Published in final edited form as:

Biochem J. ; 476(15): 2209–2219. doi:10.1042/BCJ20190207.

Discovery of novel inhibitors of ribosome biogenesis by innovative high throughput screening strategies

Catherine E. Scull¹, Yinfeng Zhang¹, Nichole Tower², Lynn Rasmussen², Indira Padmalayam², Robert Hunter², Ling Zhai², Robert Bostwick², David A. Schneider¹

¹Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A

²Southern Research, Birmingham, AL 35205, U.S.A

Abstract

Over the past two decades, ribosome biogenesis has emerged as an attractive target for cancer treatment. In this study, two high-throughput screens were used to identify ribosome biogenesis inhibitors. Our primary screen made use of the HaloTag selective labeling strategy to identify compounds that decreased the abundance of newly synthesized ribosomes in A375 malignant melanoma cells. This screen identified 5786 hit compounds. A subset of those initial hit compounds were tested using a secondary screen that directly measured pre-ribosomal RNA (pre-rRNA) abundance as a reporter of rRNA synthesis rate, using quantitative RT-PCR. From the secondary screen, we identified two structurally related compounds that are potent inhibitors of rRNA synthesis. These two compounds, Ribosome Biogenesis Inhibitors 1 and 2 (RBI1 and RBI2), induce a substantial decrease in the viability of A375 cells, comparable to the previously published ribosome biogenesis inhibitor CX-5461. Anchorage-independent cell growth assays further confirmed that RBI2 inhibits cell growth and proliferation. Thus, the RBI compounds have promising properties for further development as potential cancer chemotherapeutics.

Introduction

The number of ribosomes per cell is proportional to the growth rate of that cell. Thus, for cancer cells to grow and proliferate rapidly they must maintain a high rate of ribosome biosynthesis. Synthesis of the ribosome requires the coordination of all three nuclear RNA polymerases and the participation of more than 200 auxiliary assembly factors that interact with pre-ribosomes in the nucleolus, nucleoplasm, and cytoplasm [1-4]. Thus, ribosome biogenesis is energetically costly. To balance the demand for cell growth with the extensive

Correspondence: David A. Schneider (dschneid@uab.edu).

Author Contribution

C.E.S. wrote manuscript, designed research and methodology, performed investigations, did formal analysis, and provided funding. Y.Z. wrote the manuscript, designed research and methodology, performed investigations, and did formal analysis. N.T., L.R., I.P., R.H., and L.Z., performed investigations and did formal analysis. R.B. conceptualized and designed research and methodology, provided supervision, wrote manuscript, and provided funding. D.A.S. conceptualized and designed research and methodology, provided supervision, performed investigations, did formal analysis, wrote manuscript, and provided funding.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

metabolic cost of ribosome biogenesis, cells have evolved robust regulatory networks to control ribosome biogenesis.

In pathologies where cell growth control is disrupted, such as in cancers, there is clear evidence for dysregulation of ribosome biogenesis [5-9]. While the precise mechanisms that govern the synthesis of all of the ribosome components (rRNA, r-proteins, and assembly factors) are topics of continued investigation, it is clear that ribosome expression is directly and intimately connected to cell growth and proliferation. As a general trend, cell proliferation is positively correlated with the size of the nucleolus, the cellular compartment in which ribosomes are synthesized [10,11]. One of the earliest identified features of cancer cells was the appearance of large, irregular nucleoli [11,12]. Cancer cells appear to elevate rDNA transcription, the first step of ribosome biogenesis, in order to meet the demand for increased protein synthesis [13]. This phenomenon in cancer cells is considered to be an 'addiction' to ribosome biogenesis [14]. This addiction presents a unique vulnerability for cancer cells that can be exploited therapeutically.

In recent years, promising small molecules have been identified via high-throughput screening (HTS) to effectively inhibit cancer cell growth and proliferation by inhibiting ribosome biogenesis, including CX-5461 and BMH-21. CX-5461 is thought to specifically inhibit Pol I transcription initiation by preventing the binding of transcription initiation factor SL1 to the rDNA promoter to disrupt transcription initiation complex formation [15]. CX-5461 triggers p53-mediated cell senescence and autophagy in solid tumor cells *in vivo* [15]. Recent research indicates that CX-5461 inhibits cancer growth and proliferation through a variety of pathways such as activating ATM/ATR pathway to arrest cells at G2 phase [16], disrupting rDNA chromatin state [17], and stabilizing G-quadruplex structures [18]. Additionally, in combination with other inhibitors, such as inhibitors of the ATM/ATR pathway, CX-5461 has been reported to selectively and synergistically cause cancer cell death [17,19,20]. Pre-clinical studies demonstrated that CX-5461 robustly reduced tumor burden of myc-driven hematological cancers in mouse models [21]. This small molecule has entered into clinical trials for patients with BRCA1/2 deficient tumors [18] (Canadian trial, [NCT02719977](#)) and patients with advanced hematological malignancies (Peter Mac, Melbourne, Australia).

Another class of small molecules, the BMH-compounds, also appears to inhibit RNA polymerase I transcription and perturb nucleolar function [22]. An in-depth investigation of one of these compounds, BMH-21, found that it is a DNA intercalator, which binds preferentially to the GC rich rDNA. BMH-21 rapidly inhibits transcription by Pol I, but also induces proteasome-dependent degradation of A194, the largest catalytic subunit of human Pol I complexes [23,24]. The molecular target of BMH-21 and mechanisms of action appear distinct from CX-5461, but this compound also potently inhibits rRNA synthesis.

Early successes with CX-5461 and BMH-21 support the model that ribosome biosynthesis is an excellent target for therapeutic intervention in cancer. Since transcription of the rDNA is a dynamic process that requires sophisticated regulation at many distinct levels, many steps in the pathway are vulnerable to selective inhibition. There remains a need to identify and characterize novel inhibitors of alternate steps in ribosome biosynthesis. Here, we use a

series of novel HTS strategies to discover compounds that inhibit cancer cell growth and ribosome biosynthesis in malignant melanoma cells. In the primary screen, a small ribosomal subunit protein, Rps9, was tagged with a HaloTag epitope, a modified bacterial enzyme that covalently binds to a synthetic ligand designed to carry various functional groups (such as a fluorophore). Since Rps9 is stable only when assembled into ribosomes, monitoring the level of Rps9 after treatment with a library of small molecules served as an excellent reporter of overall ribosome synthesis rate. A secondary RT-qPCR screen was then carried out to identify inhibitors that decreased the abundance of the pre-rRNA. Two small molecule inhibitors, Ribosome Biogenesis Inhibitors 1 and 2 (RBI1 and RBI2), were identified. Cell viability analysis indicates that both RBI1 and RBI2 potentially inhibit the growth of A375 malignant melanoma cells *in vitro*. Furthermore, acute, transient treatment with RBI2 inhibits anchorage-independent cell growth and proliferation. These studies validate our screening strategy and identify two promising compounds for further pre-clinical development.

Materials and methods

Compound library collection

Southern Research maintains a collection of 470 805 unique, non-proprietary compounds with MW < 500 assembled from various commercial vendors (Enzo, Selleck, ChemBridge, Enamine, Life Sciences) for screening targets in HTS. Eight molecular properties were calculated for this collection using Accelrys Pipeline Pilot application. Analysis showed that 438 645 (92.03%) of compounds have molecular properties matching all eight criteria for lead-like molecules to serve as starting points for a drug discovery effort (Molecular mass 500; Heteroatom count 10; Number Rotatable Bonds 8; Number Aromatic Rings 4; ALog *P* 6; Molecular Polar Surface Area 200; H-bond acceptors < 10; H-bond donors < 5). Within this chemical space, the collection is diverse, containing 165 632 non-overlapping Murcko scaffolds with an average cluster size 2 to 3. (The Murcko scaffold of a molecule is determined by locating all ring systems of the molecule and all direct connections between them), 5924 individual ring systems with unique substitution pattern (average frequency 245) and 10 594 contiguous ring systems with unique substitution pattern (average cluster frequency 111). A representative subset of 153 673 compounds were selected from this collection for HTS. All compound samples were tested once at a concentration of 20 μ M or 10 μ g/ml, depending on the commercial source of the compound sample.

Primary high throughput screening strategy using HaloTag technology

A fluorescence-based assay was developed to screen for compounds that reduce ribosome biogenesis. A375 cells were obtained from ATCC (Cat #CRL-1619) and transfected with a pFC14K plasmid derivative expressing RPS9 with a C-terminal HaloTag epitope under a CMV enhancer/promoter. Stably expressing colonies were isolated and screened by Western blot for high expression of Halo-RPS9. RPS9 is a ribosomal protein and is only stable when incorporated into the ribosome, thus RPS9 abundance reflects overall ribosome levels in a cell [25,26]. To monitor ribosome synthesis, cells were grown in T-150 flasks in Dulbecco's modified Eagle's medium (DMEM) (Gibco cat# 11965-084) supplemented with 10% fetal bovine serum (FBS) and 1 mg/ml G418 (Gibco; cat# 11811-031). HaloTag® Amine O2

ligand (Promega; cat # P6711) was diluted 1 : 200 in fresh growth medium and added to the cells grown to 80% confluence after removing old media. The cells were incubated at 37°C for 15 min after which media was completely removed and cells were washed three times with 10 ml fresh media. After incubating the cells at 37°C for 30 min the third wash was removed and replaced with 2 ml of TrypLE Express (Gibco; cat#12605-010). Cells were trypsinized for 5 min at 37°C, resuspended in fresh media, counted using a ViCell and diluted to a final concentration of 400 000 cells/ml. R110 Direct ligand (Promega; cat# G3221) was added to the cells at 1 : 1000 final volume and 20 µl of the cell suspension was added to each well of a 384-well plate (Corning; cat # 3712) using a Wellmate. Wells in columns 3–22 contained 5 µl test compounds at 5× final concentration in media. All wells contained a final assay concentration of 0.2% DMSO. Wells in columns 1 and 2 contained cells only (32 wells). Rows A–L in columns 23 and 24 contained 6 µg/ml (final concentration) CX-5461 as a positive control and rows M–P of columns 23 and 24 contained 1 µg/ml (final concentration) rapamycin as a reference compound. After 24 h of incubation at 37°C the total fluorescence intensity associated with cells in each well was measured by laser scanning cytometry using a TTP Labtech Mirrorball® with a 488 nm excitation laser and the FL2 (495–540 nm) emission detector. Plates were returned to the incubator for an additional 2 days after which cell viability was determined by measuring ATP content with Promega Cell Titer-Glo®.

Secondary high throughput screening strategy using RNA Pol I TaqMan RT-qPCR

For TaqMan RT-qPCR, the human A375 cell line was obtained from ATCC (Cat #CRL-1619) and maintained in DMEM (Corning #10-013-CV) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified incubator with a 5% CO₂ atmosphere at 37°C.

A375 cells (4000 cells/well) were seeded to PDL (poly-D-Lysine)-coated 384-well plate (Corning #3845) with 20 µl DMEM containing 10% FBS and incubated at 37°C overnight. Compounds were drugged directly to the cells using a LabCytte Echo 550 acoustic dispensing system. The media was removed after 3 h incubation at 37°C, then TaqMan RT-qPCR with cell lysate was conducted using TaqManGene Expression Cells-to-Ct Kit (Life Technologies #AM1729) following the manufacturer instruction. Briefly, cells were washed with cold PBS and lysed in Lysis Solution for 5 min at ambient temperature; DNase treatment was performed simultaneously. Lysis was terminated at ambient temperature by a 2-minute incubation with Stop Solution. Each 10 µl Reverse Transcription (RT) reaction mixture contained 5 µl of 2× RT Buffer, 0.5 µl 20× RT Enzyme Mix and 2 µl cell lysis. Thermal cycling included 37°C for 60 min, followed by 95°C for 5 min. Each 10 µl qPCR reaction mixture contained 5 µl of 2× Master Mix, 0.5 µl 20× ACTB TaqMan Gene Expression Assay (Applied Biosystems #4448485) including 18 µM/each primer and 5 µM VIC-probe as housekeeping gene internal control, 0.25 µl 20 µM concentrations of pre-rRNA forward primer (5'-CCGCGCTCTACCTTACCTACCT-3') and 0.25 µl 20 µM concentrations of pre-rRNA reverse primer (5'-GCATGGCTTAATCTTTGAGACAAG-3'), as well as 0.25 µl 10 µM of its specific probe (5'-TTGATCCTGCCAGTAGC-3') [27]. The probes were synthesized using 6-carboxyfluorescein (FAM) at the 5' end and a TAMRA quencher at the 3' end (Applied Biosystems). Two microliters RT reaction (cDNA) were

thermally cycled: 50°C for 2 min, followed by 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min on LightCycler 480 (Roche). The data were analyzed according to the 2^{-CT} method [28].

Metabolic labeling

Two million A375 cells in a total volume of 2 ml were inoculated into each well of a six-well plate in phosphate-free RPMI-1640 medium and allowed to attach for 2 h. Forty microliters of phosphorus-32 radionuclide [^{32}P] 1 mCi/ml (PerkinElmer # NEX053001MC) was then added per well and cells were incubated overnight. The next day, 5 μM of RBI2 was used to treat the cells for 0, 10, 30, and 120 min time points. Cells were then harvested, and RNA was purified with TRIzol in combination with Purelink RNA mini kit. The RNA was electrophoresed on a 1% formaldehyde/MOPS : agarose gel. The gel was dried and exposed to a Phosphor-Image screen, and imaged by autoradiography.

Cell culture and compound treatment

A375, malignant melanoma cells and U2OS osteosarcoma cells were obtained from ATCC. A375 cells were cultured in DMEM (Gibco #11965-092) medium plus 10% FBS at 37°C with 5% CO_2 . HUVEC cells were cultured in F-12K media (ATTC 30-2004) supplemented with 0.1 mg/ml heparin, 1% endothelial cell growth supplement (Sigma E2759-15MG) and 10% FBS (ATCC #30-2020) at 37°C with 5% CO_2 .

Cell growth curve

Cell growth curves for A375 cells and human umbilical vein epithelial cells (HUVECs) were generated before performing cell viability assays to insure that cells were still in the exponential growth phase at a linear range of detection by Alamar Blue dye during compound treatment (A375 growth curves can be found in Supplementary Figure S1). Exponentially growing cells were serially diluted in fresh medium. The number of cells per well in 96-well plate (Falcon #353072) ranged from 80 to 40 000 cells in 100 μl of the medium. A control well only containing media was included. The wells on the edge of 96-well plates were filled with sterilized distilled water to prevent evaporation. Cells were incubated at 37°C for 3 days. The proliferative status of the cells was monitored by Alamar Blue assay. Ten microliters of Alamar Blue was added into each well and incubated at 37°C with 5% CO_2 . The fluorescent signal of reduced Alamar Blue was quantified using BioTek Synergy 2 Multi-Mode Reader. Fluorescence was measured as a function of incubation time for up to 7 h.

Cell viability assay

A375 cells and HUVECs were grown to exponential phase. After trypsinization, cells were counted via hemocytometer. The appropriate number of cells were seeded based on the previously calculated growth curve (for example, for A375 cells 5000 cells/well were used as shown in Supplementary Figure S1) to a total volume of 90 μl into a 96-well plate and incubated overnight. The next day, 10 μl of RBI1, RBI2, or RBIX was added per well to a final concentration ranging from 10 nM to 10 000 nM in triplicate. Cells were incubated at 37°C with 5% CO_2 for 72 h. Cell viability was examined by Alamar Blue assay. Ten

microliters of Alamar Blue were added per well and incubated at 37°C with 5% CO₂ for 2–3 h. The fluorescent signal was quantified using a BioTek Synergy 2 Multi-Mode Reader. Cell viability curves were generated using GraphPad Prism.

Anchorage-independent growth assay

For anchorage-independent growth, A375 cells were treated with 10 μ M RBI2 for 24 h. Cells were then trypsinized and counted. Five thousand cells were mixed with 0.4% agarose in 1 $\times$ RPMI medium. One milliliter of cell suspension was laid over the base layer (0.8% agarose in 1 $\times$ RPMI). Cells were incubated at 37°C, 5% CO₂ for 7–14 days. Colonies were stained with 5 μ l of 0.5 mg/ml MTT solution in 1 $\times$ PBS. After overnight incubation, plates were scanned and colonies were counted using ImageJ software.

Results

Primary HTS identifies novel ribosome biogenesis inhibitors

We developed and implemented a high-throughput screen that identified compounds that inhibit ribosome biosynthesis in A375 malignant melanoma cells in culture using Promega's HaloTag technology. For the screen, we constructed cell lines stably expressing HaloTagged ribosomal protein S9. To determine whether Halo-RPS9 was incorporated into ribosomes and whether its incorporation reflects the kinetics of ribosome synthesis, we used a pulse:chase labeling strategy using fluorescent halo-ligands. To test whether nucleolar and cytoplasmic ribosome populations could be visualized we used U2OS cells expressing Halo-RPS9. Due to their relatively large size, U2OS cells were ideal for testing this strategy microscopically. Cells were treated first with TMR Red halo-ligand. This resulted in covalent-binding of the ligand to all pre-existing ribosomes. This label was then washed away with fresh media and then cells were grown three hours and subsequently treated with Oregon Green halo-ligand. At this point, cells were fixed and imaged by using standard fluorescent microscopy. As shown in Figure 1A, the red label is found almost exclusively in the cytoplasm, where mature ribosomes are localized. However, transient treatment with the green ligand prior to fixing cells reveals primarily nucleolar signal. These data demonstrate that pre-existing ribosomes can be 'blocked' with one halo-ligand and new synthesis can be monitored, validating our screening strategy.

We used A375 cells stably expressing halo-RPS9 for high throughput screening. These cells were chosen due to their previously demonstrated sensitivity to inhibitors of RNA polymerase I [15,22] and because they bind tightly to the culture plates, aiding robotic screening. As described in Figure 1B, cells were treated with excess HaloTag[®] Amine O2 ligand, washed and dispensed into 384 well cell culture plates. The non-fluorescent HaloTag[®] Amine O2 ligand essentially blocked all pre-existing ribosomes. After incubating cells for 24 h in the presence of test compounds, cells were treated with a green fluorescent halo-ligand (R110), which covalently bound newly synthesized ribosomes. Reduction in fluorescence indicated inhibition of one or more steps in ribosome biosynthesis.

The HTS campaign comprised 499 plates, each containing 32 negative control (cells only) wells, 24 positive control wells treated with CX-5461 (6 μ g/ml) and six wells treated with

rapamycin (1 µg/ml) as a reference compound. The fluorescent signal of these controls were normalized as a percent of the average negative control values for each plate to determine assay performance. Rapamycin and CX5461 treatment resulted in an average reduction in signal to 21.4% and 6.6%, respectively, of the untreated controls (Supplementary Figure S2A). The signal separation of the positive and negative controls on each plate was determined by calculating the Z' factor [29] according to the following formula:

$$Z' \text{ factor} = 1 - \frac{3(SD_t + SD_b)}{|m_t - m_b|}$$

Where

m_t = Mean of negative controls (maximum signal)

m_b = Mean of positive controls (minimum signal)

SD_t = Standard deviation of the negative controls

SD_b = Standard deviation of the positive controls

Average Z' factor = 0.68 (ranging from 0.42 to 0.85) for all 499 plates (Supplementary Figure S2B). A Z' factor > 0.4 indicates that the assay performance is robust enough for HTS [30].

The effect of each compound was calculated using the positive and negative controls on the same plate by the following formula:

$$\% \text{ inhibition} = 100 \times 1 - \frac{(\text{Well value} - \text{Avg positive control})}{(\text{Avg negative control} - \text{Avg positive control})}$$

Since inhibition of ribosome synthesis results in reduction in cell growth, compounds working through this mechanism should reduce the level of cell viability compared with untreated controls. However, compounds exerting cytotoxic effects through other mechanisms will also be detected in an assay measuring cell viability. We reasoned that compounds that inhibited ribosome synthesis during the initial 24 h of treatment will exert a cytostatic rather than cytotoxic effect and reduce, but not eliminate, cell viability levels compared with controls when measured at 72 h. Indeed, this is the activity profile of CX-5461 which showed on average ~95% inhibition of fluorescent signal at 24 h and 55% cell viability at 72 h. Using this profile as a guide to set criteria for selecting hits in the primary screen we identified 5786 compounds showing >80% inhibition and >60% cell viability in the HTS.

Secondary screening

Hits identified in the primary screen were tested in a secondary RT-qPCR assay to identify those that inhibited pre-rRNA synthesis. The assay employed oligonucleotide primers specific for short-lived pre-rRNA species (Figure 2, specifically we probed for 5' external transcribed spacer, 5'-ETS, RNA). Cells were exposed to each compound to a final

concentration of 10 μ M 3 h prior to lysis and RT-qPCR. Short-lived 5'ETS rRNA transcripts were quantified relative to Pol II-transcribed GAPDH or Actin mRNAs. Since we did not know what level of cell viability would be exhibited by compounds that inhibit pre-rRNA synthesis, we triaged hits from the primary screen for testing in the RT-qPCR assay by binning them according to the combined effects on fluorescence inhibition and cell viability as follows: Bin 1: 80% inhibition and 80% cell viability; Bin 2: 80% inhibition and 60–79% cell viability; Bin 3: 60–79% inhibition and 80% cell viability; Bin 4: 60–79% inhibition and 60–79% cell viability. A total of 320 compounds (80 from each bin) were selected for retest using RT-qPCR. Two structurally related compounds, which we named ribosome biogenesis inhibitors 1 and 2, RBI1 and 2 (Figure 2) were the only compounds that showed robust activity from this analysis and were contained in Bin 2 with activity profiles of 100% inhibition and 60% cell viability. Since the control compound CX-5461 had a similar activity profile (95% inhibition and 55% cell viability) we tested another 320 compounds with activity profile >95% inhibition and 55–65% cell viability but did not find any additional active compounds in the RT-qPCR assay. Within our library, we also identified a structurally related, but inactive compound termed RBIX. To confirm compound structural integrity, the molecular mass of RBI1, RBI2 and RBIX in the stock solutions of our HTS collection were determined by LC/MS. These values matched the calculated values (RBI1 = 429.49, RBI2 = 459.52, RBIX = 570.46). In addition, we ordered fresh dry powder samples of each compound from commercial vendors (RBI1: Zelinsky cat.# UZI/1914004; RBI2: ChemBridge cat# 5710183; RBIX: ChemBridge cat# 5727417) and also confirmed the molecular mass of these samples by LC/MS. Using the resupplied fresh powder samples, we assessed the potency of these compounds for inhibition of rRNA synthesis by exposing cells to a range of concentrations, incubating cells for 3 h and measuring 5'-ETS pre-rRNA by RT-qPCR. We observed nanomolar IC₅₀ values for RBI1 and RBI2, but no inhibition by RBIX (Figure 2). For further verification, RBI1 was resynthesized and shown to be active in the qPCR assay (IC₅₀ = 470 nM).

Secondary screen hits were validated as inhibitors for pre-rRNA inhibition through metabolic labeling with radioactive, ³²P-ortho-phosphate. We observed the complete loss of 45S pre-rRNA after 30 min of treatment with IC₉₀ concentrations of RBI2 (Supplementary Figure S3). These findings validate that the observed loss of pre-rRNA was not due to inhibition of RT activity due to interactions with the small molecule inhibitor. Collectively, these data demonstrate that our screening strategy was effective and we have identified a new class of inhibitors of ribosome synthesis.

RBI2 inhibits cell growth and proliferation in cell viability assays in A375 cells

A375 malignant melanoma cells were exposed to a range of concentrations of RBI1, RBI2, and RBIX and cell viability was measured. Alamar Blue dye, a fluorescent indicator of the oxidative activity of cells, was used to measure relative cell proliferation. We observed that RBIX has no impact on A375 cell viability, whereas RBI1 and RBI2 cause a substantial decrease in cell viability (Figure 3). The IC₅₀ values for inhibition of cell viability by RBI1 and RBI2 were 3375 nM and 680 nM, respectively. Importantly, we see a limited effect of RBI2 on the proliferation of our non-cancer cell control, HUVECs. These results suggest

that RBI2 inhibits the growth of rapidly proliferating cancer cells and is a viable candidate for further development as a selective inhibitor of cancer cell growth.

Anchorage-independent cell growth assay

Metastatic cancer cells possess characteristics that allow them to grow and proliferate in foreign environments. One of these well-described characteristics is the ability to grow without attachment/adherence. We used anchorage-independent cell growth assays to test the metastatic potential of cancer cells. A375 cells were transiently treated with 10 μ M RBI2 or vehicle for 24 h. Notably, this treatment condition does not kill cells. Cells were then trypsinized and resuspended into DMEM media containing 0.4% agarose and grown in low retention plates for 14 days (Figure 4). The number of colonies observed for RBI2 treated cells was reduced to ~50% of the DMSO control cells, thus it is clear that even brief exposure to RBI2 negatively impacts anchorage-independent growth of cells. These results suggest that not only does RBI2 negatively impact cell viability, it also decreases the metastatic potential of A375 malignant melanoma cells.

Taken together, all of these data emphasize the value of ribosome synthesis as a target for efficient control of cancer cell growth and identify a new chemical scaffold that potentially inhibits ribosome synthesis. The molecular target and mechanism of action of RBI1 and RBI2 remain a topic of continued investigation.

Discussion

Ribosome biogenesis as a target for generalized cancer chemotherapeutic

Ribosome biogenesis is critical for cell growth and proliferation, especially for fast growing cancer cells. It is known that rapidly dividing yeast cells can devote up to 60% of total transcription to ribosomal RNA [4]. As the rate-limiting step of ribosome biogenesis, rRNA synthesis is extensively activated in most cancers. Thus, rRNA synthesis is an excellent target for therapeutic intervention in cancer. Here we described a new class of compounds that potentially inhibits rRNA synthesis, impairs cells viability, and reduces anchorage-independent growth. Compounds with these properties should hold promise in the treatment of a wide array of tumor cell types.

Until recently, very little effort was invested in defining selective inhibitors of ribosome biosynthesis [15,22]. However, retrospective analysis of known chemotherapeutic agents found that many target ribosome biogenesis (directly or indirectly) to elicit anti-tumor effects [31]. Thirty-six chemotherapeutic drugs were found to have off-target effects that illicit inhibition of Pol I transcription and processing of rRNA. Cytostatic drugs cisplatin, oxaliplatin, doxorubicin, and mitoxantrone have also been shown to inhibit rDNA transcription non-specifically. Topoisomerase inhibitors like camptothecin and kinase inhibitors, such as benzimidazole (DRB), affect early rRNA processing. The proteasome inhibitor MG132 and translation inhibitors such as cycloheximide impair late rRNA processing. All of these examples suggest that targeting ribosome biogenesis as a cancer therapeutic is not only valid, but also a proven effective strategy that is currently being used, and will continue to be developed in the future.

Implementation of novel HTS strategies

For high throughput screening, we utilized HaloTag[®] technology in a pulse-chase manner as a reporter system to measure the production of newly synthesized ribosomes. High throughput quantification of cell-associated fluorescent signals in 384 well plate format was achieved by using laser scanning cytometry (TTP Labtech Mirrorball[®]). To our knowledge, this is the first report of using HaloTag[®] technology for HTS. This screening strategy and derivatives thereof can be applied to a wide array of biological systems.

To focus our progress of compounds that inhibit rRNA synthesis, rather than later stages in ribosome biosynthesis, we implemented a secondary screen using RT-qPCR. This approach detected short-lived precursors of rRNA, approximating rRNA synthesis rate. The tandem use of these two screens enabled us to identify two highly potent compounds.

We note that our data do not reveal the molecular target of the RBI compounds. Previous studies with both CX-5461 and BMH-21 have revealed effects of the compounds in cell-free or fully reconstituted transcription assays [15,24]. In a fully reconstituted transcription assay using yeast Pol I and associated factors, we see no effect of the RBI compounds. Thus, these compounds either selectively bind mammalian Pol I transcription factors, or they induce indirect effects on Pol I transcription. The latter explanation is more likely.

The need for novel ribosome biogenesis inhibitors

Inhibition of ribosome synthesis is a clear vulnerability in cancer cells. In fact, there is evidence that inhibition of rRNA synthesis may hold great promise in treating chemoresistant cells or cells that are refractory to treatment with the standard of care. Recently we showed that ovarian cancer cells which have evolved resistance to treatment with carboplatin and paclitaxel were hyper-sensitive to CX-5461. Our data suggest that in the process of gaining chemoresistance, cells induced rRNA synthesis, rendering them hyper-sensitive to ribosome biosynthesis inhibitors [32]. This remarkable finding was described in ovarian cancers, but it is reasonable to expect other tumor types will be equally vulnerable. Thus, there is a need for effective and selective inhibitors of ribosome biosynthesis.

Excellent studies from two independent groups have identified potentially selective inhibitors of ribosome biosynthesis [15,22]. Development and testing of CX-5461 and BMH-21 are ongoing, and pre-clinical data are promising. However, the identification of these inhibitors does not eliminate the need for further discovery and development. First, it is well-established that cancer cells are genetically unstable and resistance can be rapidly acquired or selected during treatment. Furthermore, all compounds have limitations in various tumor types, thus there is a need to target unique steps in ribosome synthesis with chemically distinct scaffolds.

Here, we describe a screening strategy to discover novel inhibitors of ribosome biosynthesis. This approach can be applied to many different cells types, using a variety of compound libraries. The two inhibitors described here reflect a unique chemical scaffold that induces potent inhibition of rRNA synthesis, cell viability, and anchorage-independent growth.

Further characterization of these compounds is required to determine their potential utility in cancer chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank present and past members of the Schneider laboratory for their insight and suggestions for this work. We also thank the many members of the Alabama Drug Discovery Alliance Advisory Committee for their creative inspiration and guidance during the synthesis of this screening strategy.

Funding

This project was supported by grants from the National Institute of Health to DAS [GM084946] and CES [T32GM109780 and T32GM008111]. This work was supported by the Alabama Drug Discovery Alliance — a collaboration between the University of Alabama at Birmingham and Southern Research.

Abbreviations

DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
HTS	high-throughput screening
HUVECs	human umbilical vein epithelial cells
RT	reverse transcription

References

1. Fromont-Racine M, Senger B, Saveanu C and Fasiolo F (2003) Ribosome assembly in eukaryotes. *Gene* 313, 17–42 10.1016/S0378-1119(03)00629-2 [PubMed: 12957375]
2. Henras AK, Soudet J, Grus M, Lebaron S, Caizergues-Ferrer M, Mougn A et al. (2008) The post-transcriptional steps of eukaryotic ribosome biogenesis. *Cell. Mol. Life Sci* 65, 2334–2359 10.1007/s00018-008-8027-0 [PubMed: 18408888]
3. Kressler D, Hurt E and Bassler J (2010) Driving ribosome assembly. *Biochim. Biophys. Acta, Mol. Cell Res* 1803, 673–683 10.1016/j.bbamcr.2009.10.009
4. Warner JR (1999) The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* 24, 437–440 10.1016/S0968-0004(99)01460-7 [PubMed: 10542411]
5. Bolze A, Mahlaoui N, Byun M, Turner B, Trede N, Ellis SR et al. (2013) Ribosomal protein SA haploinsufficiency in humans with isolated congenital asplenia. *Science* 340, 976–978 10.1126/science.1234864 [PubMed: 23579497]
6. Freed EF, Bleichert F, Dutca LM and Baserga SJ (2010) When ribosomes go bad: diseases of ribosome biogenesis. *Mol. Biosyst* 6, 481–493 10.1039/b919670f [PubMed: 20174677]
7. Landowski M, O'Donohue M-F, Buros C, Ghazvinian R, Montel-Lehry N, Vlachos A et al. (2013) Novel deletion of RPL15 identified by array-comparative genomic hybridization in Diamond-Blackfan anemia. *Hum. Genet* 132, 1265–1274 10.1007/s00439-013-1326-z [PubMed: 23812780]
8. Narla A and Ebert BL (2010) Ribosomopathies: human disorders of ribosome dysfunction. *Blood* 115, 3196–3205 10.1182/blood-2009-10-178129 [PubMed: 20194897]
9. Teng T, Thomas G and Mercer CA (2013) Growth control and ribosomopathies. *Curr. Opin. Genet. Dev* 23, 63–71 10.1016/j.gde.2013.02.001 [PubMed: 23490481]

10. Derenzini M, Trerè D, Pession A, Govoni M, Sirri V and Chieco P (2000) Nucleolar size indicates the rapidity of cell proliferation in cancer tissues. *J. Pathol* 191, 181–186 10.1002/(SICI)1096-9896(200006)191:2<181::AID-PATH607>3.0.CO;2-V [PubMed: 10861579]
11. Pianese G (1896) Beitrag zur Histologie und Aetiologie des Carcinoms, Fischer G, Jena 193 p., 8 leaves of folded plates p
12. Gani R (1976) The nucleoli of cultured human lymphocytes: I. Nucleolar morphology in relation to transformation and the DNA cycle. *Exp. Cell Res* 97, 249–258 10.1016/0014-4827(76)90614-5 [PubMed: 1248517]
13. Laribee RN (2018) Transcriptional and epigenetic regulation by the mechanistic target of rapamycin complex 1 pathway. *J. Mol. Biol* 430, 4874–4890 10.1016/j.jmb.2018.10.008 [PubMed: 30359581]
14. Ruggero D (2012) Revisiting the nucleolus: from marker to dynamic integrator of cancer signaling. *Sci. Signal* 5, pe38 10.1126/scisignal.2003477
15. Drygin D, Lin A, Bliesath J, Ho C, O'Brien S, Proffitt C et al. (2010) Targeting RNA polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA synthesis and solid tumor growth. *Cancer Res.* 71, 1418–1430 10.1158/0008-5472.CAN-10-1728 [PubMed: 21159662]
16. Negi SS and Brown P (2015) rRNA synthesis inhibitor, CX-5461, activates ATM/ATR pathway in acute lymphoblastic leukemia, arrests cells in G2 phase and induces apoptosis. *Oncotarget* 6, 18094–18104 10.18632/oncotarget.4093 [PubMed: 26061708]
17. Quin J, Chan KT, Devlin JR, Cameron DP, Diesch J, Cullinane C et al. (2016) Inhibition of RNA polymerase I transcription initiation by CX-5461 activates non-canonical ATM/ATR signaling. *Oncotarget* 7, 49800–49818 10.18632/oncotarget.10452 [PubMed: 27391441]
18. Xu H, Di Antonio M, McKinney S, Mathew V, Ho B, O'Neil NJ et al. (2017) CX-5461 is a DNA G-quadruplex stabilizer with selective lethality in BRCA1/2 deficient tumours. *Nat. Commun* 8, 14432 10.1038/ncomms14432 [PubMed: 28211448]
19. Devlin JR, Hannan KM, Hein N, Cullinane C, Kusnadi E, Ng PY et al. (2016) Combination therapy targeting ribosome biogenesis and mRNA translation synergistically extends survival in MYC-driven lymphoma. *Cancer Discov.* 6, 59–70 10.1158/2159-8290.CD-14-0673 [PubMed: 26490423]
20. Rebello RJ, Kusnadi E, Cameron DP, Pearson HB, Lesmana A, Devlin JR et al. (2016) The dual inhibition of RNA Pol I transcription and PIM kinase as a new therapeutic approach to treat advanced prostate cancer. *Clin. Cancer Res* 22, 5539–5552 10.1158/1078-0432.CCR-16-0124 [PubMed: 27486174]
21. Bywater MJ, Poortinga G, Sanij E, Hein N, Peck A, Cullinane C et al. (2012) Inhibition of RNA polymerase I as a therapeutic strategy to promote cancer-specific activation of p53. *Cancer Cell* 22, 51–65 10.1016/j.ccr.2012.05.019 [PubMed: 22789538]
22. Peltonen K, Colis L, Liu H, Jäämaa S, Zhang Z, Af Hällström T et al. (2014) Small molecule BMH-compounds that inhibit RNA polymerase I and cause nucleolar stress. *Mol. Cancer Ther* 13, 2537–2546 10.1158/1535-7163.MCT-14-0256 [PubMed: 25277384]
23. Peltonen K, Colis L, Liu H, Trivedi R, Moubarek MS, Moore HM et al. (2014) A targeting modality for destruction of RNA polymerase I that possesses anticancer activity. *Cancer Cell* 25, 77–90 10.1016/j.ccr.2013.12.009 [PubMed: 24434211]
24. Wei T, Najmi SM, Liu H, Peltonen K, Kucerova A, Schneider DA et al. (2018) Small-molecule targeting of RNA polymerase I activates a conserved transcription elongation checkpoint. *Cell Rep.* 23, 404–414 10.1016/j.celrep.2018.03.066 [PubMed: 29642000]
25. Agrawal MG and Bowman LH (1987) Transcriptional and translational regulation of ribosomal protein formation during mouse myoblast differentiation. *J. Biol. Chem* 262, 4868–4875 PMID:3558374 [PubMed: 3558374]
26. Maicas E, Pluthero FG and Friesen JD (1988) The accumulation of three yeast ribosomal proteins under conditions of excess mRNA is determined primarily by fast protein decay. *Mol. Cell. Biol* 8, 169 10.1128/MCB.8.1.169 [PubMed: 3275866]
27. Drygin D, Siddiqui-Jain A, O'Brien S, Schwaebe M, Lin A, Bliesath J et al. (2009) Anticancer activity of CX-3543: a direct inhibitor of rRNA biogenesis. *Cancer Res.* 69, 7653–7661 10.1158/0008-5472.CAN-09-1304 [PubMed: 19738048]

28. Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-CT} method. *Methods* 25, 402–408 10.1006/meth.2001.1262 [PubMed: 11846609]
29. Zhang J-H, Chung TDY and Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen* 4, 67–73 10.1177/108705719900400206 [PubMed: 10838414]
30. Iversen PW, Beck B, Chen YF, Dere W, Devanarayan V, Eastwood BJ et al. (2004) HTS Assay Validation In Assay Guidance Manual (Sittampalam GS, Coussens NP, Brimacombe K, Grossman A, Arkin M, Auld D et al., eds), Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda, MD
31. Burger K, Muhl B, Harasim T, Rohrmoser M, Malamoussi A, Orban M et al. (2010) Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. *J. Biol. Chem* 285, 12416–12425 10.1074/jbc.M109.074211 [PubMed: 20159984]
32. Cornelison R, Dobbin ZC, Katre AA, Jeong DH, Zhang Y, Chen D et al. (2017) Targeting RNA-polymerase I in both chemosensitive and chemoresistant populations in epithelial ovarian cancer. *Clin. Cancer Res* 23, 6529–6540 10.1158/1078-0432.CCR-17-0282 [PubMed: 28778862]

Figure 1. Primary screening strategy narrowed a library of 150 000 compounds down to 5786 hits.

(A) Ribosome labeling strategy was validated in U2OS cells by pulse labeling with two distinct fluorescent HaloTag ligands with (TMR Red) and without (Oregon Green) a 3 h chase period prior to fixation and imaging. Images are displayed without false color. (B) Primary HTS screen strategy deployed the HaloTag technology for a fluorescent pulse labeling of newly synthesized ribosomes.

Figure 2. RBI1 and RBI2 decrease pre-rRNA levels as compared with related inactive control (RBIX).

(A) Diagram of yeast rDNA repeat. The probes for qPCR annealed to 5'-ETS region, which is rapidly degraded during pre-rRNA processing. (B) A375 (malignant melanoma) cells were treated with titrations of RBI1, RBI2 and RBIX for 3 h. Precursor rRNA abundance was quantified via RT-qPCR of the immature pre-rRNA species (5'-ETS). The degree of inhibition was plotted versus compound concentration and the concentration resulting in 50% inhibition (IC₅₀) was calculated from the resultant curves. Structures of compounds used to treat cells are displayed above each plot.

Figure 3. RBI1 and RBI2 inhibit cell viability in A375 malignant melanoma cells, and RBI2 has minimal impact on cell viability of non-cancerous HUVEC cells.

Cell viability curve was determined by incubation of cells with serially diluted compounds ranging from 10 nM to 0 μ M in A375 cells in the presence of RBI2 (orange squares), RBI1 (maroon circles), and RBIX (light gray triangles). In A375 cells, RBI1 and RBI2 treatment resulted in IC_{50} values of 3375 nM and 680 nM, respectively for cell viability. Cell viability of HUVEC cells in the presence of RBI2 was also tested (black triangles). IC_{50} values for cell viability for A375 cells in the presence of RBI1 and RBI2 were calculated using GraphPad Prism.

Figure 4. RBI2 inhibits anchorage independent and clonogenic growth.

Representative images of the wells with colonies developed after 2 weeks growth inside soft agar. Colonies were stained with MTT and counted using ImageJ software. Error bars represent one standard deviation $\pm$ from three independent wells.