Targeting RNA Polymerase I with an Oral Small Molecule CX-5461

Inhibits Ribosomal RNA Synthesis and Solid Tumor Growth

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Abstract

Deregulated ribosomal RNA synthesis is associated with uncontrolled cancer cell proliferation. RNA Polymerase I (Pol I), the multi-protein complex that synthesizes rRNA, is activated widely in cancer. Thus, selective inhibitors of RNA Pol I may offer a general therapeutic strategy to block cancer cell proliferation. Coupling medicinal chemistry efforts to tandem cell and molecular based screening led to the design of CX-5461, a potent small molecule inhibitor of rRNA synthesis in cancer cells. CX-5461 selectively inhibits Pol I-driven transcription relative to RNA Polymerase II (Pol II)-driven transcription, DNA replication and protein translation. Molecular studies demonstrate that CX-5461 inhibits the initiation stage of rRNA synthesis and induces both senescence and autophagy, but not apoptosis, through a p53-independent process in solid tumor cell lines. CX-5461 is orally bioavailable and demonstrates in vivo antitumor activity against human solid tumors in murine xenograft models. Our findings position CX-5461 for investigational clinical trials as a potent, selective, and orally administered agent for cancer treatment.
Introduction

The rate of ribosome biogenesis controls cellular growth and proliferation (reviewed in (1)). It therefore is tightly regulated in mammalian cells and is tuned to respond to extracellular stimuli such as nutrient availability and stress. During tumorigenesis, the tightly regulated relationship between extracellular signaling and ribosome biosynthesis is disrupted, and cancer cells begin the excessive production of ribosomes necessary for the protein synthesis associated with unbridled cancer growth. rRNA is a major component of the ribosome and, as such, carcinogenesis requires an increase in its synthesis (reviewed in (2-5)). Indeed, an increase in the synthesis of rRNA, which is transcribed in the nucleolus by Pol I, correlates with an adverse prognosis in cancer (6). Moreover, enlarged nucleoli, reflective of accelerated rRNA synthesis, have long been recognized as a marker for aggressive tumor cells (7, 8). A number of approved cancer therapeutics reportedly act through inhibition of rRNA synthesis, but none directly target the Pol I multi-protein enzyme complex (4, 9).

The potential therapeutic benefit of selectively inhibiting the Pol I target so fundamental to cancer cell survival prompted the need for identification of small molecule drugs that selectively inhibit rRNA synthesis. For this purpose we fashioned a cell-based qRT-PCR assay that differentiates between the effects of compounds on Pol I and RNA Polymerase II (Pol II) – driven transcription. In this paper, we describe the discovery and characterization of CX-5461, a potent and selective inhibitor of Pol I-mediated rRNA synthesis in cancer cells that does not inhibit DNA, mRNA or protein synthesis. We reveal that CX-5461 induces autophagic cell death in cancer cells but not normal cells and exhibits potent in vivo antitumor activity in murine xenograft models of cancer.
human solid tumors with a favorable safety profile. CX-5461 represents a fundamentally new class of small molecule targeted anticancer therapeutics.
Materials and Methods

**Materials:** CX-5461 and CX-5447 were synthesized by Cylene Pharmaceuticals as off-white solid materials (99.2-99.5% pure) and stored at room temperature as 10 mM stock solutions in 50 mM NaH$_2$PO$_4$ (pH 4.5). The compounds were diluted directly in growth media prior to treatment.

**Cell lines:** hTERT- immortalized BJ-hTERT human fibroblasts were a gift from Dr. William Hahn, Harvard Medical School (10). Human inflammatory breast cancer cell lines SUM 149PT and SUM 190PT were obtained from Asterand (Detroit, MI). Human eosinophilic leukemia cell line EOL-1, human B cell precursor leukemia cell line SEM and human acute monocytic leukemia cell line THP-1 were obtained from DSMZ (Braunschweig, Germany). Other cell lines were purchased from American Tissue Culture Collection (Manassas, VA). All cell lines were used within five to ten passages from their acquisition. The internal authentication has been performed by monitoring growth rate and tracking the changes in morphology.

**qRT-PCR.** qRT-PCR assays were performed as previously described (11) with HCT-116 colorectal carcinoma cells and then confirmed with A375 and MIA PaCa-2 cells.

**Cell-free Pol I transcription assay.** A reaction mixture consisting of 30 ng/μL DNA template corresponding to (-160/+379) region on rDNA and 3 mg/mL nuclear extract isolated from HeLa S3 cells in a buffer containing 10 mM Tris HCl pH 8.0, 80 mM KCl, 0.8% polyvinyl alcohol, 10 mg/mL α-amanitin was combined with different amounts of test compounds and incubated at ambient for 20 min. Transcription was
initiated by addition of rNTP mix (New England Biolabs, Ipswich, MA) to a final concentration of 1 mM and was incubated for one hour at 30°C. Afterwards DNase I was added and the reaction was further incubated for 2 hr at 37°C. DNase digestion was terminated by the addition of EDTA to final concentration of 10 mM, followed immediately by 10 min incubation at 75°C, and then samples were transferred to 4°C. The levels of resultant transcript were analyzed by qRT-PCR on 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA).

**Chromatin immunoprecipitation.** Cells were treated with 2 μM of CX-5461 for 1 hour and chromatin immunoprecipitation assay was performed as previously described (11, 12).

**Electrophoretic mobility shift assay.** 32P-labeled DNA probe corresponding to the rDNA promoter was produced by PCR using pRu3 plasmid as a template. The SL1 complex was isolated from HeLa S3 cells nuclear extract as described previously (13). For the competition studies, a mixture of 5 nM of DNA probe and 0.6 μg SL1 complex was incubated in binding buffer for 15 min at ambient temperature with 0.4 – 12.5 μM CX-5461 or CX-5447. The resulting complexes were resolved using Novex TBE DNA retardation PAGE (Invitrogen, Carlsbad, CA). The gels were dried and exposed to X-ray film (Kodak, Rochester, NY).

**Gene Expression analysis.** For MIA PaCa-2 cells gene array analyses were performed at Expression Analysis (Durham, NC) using Illumina Whole Genome Human -6 v2 beadchips. For A375 cells the expression analysis was performed using Human Cancer Pathway Finder qRT-PCR array from SABiosciences (Frederick, MD).
Cell viability assay. Cells were plated on 96-well plates and treated the next day with dose-response of drugs for 96 hours. Cell viability was determined using Alamar Blue and CyQUANT assays (Invitrogen, Carlsbad CA).

Detection and Quantification of Acidic Vesicular Organelles (AVO) with Acridine Orange Staining. Cells were plated on 10 cm dishes and treated the next day with indicated doses of CX-5461 for 24 or 48 hours. At the end of treatment, cells were stained for 15 minutes with 1 μg/mL of acridine orange, trypsinized, washed twice with ice-cold PBS, and analyzed on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA). The analysis was performed as previously described (14).

Immunocytochemistry-based autophagy detection assay. Immunofluorescent analysis was performed as previously described (11) using 1:100 dilution of rabbit polyclonal anti-LC3B-II antibody (Cell Signaling Technology, Danvers, MA).

Immunocytochemistry-based senescence detection assay. The assay was performed using Senescence Detection Kit (Calbiochem, San Diego, CA) according to manufacturer’s instructions.

In vivo efficacy in murine xenograft model. Animal experiments were performed with five to six week old female athymic (NCr nu/nu fisol) mice of Balb/c origin (Taconic Farms, Germantown, NY) in accordance with approved standard operating protocols of Cylene Pharmaceuticals that were approved by the Institutional Animal Care and Use Committee. Mice were inoculated with 5x10^6 in 100 μL of cell suspension subcutaneously in the right flank. Tumor measurements were performed by caliper analysis, and tumor volume was calculated using the formula \((l \times w^2)/2\), where \(w\) = width and \(l\) = length in mm of the tumor. Established tumors (~ 110-120mm^3) were randomized.
into vehicle (50 mM NaH$_2$PO$_4$ (pH 4.5)), gemcitabine or CX-5461 treatment groups. Tumor Growth Inhibition (TGI) was determined on the last day of study according to the formula: TGI (%) = (100-(V$_{fD}$ - V$_{iD}$) / (V$_{fV}$ - V$_{iV}$)*100), where V$_{iV}$ is the initial mean tumor volume in vehicle-treated group, V$_{fV}$ is the final mean tumor volume in vehicle-treated group V$_{iD}$ is the initial mean tumor volume in drug-treated group and V$_{fD}$ is the final mean tumor volume in drug-treated group.
Results

**Discovery of CX-5461.** To screen for inhibitors of rRNA synthesis, we developed a cell-based screening assay capable of identifying agents that selectively inhibit Pol I transcription relative to Pol II transcription. Two short-lived RNA transcripts (half-lives ~20-30 minutes), one produced by Pol I and another by Pol II, were quantitated by qRT-PCR as a measure of drug-related effects on transcription. The 45S pre-rRNA served as the Pol I transcript and the mRNA for the proto-oncogene *c-myc* served as the comparator Pol II transcript. Both Pol I and Pol II transcription are known to be affected by general cellular stress. To minimize the potential effects of such stress, cells were exposed to test agents for only a short period of time (2 hours). This is sufficient time for these transcripts to be reduced by > 90% if a drug affects their synthesis.

Among numerous molecules screened, CX-5461 (Fig. 1A) was found to selectively inhibit rRNA synthesis (Pol I IC$_{50}$ = 142 nM; Pol II IC$_{50}$ > 25 μM; Selectivity ~ 200-fold) in the HCT-116 cells (Fig. 1B). Selective inhibition of rRNA synthesis by CX-5461 was confirmed in two other human solid tumor cell lines; melanoma A375 (Pol I IC$_{50}$ = 113 nM; Pol II IC$_{50}$ > 25 μM) and pancreatic carcinoma MIA PaCa-2 (Pol I IC$_{50}$ = 54 nM; Pol II IC$_{50}$ ~ 25 μM) (Fig. 1C).

**Characterization of CX-5461.** To determine if CX-5461 directly targets the Pol I machinery and to identify which specific step in Pol I transcription it may affect, we performed cell-free transcription “order of addition” studies (Fig. 1D). CX-5461 was pre-incubated with either nuclear extract [NE] or with DNA template [Template] prior to the pre-initiation complex (PIC) formation, after the PIC formation but prior to initiation of transcription [NE/Template], or after the initiation of the transcription.
[NE/Template/NTP]. Analysis of the resulting rRNA transcripts by qRT-PCR (Fig. 1D) revealed that addition of 80 nM CX-5461 prior to PIC formation resulted in considerable inhibition of Pol I transcription, while addition after PIC formation only minimally affected Pol I transcription. These data indicate CX-5461 directly targets the Pol I machinery and inhibits Pol I transcription at the initiation stage.

CX-5461 inhibits Pol I via disruption of the SL1-rDNA complex. ChIP analysis was employed to investigate the effects of CX-5461 on the association of various components of the Pol I multi-protein complex with the rDNA promoter. Treatment of HCT-116, A375 or MIA PaCa-2 with 2 μM CX-5461 resulted in 40-60% reduction of the Pol I enzyme association with the rDNA promoter (Fig. 2A). Further, CX-5461 significantly depleted the binding of Pol I transcription factors (TF) to the rDNA promoter in HCT-116 cells, with the TBP and TAF110 subunits of SL1 being most overtly affected (Fig. 2B). As SL1 is required for stabilization of UBF on and recruitment of Pol I to the rDNA promoter, the effect of CX-5461 on the binding of UBF and Pol I to rDNA is most likely secondary to the compound’s effect on SL1 (15).

Since TBP is a member of both Pol I and Pol II transcription machineries (as a unit of the TFIID transcription factor), we compared the effects of CX-5461 on binding of TBP to the Pol I promoter on rDNA relative to its binding to the promoter of two genes transcribed by Pol II (p21 and histone H2B). Treatment with CX-5461 had no effect on the binding of TBP to the Pol II promoter regions in HCT-116 cells, but did inhibit binding of TBP to the Pol I promoters (Figure 2C). To test if CX-5461 could directly compete with SL1 for the rDNA promoter, we performed an electrophoretic mobility shift assay (EMSA) measuring the interaction of purified human SL1 with a
radiolabeled double-stranded DNA fragment corresponding to the promoter region of rDNA. As a negative control we used a close analog of CX-5461, CX-5447, that is inactive against Pol I transcription (IC$_{50}$ > 25 μM). While CX-5447 had no detectable effect on the stability of the SL1/rDNA complex, CX-5461 clearly disrupted the SL1/rDNA complex (Fig. 2D). Such dissociation was likely due to disruption of the protein-rDNA interaction rather than dissociation of SL1 itself, since we detected no decrease in protein-protein interactions within SL1 after treatment with CX-5461 (data not shown).

**Biological characterization of CX-5461.** To ensure that CX-5461 selectively inhibits rRNA transcription but not DNA or protein synthesis, HCT-116, A375 and MIA PaCa-2 cells were pre-incubated with 10 μM CX-5461 for 60 minutes and pulsed for an additional 120 minutes with modified precursors BrdU or $^{35}$S-methionine. Mitoxantrone and cyclohexemide served as positive controls for inhibition of DNA and protein synthesis, respectively. At this concentration, which is ~100-times higher than its IC$_{50}$ for the inhibition of Pol I transcription, CX-5461 had only modest effects on global DNA or protein synthesis (Fig. 3A). In dose-response studies, the IC$_{50}$ for inhibition of DNA synthesis in A375 and MIA PaCa-2 cell lines ranged from 16.8 μM to 27.9 μM, while a high test dose of 30 μM CX-5461 had no significant effect on proteins synthesis (Supplementary Fig. S1). Thus, CX-5461 possesses 250-300 fold selectivity for inhibition of rRNA transcription versus DNA replication and protein translation.

Using Gene Expression Arrays (Illumina or qRT-PCR based) as a tool to probe for any potential effect of CX-5461 on Pol II transcription, we observed that 1 hour exposure of MIA PaCa-2 (Fig. 3B) or A375 (Fig. 3C) cells to 300 nM CX-5461 (that
resulted in 63% and 55% reductions in pre-rRNA, respectively), an equal number of Pol II-transcribed genes were significantly up-regulated as were down-regulated. These changes in Pol II transcription were most likely secondary to the inhibition of Pol I by CX-5461, as an inhibition of Pol II transcription results in a distribution significantly skewed to the negative (16). To further illustrate selectivity of CX-5461 for Pol I vs Pol II transcription, we compared it to another drug (actinomycin D) known to inhibit Pol I transcription. Treatment of MIA PaCa-2 cells with either 1 μM CX-5461 or 1 μM actinomycin D causes rapid inhibition of Pol I transcription, with an observed half-life of 21 minutes for 45S pre-rRNA in both cases (Fig. 3D). However, actinomycin D inhibited Pol II transcription as well, as judged by the depletion of c-myc mRNA (half-life equal to 35 minutes), while CX-5461 had no major effect on Pol II transcription even after 24 hour treatment (Fig. 3D).

In vitro antiproliferative activity of CX-5461. To evaluate the range of antiproliferative activity of CX-5461, we measured the antiproliferative EC50 across a panel of 50 human cancer cell lines and five non-transformed cell lines. The EC50 values ranged from 3 nM against the EOL-1 eosinophilic leukemia cell line to 5,500 nM against the LNCaP prostate carcinoma cell line (Fig. 4A). The median EC50 across all tested cell lines was 147 nM, yet all normal cell lines had EC50 values of approximately 5,000 nM. Evaluation of the antiproliferative dose response (Fig. 4B, left panel) for HCT-116, A375 and MIA PaCa-2 cell lines yielded EC50 values of 167 nM, 58 nM and 74 nM, respectively. In contrast, an EC50 of approximately 5,000 nM was observed for the BJ-hTert normal cell line (non-transformed hTERT-immortalized human foreskin fibroblasts). The lesser sensitivity of the normal cells was not due to a reduced uptake of
CX-5461, as the IC$_{50}$ for rRNA synthesis for the BJ-hTert normal cell line of 74 nM correlated well with the IC$_{50}$ values of the solid tumor cell lines (IC$_{50} = 142$ nM, 113 nM and 54 nM, respectively) (see Fig. 4B, right panel). These findings illustrate that CX-5461 can equivalently inhibit Pol I transcription in cancer and normal cells, but the normal cells can tolerate reductions in RNA synthesis without induction of cell death whereas the cancer cells cannot.

Inhibition of Pol I transcription has been previously demonstrated to cause nucleolar stress that leads to stabilization of p53 and induction of p53-dependent apoptosis (17). We therefore wanted to determine if CX-5461 promotes stabilization of p53. For this purpose we performed a dose-response analysis of CX-5461 and measured levels of p53 at 24 and 48 hours in wt $p53$ A375 cells. As shown in Fig. 4C, while CX-5461 slightly increased p53 levels, it did so at concentrations three to ten-fold higher than its IC$_{50}$ for Pol I transcription. Actinomycin D, used as a positive control, caused a more robust increase in p53 levels. In addition, we analyzed the correlation between the sensitivity of cell lines to CX-5461 and their $p53$ genetic status. Among the 44 cell lines with known $p53$ status, 18 had wild type (wt) $p53$ and 26 carried some form of mutation in the $p53$ gene (18). The analysis revealed that across all tested cell lines CX-5461 exhibits similar sensitivity in wild-type $p53$ cell lines (median IC$_{50} = 144$ nM) as it does in cell lines with mutant $p53$ (median IC$_{50} = 138$ nM), $P = 0.56$ (Fig. 4D). Interestingly, the three cell lines most sensitive to CX-5461 were derived from hematologic malignancies that had wt $p53$. When we then further analyzed data for correlations of $p53$ status with sensitivity to CX-5461 among hematologic cancers, we did observe a significant difference ($P = 0.003$) between the activity of CX-5461 in cell lines with wild
type \( p53 \) (3 cell lines, median IC\(_{50} = 5 \) nM) and those with mutant \( p53 \) (8 cell lines, median IC\(_{50} = 94 \) nM). While the sample size is too limited to make strong conclusions, it does hint at an intriguing possibility that nucleolar stress signaling in hematological cancers is more dependent on p53 function and is worthy of future investigation.

**CX-5461 induces autophagy and senescence in cancer cells.** Next, we investigated the mode of cell death promoted by CX-5461 in the A375 and MIA PaCa-2 cells. Previously, inhibition of rRNA synthesis using “semi-selective” drugs such as actinomycin D was shown to induce apoptosis (19). Not surprisingly, we found that actinomycin D induced apoptosis, as judged by western blot analysis of PARP cleavage and caspases-3/7/9 cleavage (Fig. 5A), as well as caspase activation analysis and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Supplementary Fig. S2). However, no changes in these apoptotic markers were observed for CX-5461, with the exception of very modest cleavage of PARP after 48 hr treatment with 1 \( \mu \)M of CX-5461 in MIA PaCa-2 (Fig. 5A). These data indicated that apoptosis is not the primary pathway through which CX-5461 affects cellular viability. In addition, no evidence of necrotic cell death was detected, as measured by lactate dehydrogenase (LDH) release as late as 48 hours after the initiation of treatment (data not shown). These data led us to consider the possibility that CX-5461 might be inducing autophagy to kill these solid tumor cell lines.

Using immunofluorescence cytometry to monitor the production of LC3B-II, a known marker of autophagy (20), we observed that a 24 hour treatment with 300 nM CX-5461 caused a significant increase from 15 ± 3% to 67 ± 2% in LC3B-II staining (\( P = 0.0041 \)) in A375 and from 19 ± 2% to 62 ± 3% in LC3B-II staining (\( P = 0.002 \)) in MIA PaCa-2.
PaCa-2 cells (Fig. 5B). In addition, CX-5461 promoted a concentration-dependent increase in acidic vesicular organelles (AVO) including autophagolysosomes, another marker of autophagic response (14, 21) (Fig. 5C, see Supplementary Materials and Methods). To exclude the possibility that induction of autophagy by CX-5461 resulted from a decrease in the protein translation rate, we performed a time-course analyses to monitor the effects of CX-5461 on protein synthesis in A375 and MIA PaCa-2 cells (Supplementary Fig. S3). While twenty-four hours treatment of A375 or MIA PaCa-2 cells with CX-5461 dramatically reduced Pol I transcription (Fig. 3D; data not shown), it had only a minor effect on overall protein synthesis (13-19% reduction at 300 nM and 24-27% reduction at 1 μM). Several reports that have investigated the effects of overall protein synthesis inhibition on the viability of cancer cells demonstrated that such a reduction (i.e. < 30%) should not have a significant effect on cell viability (22). As autophagic cells can themselves have reduced protein synthesis rate (23), the observed effect of CX-5461 on protein synthesis is likely secondary to the induction of autophagy and not vice versa.

Several recent reports link autophagy to the induction of cellular senescence (24). The analysis of senescence-associated marker β-galactosidase demonstrated that, in addition to autophagy, CX-5461 is able to induce senescence in both cell lines. Treatment with 300 nM CX-5461 for 24 hours caused an increase in β-galactosidase staining from 6 ± 1% to 79 ± 1% (P < 0.0001) in A375 cells and from 14 ± 2% to 55 ± 3% (P < 0.0001) in MIA PaCa-2 cells (Fig. 6). Together, the mechanistic data demonstrate that treatment of solid tumor cell lines with CX-5461 induces cellular senescence and autophagy through selective inhibition of rRNA synthesis.
**In vivo anti-tumor activity of CX-5461.** The antitumor activity of CX-5461 was evaluated in two murine xenograft models of human cancers, pancreatic carcinoma (MIA PaCa-2) and melanoma (A375). In these xenograft models, CX-5461 was administered orally (50 mg/kg) either once daily or every three days. Untreated animals received vehicle orally on the equivalent schedule, while the positive control gemcitabine was administered intraperitoneally once every three days at 120 mg/kg. CX-5461 demonstrated significant MIA PaCa-2 tumor growth inhibition with TGI equal to 69% on day 31 (Fig. 7A), comparable to that of gemcitabine (63% TGI). Likewise, CX-5461 demonstrated significant A375 tumor growth inhibition (Fig. 7B) with TGI equal to 79% on day 32. Unpaired t test revealed statistically significant differences between the vehicle-treated and CX-5461-treated groups throughout both studies. CX-5461 was well tolerated at all tested schedules as judged by the absence of significant changes in animal body weights. These data demonstrate that a selective inhibitor of Pol I transcription can produce *in vivo* antitumor responses against solid tumors, with a favorable therapeutic window.
Discussion

Deregulated rRNA synthesis plays a fundamental role in tumorigenesis (reviewed in (1-5)), and Pol I is the key regulatory enzyme required for production of rRNA (reviewed in (25-27)). Herein we present the results from a discovery program that identified CX-5461, a small molecule that selectively inhibits Pol I-driven transcription of rRNA but does not inhibit Pol II-driven mRNA synthesis or DNA or protein synthesis. CX-5461 must be viewed globally as targeting a specific transcriptional event. A host of currently marketed drugs target transcription, including tamoxifen, cyclosporins, salicylates and others (28). In the case of CX-5461, it disrupts the binding of the SL1 transcription factor to rDNA promoter, which inhibits initiation of rRNA synthesis by the Pol I multi-protein complex. The inhibition of rRNA synthesis leads to induction of senescence and autophagy in a p53-independent manner in solid tumor cell lines. This response is not driven by reductions in ribosomes or protein synthesis, as the cancer cell induces the death pathway signaling long before any reduction in ribosomes or protein content can occur (29).

SL1, a protein complex containing TATA binding protein-associated factors, is responsible for Pol I promoter specificity (27, 30, 31). SL1 performs important tasks in transcription complex assembly, mediating specific interactions between the rDNA promoter region and the Pol I enzyme complex, thereby recruiting Pol I, together with a collection of Pol I-associated factors, to rDNA. Our data indicate that disruption of the SL1/rDNA complex by CX-5461 results from the interference between SL1 and rDNA and not through dissociation of protein-protein interactions, as was shown for several known tumor-suppressors (p53, Rb, PTEN) (32-34).
Numerous reports have linked the inhibition of rRNA synthesis to induction of apoptosis. Actinomycin D, a relatively selective inhibitor of rRNA synthesis, inhibits Pol I transcription during the elongation step (35) and induces apoptosis in cancer cell lines (36). Inhibition of Pol I transcription at the elongation step leads to the formation of truncated pre-rRNA, which can be interpreted by the cell as breaks in rDNA. The cell attempts to repair such breaks and ultimately triggers the apoptotic response (37). In contrast, CX-5461 inhibits Pol I transcription prior to/during the PIC formation step and induces autophagy in solid tumor cell lines. When Pol I transcription is inhibited at the initiation step, cells may interpret this as decreased nutrient availability. The process of autophagy is known to be used by cancer cells to survive times of limited nutrient availability, but when driven to the extreme it causes cell death (38). Cancer cells are continuously subjected to autophagy inducing stress (for example, unfolded protein response, (reviewed in (39)), and this may prime them to become more susceptible to certain types of metabolic stress, such as inhibition of rRNA synthesis. This may be one of the reasons why rapamycin that targets mTOR, a protein kinase that serves as a sensor for nutrient availability and in turn controls both rRNA as well as protein synthesis (40), can also induce autophagy (reviewed in (41)). Likewise, this might illustrate how CX-5461 takes advantage of the unique qualities of cancer cells and elicits potent and selective antitumor activity, while having minimal effect on non-transformed cells.

CX-5461 exhibited a broad spectrum antiproliferative activity of cancer cells in vitro, while having a lesser effect on the viability of non-transformed cells, indicating that certain cancer cells are significantly more susceptible to inhibition of rRNA synthesis than non-transformed cells. Further, we analyzed if sensitivity of CX-5461 correlates
with the \( p53 \) mutational status of cells, as inhibition of Pol I was previously reported to cause nucleolar stress that leads to stabilization of \( p53 \) and induction of \( p53 \)-dependent apoptosis (17). Across all cell lines, CX-5461 exhibited similar sensitivity in wt \( p53 \) and mutant \( p53 \) cell lines. However, wt \( p53 \) cell lines derived from hematologic malignancies appeared highly sensitive to CX-5461 and this apparent trend is the focus of additional investigations.

Herein we present the results of a discovery and development program that identified CX-5461, a small molecule that selectively inhibits Pol I transcription of rRNA. CX-5461 does not inhibit Pol II transcription of mRNA or inhibit the synthesis of DNA or proteins. CX-5461 targets the SL1 transcription factor of the Pol I complex and induces autophagy and senescence among solid tumor cell lines and selectively kills cancer cells relative to normal cells. Studies with human solid tumors grown in murine xenograft models revealed that CX-5461 can be orally administered with favorable pharmacokinetics and an antitumor efficacy without changes in body weight or overt toxicity. In addition, our data demonstrate that the anticancer activity of CX-5461 against solid tumors does not depend on the \( p53 \) pathway that is frequently mutated in many types of cancer. Thus, CX-5461 represents a first-in-class oral small molecule therapeutic agent that selectively targets Pol I transcription and induces autophagy in solid tumors.

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References


Figure Legends

Figure 1. CX-5461 selectively inhibits rRNA synthesis. (A) Structure of CX-5461. (B) Inhibition of Pol I (pre-rRNA) versus Pol II (c-Myc mRNA) transcription in HCT-116 cells by CX-5461. (C) qRT-PCR analysis of rRNA, c-myc mRNA and β-actin mRNA transcription after 2 hr treatment with CX-5461 in A375 and MIA PaCa-2 cells. (C) Order of addition study setup and results.

Figure 2. CX-5461 selectively competes SL1 from the Pol I promoter on rDNA. (A) ChiP analysis of Pol I associations with Pol I promoter in HCT-116, A375 and MIA PaCa-2 cells. (B) ChiP analysis of Pol I transcription factors (TF) SL1 (TBP, TAFI110, TAFI63) and UBF associations with Pol I promoter in HCT-116 cells. (C) ChiP analysis of TBP associations with Pol I, histone H2B and p21 promoters in HCT-116 cells. (D) EMSA of SL1-rDNA complex in presence of increasing concentrations of CX-5461 and CX-5447.

Figure 3. CX-5461 inhibits Pol I transcription without having an effect on DNA replication, protein translation and general cellular transcription. (A) Effect of CX-5461 on total DNA and protein synthesis in HCT-116, A375 and MIA PaCa-2 cells. Histogram showing the proportion of genes that were up- or down-regulated upon treatment of (B) MIA PaCa-2 or (C) A375 cells with 300nM of CX-5461 for 1 hour. Each column represents a bin of 0.25 units (log2 change in gene expression) and its height is proportional to the number of genes showing such a change in detected mRNA levels. (D) Comparative analysis of the effects of CX-5461 and actinomycin D on Pol I- and Pol II-driven transcription.
Figure 4. **CX-5461 exhibits broad anti-proliferative potency in a panel of cancer cell lines in p53-independent manner, but has minimal effect on viability of non-transformed human cells.** (A) Panel of cancer and normal cell lines were treated with various doses of CX-5461 for 96 hours and the resulting effects on cell viability were measured with CyQUANT assay. (B) Effect of CX-5461 on cell viability and Pol I transcription of HCT-116, A375, MIA PaCa-2 and BJ-hTERT cells. (C) Effect of CX-5461 on p53 stabilization in A375 cells. (D) CX-5461 exhibit similar activity against p53 wild type and p53 mutant solid cancer cell lines.

Figure 5. **CX-5461 induces autophagy but not apoptosis in A375 and MIA Paca-2 cancer cells.** (A) A375 and MIA PaCa-2 cells were treated with various doses of CX-5461 for 24 hours and the induction of apoptosis was measured by monitoring activation of caspases-3/7/8/9 and PARP cleavage. Actinomycin D (Act D) was used as a positive control. (B) A375 and MIA PaCa-2 cells were treated with 300 nM of CX-5461 for 24 hours and the induction of autophagy was measured by detection of LC3B-II using fluorescent microscopy. (C) A375 and MIA PaCa-2 cells were treated with a dose-response of CX-5461 for 24 or 48 hours and the induction of autophagy was measured by acridine orange staining followed by flow cytometry. Statistically significant differences from UTC are marked by asterisks (* indicates P < 0.05; ** indicates P < 0.01).

Figure 6. **CX-5461 induces senescence in A375 and MIA Paca-2 cancer cells.** A375 and MIA PaCa-2 cells were treated with 300 nM of CX-5461 for 24 hours and the induction of senescence was measured by β-galactosidase staining.

Figure 7. **CX-5461 demonstrates in vivo anticancer activity.** (A) MIA PaCa-2 human pancreatic cancer and (B) A375 human melanoma xenograft models in which mice were
treated with vehicle, CX-5461 or gemcitabine. The resulting changes in tumor volumes and body weights are demonstrated. Statistical significance in tumor volume between vehicle-treated and CX-5461 treated groups is marked by asterisks (* indicates P < 0.05; ** indicates P < 0.01).
Figure 1
Figure 2

(A) UTC and CX-5461 Relative Pol I ChIP Recovery

(B) UTC and CX-5461 Relative TF ChIP recovery

(C) UTC and CX-5461 Relative TBP ChIP recovery

(D) DMSO, CX-5461, and CX-5447 at different concentrations (% Untreated)

Figure 2
Figure 3
Figure 7
Targeting RNA Polymerase I with an Oral Small Molecule CX-5461 Inhibits Ribosomal RNA Synthesis and Solid Tumor Growth

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