation (Supplementary Fig. S2) and was in this regard similar to BMH-9, BMH-21 and BMH-22 (8).

Bioactivity of BMH-9 and BMH-22 in *ex vivo*-cultured human prostate tissue

We next studied the nucleolar stress response of the compounds in a human prostate tissue model (30, 31). Tissue biopsies obtained from volunteers undergoing radical prostatectomy were sectioned and maintained under culture. The tissues were treated with BMH-9 and BMH-22, fixed and processed for immunohistochemical staining of NPM. BMH-9 and BMH-22 caused prominent translocation of NPM from the nucleolus to nucleoplasm (Fig. 4), in line with that observed in cultured cells (Fig. 1C). This indicates that BMH-9 and BMH-22 are tissue permeable and cause relevant bioactivity reflecting the nucleolar stress response.

Chemical constraints of BMH-9 and BMH-22/23 analogs

The small molecule libraries chosen for our initial cell-based p53 activation screen presented high chemical diversity. We subsequently identified two structurally close analogs of BMH-9 (BMH-9_A1, BMH-9_A2) and one for BMH-22 and BMH-23 (BMH-22_A1) (Fig. 5A), and used them to assess chemical characteristics associated with their activity. BMH-9_A1, BMH-9_A2 did not affect the localization or stability of NCL and RPA194 (Fig. 5B), or that of p53 (not shown) indicating that they are inactive. BMH-22_A1, in which a methyl –group was substituted with an ethyl group, retained some activity as compared to the parent molecules BMH-22 and BMH-23 (Fig. 5B–D). These findings suggested that the parent core structures may be relatively tightly constrained.

p53 activator Nutlin-3 does not affect nucleolar integrity and is dispensable for BMH-9, BMH-22 and BMH-23 nucleolar effects

p53 has been shown to inhibit Pol I transcription by interfering with the preinitiation complex formation (33, 34). On the other hand, ample studies show that inhibition of ribosome biogenesis leads to p53 activation, placing p53 activation downstream of Pol I transcription blocks (25, 26). We studied the p53-dependency of the nucleolar stress response utilizing Nutlin-3. Although Nutlin-3 increased p53 protein it did not affect the localization of nucleolar proteins UBF, FBL, NPM and NCL (Supplementary Fig. S3A and S3B), RPA194 (Supplementary Fig. S3C and D), or decrease nascent rRNA synthesis (Supplementary Fig. S3E and F).

To analyze the dependency of the nucleolar responses by BMH-9, BMH-22 and BMH-23 on p53, we monitored these responses in a p53 null cell line SaOS-2. As shown in Fig. 6A, the BMH-compounds caused UBF cap formation, translocation of NCL from the nucleolus and degradation of RPA194 consistent with segregation of the nucleolus. Thus, the nucleolar stress response by the BMH-compounds was evident in the absence of p53.

We have earlier shown using *TP53* isogenic HCT116 cells that the compound cytotoxic activities are independent of p53, whereas BMH-9 demonstrated partial dependency (8). This was further tested here in a kinetic study where BMH-compounds were used at their near IC_{50} doses and cells were counted after 24 h, 72 h and 120 h. As shown in Fig. 6B, BMH-22 and BMH-23 decreased the number of HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells in a similar manner, whereas BMH-9 and Nutlin-3 were less effective in the $p53^{-/-}$ cells. In addition, we analyzed whether BMH-9 and BMH-22 affect cell cycle in p53 null SaOS-2 cells. Cells were treated with the compounds and incubated for 72 h. In comparison, cells were treated with Nutlin-3 and ionizing radiation (IR). Nutlin-3 had no discernible effect on the cell cycle distribution, whereas BMH-9 and BMH-22 increased the sub- G_1 fraction of the cells and altered the distribution of S and G_2/M phase cells (Fig. 6C). BMH-22 had more prominent effects in this regard. IR caused a profound G_2/M phase arrest, as expected. These findings demonstrated that BMH-22, and BMH-23 in those assays that it was tested, acted in a p53 independent manner. To assess whether activation of p53 by Nutlin-3 synergizes with the BMH-compounds we co-treated the cells with increasing doses of the compounds and Nutlin-3, analyzed cell viability and determined the Chou-Talalay combination index (CI)

(35). Synergism was detected between Nutlin-3 and BMH-23 (CI 0.628), and moderately with BMH-22 (0.776) (Fig. 6D).

Discussion

This paper describes novel small molecule lead structures for inhibition of RNA Pol I. BMH-9, a quinolinecarboxylate and BMH-22 and BMH-23, benzonaphthyridins, cause nucleolar stress represented by relocalization of nucleolar proteins, inhibition Pol I transcription and loss of RPA194. These activities are strikingly similar to the structurally distinct pyridoquinazolinecarboxamide BMH-21 that we described as first-in-kind Pol I inhibitor that activates RPA194 destruction (9). All molecules elicit broad anticancer activity across the NCI60 cancer lines. These findings support the notion that Pol I targeting effectively restricts cancer cell growth. The present study concludes the identification of activities of the six p53 activating molecules discovered in our screen (8) and shows that 4 of these act as Pol I inhibitors and share the exceptional property to destabilize RPA194.

Pol I transcription block, such as elicited by BMH-21, causes rapid segregation of the nucleolus and relocalization of nucleolar proteins (9). These changes occur temporally faster than stabilization of p53 (9). p53 is potentially activated as a consequence of ribotoxic stress response by ribosomal and other nucleolar proteins (20, 23, 24–26). We show here that p53 is not required for the nucleolar stress responses by BMH-9, BMH-22 and BMH-23. Conversely, activation of p53 by Nutlin-3 did not affect Pol I transcription activity, stability of RPA194 or integrity of the nucleolus. Furthermore, there were marked cell cycle changes and increased apoptosis in p53 defective cells by the compounds. These findings indicate that first, p53 activation occurs downstream of Pol I inhibition (Fig. 6E), and secondly, that p53 provides little contribution to the compound cytotoxic activity *in vitro* except for BMH-9. However, considering that p53 has a multitude of potential tumor suppressive activities such as modulation of the innate immune system and inhibition of tumor metastasis, some of these activities may provide a significant benefit in the *in vivo* setting. Interestingly, co-treatment of cells with BMH-9, BMH-22 and BMH-23 and Nutlin-3 showed synergism, especially with BMH-23, suggesting the p53 pathways activated by inhibition of Pol I and MDM2 may not fully overlap.

In our earlier studies we showed that BMH-22 significantly represses B cell lymphoma tumor growth and that BMH-9 and BMH-22 did not alter the histology of normal mouse tissues or reduce colony formation of hematopoietic progenitors (8). We show here that treatment of human prostate tissues derived from radical prostatectomies with BMH-9 and BMH-22 cause efficient nucleoplasmic translocation of NPM, indicating that highly expressed nucleolar proteins, such as NPM and NCL may serve as useful and sensitive biodynamic markers for on-target monitoring. These are good indicators for the BMH-9 and BMH-22 bioavailability, potential tolerance and support the design of preclinical studies testing the compound efficacy and bioactivity. BMH-23, however, was not well tolerated in normal cells.

Interestingly, data collected on small molecule bioactivity screens and available in the NCBI PubChem Bioassays show that BMH-22 and BMH-23 have been tested in over 600 assays

each, and scored as hits in 55 and 89 assays, respectively. In assays for cancer-relevant pathways, both have frequently scored as hits in assays where the readouts have involved cell viability or DNA binding. However, the compounds have not scored positive when they have progressed to secondary, validation or confirmatory screens. In other words, to the best of our knowledge, BMH-22 and BMH-23 are yet to be ascribed with other properties besides their action as DNA intercalators, cytotoxicity towards cancer cells, and, as shown by our studies, inhibition of Pol I.

BMH-22 and BMH-23 differ chemically by only a single methyl group, and BMH-22_A1 by an ethyl group. Of these, BMH-23 demonstrated higher activity than either BMH-22 or BMH-22_A1, indicating that it is more potent, and also more toxic. BMH-9 represents a chemically distinct molecule but has a dimethylaminopropylamino arm resembling that of the dimethylcarboxamide arm in BMH-21. BMH-9 analogs A_1 and A_2, in which the arm was modified by shortening the carbon linker or by introducing a bulky pyridine ring were both inactive in their ability to activate the nucleolar stress response. This suggests that the arm imparts significant bioactivity of the molecule.

The present study identifies two structurally new molecules, BMH-9 and close homologs BMH-22 and BMH-23 for inhibition of Pol I. Earlier efforts have brought forward the molecule CX-5461, which appears to inhibit Pol I preinitiation complex formation and is structurally and mechanistically distinct from the BMH-compounds (9, 37). It has also shown promising activity in preclinical trials and has entered a phase I clinical trial in Australia (36, 37). In addition, many cancer therapeutics, especially topoisomerase I and II poisons inhibit Pol I transcription by causing torsional stress of the rDNA (38). These activities will be relevant to consider and monitor for their potential therapeutic advantage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

BMH-9, BMH-22 and BMH-23 cause nucleolar segregation and RPA194 degradation. A, Chemical structures of BMH-9, BMH-22 and BMH-23. B and C, A375 melanoma cells were incubated for 3 hours with BMH-9, BMH-22 and BMH-23 (10 μ M), and ActD (50 ng/ml), fixed and stained for B, UBF and FBL and C, NPM and NCL. Representative single cell images of N = 5 experiments are shown. D, Cells were incubated for 3 hours with BMH-9, BMH-22 and BMH-23 (10 μ M), and ActD (50 ng/ml), or were pretreated with proteasome inhibitor MG132 (10 μ M) for 30 minutes, fixed and stained for RPA194. Representative images of N = 3 experiments are shown. Scale bars, 10 μ m. E, Cells were

treated as in D, and additionally, with Nutlin-3 (10 μ M). Cell lysates were analyzed by Western blotting for RPA194, TAF_I110 and TIF-IA. Representative experiment of N = 4 is shown. F, A375 cells were treated with cycloheximide (CHX) in the presence or absence of the BMH-molecules (10 μ M) for the indicated times, analyzed by western blotting and quantified. RPA194 signals were normalized to NCL used as a loading control.

Figure 2.

BMH-9, BMH-22 and BMH-23 inhibit RNA polymerase I transcription. A, A375 cells were incubated for 3 hours with the indicated BMH-compounds (10 μ M) and ActD (50 ng/ml) and de novo rRNA synthesis was detected by labeling the cells with FURd for the last 30 minutes. B, Quantitative image analysis of FURd incorporation. Fold change as compared to control set as 1 is shown. Representative experiment of N = 4 is shown. C, Metabolic labeling of the cells using 3 H-uridine. A375 cells were treated with the compounds at concentrations indicated in A for 4 hours and labeled with 3 H-uridine for the last 2 hours. Total RNA was isolated and mature and precursor rRNA forms were separated by electrophoresis. 45S form represents the precursor rRNA transcript. Total 18S is shown as loading control. D, Quantification of 45S rRNA precursor of N = 2 independent experiments.

Figure 3.

BMH-9, BMH-22 and BMH-23 activities in the NCI60 cancer cell panel. A, NCI Developmental Therapeutics Program NCI60 screen. Compound activities are presented as 50% growth inhibitory concentration (GI₅₀) using the median GI₅₀ value as y-axis. The respective median GI₅₀ concentrations are shown in the panels. B, Box plot of GI₅₀ values of BMH-9 and BMH-22 in NCI60 cell lines and normal human primary cells (as adopted from ref. 8).

Figure 4.

Bioactivity of BMH-9 and BMH-22 in *ex vivo* cultured human prostate tissue. *Ex vivo*-cultured tissues were treated with BMH-9 and BMH-22 (20 μ M) for 8 hours. Tissues were stained for NPM (green) and DNA (blue). Inset, 3x enlarged field. Scale bar 50 μ m. Representative of N = 3 independent prostate tissues.

Figure 5. RPA194 degradation is a biological response unique to BMH-parent molecules. A, Chemical structures of BMH-9 and BMH-22/23 analogs. B, A375 cells were treated with the parent molecules and their analogs (10 μ M) and incubated for 3 hours. Cells were stained for NCL and RPA194. Scale bar 10 μ m. C and D, Dose-responses. A375 cells were treated with 2.5 and 10 μ M concentrations of the parent molecules and analogs, fixed and stained for NCL and RPA194 followed by quantitative image analysis. Fold change as compared to control, set as 1, is shown.

Figure 6.

p53 is dispensable for nucleolar stress. A, SaOS-2 cells were treated with BMH-9, BMH-22 and BMH-23 (10 μ M) and ActD (50 ng/ml) for 3 hours and stained for UBF, NCL and RPA194. Scale bars 10 μ m. B, p53 isogenic HCT116 cells were treated with BMH-9, BMH-22, BMH-23 and Nutlin-3 (5 μ M) for the indicated times and cells were counted. N = 2 experiments conducted in triplicate. Fold change to control are shown as mean $\pm$ SEM. C, SaOS-2 cells were treated with BMH-9, BMH-22, BMH-23 and Nutlin-3 (10 μ M) or irradiated (IR) with 10 Gy and incubated for 72 hours. Cells were fixed and stained with propidium iodide and analyzed by flow cytometry. Cell cycle distribution was analyzed, and is shown in the inset. Separately, the fraction of sub-G₁ cells was analyzed and is shown as percentage of cells in the total population. D, A375 cells were treated with BMH-9, BMH-22 and BMH-23 (0.5 μ M and 5 μ M) and in combination with Nutlin-3 (0.5 μ M and 5 μ M) and incubated for 24 hours. Viability was determined using the WST-1 assay. N = 2 experiments conducted in triplicate. Combination index (CI) was determined according to

Chou-Talalay (35). Dashed line indicates additive effect, and values < 1 represent synergism. E, Schematic drawing of BMH-compound effects.