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 (Pol) I, the multiprotein complex that synthesizes rRNA, is activated widely in cancer. Thus, selective inhibitors of 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 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 ref. 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 refs. 2–5). Indeed, an increase in the synthesis of rRNA, which is transcribed in the nucleolus by RNA polymerase (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 multiprotein 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 quantitative reverse-transcription PCR (qRT-PCR) assay that differentiates between the effects of compounds on Pol I- and Pol II–driven transcription. In this article, 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 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 mmol/L stock solutions in

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50 mmol/L NaH2PO4 (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. 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. Other cell lines were purchased from American Type Culture Collection. All cell lines were used within 5 to 10 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 mmol/L Tris-HCl, pH 8.0, 80 mmol/L KCl, 0.8% polyvinyl alcohol, 10 mg/mL α-amanitin was combined with different amounts of test compounds and incubated at ambient for 20 minutes. Transcription was initiated by addition of rNTP mix (New England Biolabs) to a final concentration of 1 mmol/L and was incubated for 1 hour at 30°C. Afterward, DNase I was added and the reaction was further incubated for 2 hours at 37°C. DNase digestion was terminated by the addition of EDTA to final concentration of 10 mmol/L, followed immediately by 10-minute 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).

Chromatin immunoprecipitation

Cells were treated with 2 μmol/L of CX-5461 for 1 hour and chromatin immunoprecipitation (ChIP) 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 prHu3 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 mmol/L of DNA probe and 0.6 μg SL1 complex was incubated in binding buffer for 15 minutes at ambient temperature with 0.4 to 12.5 μmol/L CX-5461 or CX-5447. The resulting complexes were resolved using Novex TBE DNA retardation PAGE (Invitrogen). The gels were dried and exposed to X-ray film (Kodak).

Gene expression analysis

For MIA PaCa-2 cells, gene array analyses were performed at Expression Analysis 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 SABioscences.

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).

Detection and quantification of acidic vesicular organelles 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). 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).

Immunocytochemistry-based senescence detection assay

The assay was performed using Senescence Detection Kit (Calbiochem) according to manufacturer’s instructions.

In vivo efficacy in murine xenograft model

Animal experiments were performed with 5- to 6-week-old female athymic (Ncr nu/nu fisol) mice of Balb/c origin (Taconic Farms) 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 5 × 106 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 \( V = \frac{w^2}{2} \), where \( w \) = width and \( l \) = length in mm of the tumor. Established tumors (~110–120 mm³) were randomized into vehicle (50 mmol/L NaH2PO4, 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: \( \text{TGI} \% = \frac{[100 - (V_i^\text{f} - V_i^\text{V})/(V_i^\text{f} - V_i^\text{V}) \times 100]}{\text{V}_{i\text{V}}} \), where \( V_i^\text{f} \) is the initial mean tumor volume in vehicle-treated group, \( V_i^\text{f} \) is the final mean tumor volume in drug-treated group, and \( V_i^\text{V} \) is the initial 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 protooncogene 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 greater than 90% if a drug affects their synthesis.

Among numerous molecules screened, CX-5461 (Fig. 1A) was found to selectively inhibit rRNA synthesis (Pol IC₅₀ = 142 nmol/L; Pol II IC₅₀ > 25 μmol/L; 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 IC₅₀ = 113 nmol/L; Pol II IC₅₀ > 25 μmol/L) and pancreatic carcinoma MIA PaCa-2 (Pol I IC₅₀ = 54 nmol/L; Pol II IC₅₀ ~ 25 μmol/L; 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 preincubated with either nuclear extract (NE) or with DNA template (Template) prior to the preinitiation 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 RNA transcripts by qRT-PCR (Fig. 1D) revealed that addition of 80 nmol/L CX-5461 prior to PIC formation resulted in considerable inhibition of Pol I transcription, whereas 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 multiprotein complex with the rDNA promoter. Treatment of HCT-116, A375, or MIA PaCa-2 with 2 μmol/L CX-5461 resulted in 40% to 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 overly 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).

Because 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 with the Pol I promoter on rDNA relative to its binding to the promoter of 2 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 (Fig. 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 analogue of CX-5461, CX-5447, that is inactive against Pol I transcription (IC₅₀ > 25 μmol/L). Although 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, as 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 preincubated with 10 μmol/L CX-5461 for 60 minutes and pulsed for an additional 120 minutes with modified precursors BrdU or 35S-methionine. Mitoxantrone and cycloheximide served as positive controls for inhibition of DNA and protein synthesis, respectively. At this concentration, which is approximately 100-times higher than its IC₅₀ 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 IC₅₀ for inhibition of DNA synthesis in A375 and MIA PaCa-2 cell lines ranged from 16.8 to 27.9 μmol/L, whereas a high-test dose of 30 μmol/L CX-5461 had no significant effect on proteins synthesis (Supplementary Fig. S1). Thus, CX-5461 possesses 250- to 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 nmol/L CX-5461 (that resulted in 63% and 55% reductions in pre-rRNA, respectively), an equal number of Pol II–transcribed genes were significantly upregulated as were downregulated. 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 versus Pol II transcription, we compared it with another drug (actinomycin D) known to inhibit Pol I transcription. Treatment of MIA PaCa-2 cells with either 1 μmol/L CX-5461 or 1 μmol/L 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
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-hour treatment with CX-5461 in A375 and MIA PaCa-2 cells. D, order of addition study setup and results.
transcription as well, as judged by the depletion of c-myc mRNA (half-life = 35 minutes), whereas 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 5 nontransformed cell lines.

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*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 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. UTC, untreated control.*
The EC50 values ranged from 3 nmol/L against the EOL-1 eosinophilic leukemia cell line to 5,500 nmol/L against the LNCaP prostate carcinoma cell line (Fig. 4A). The median EC50 across all tested cell lines was 147 nmol/L, yet all normal cell lines had EC50 values of approximately 5,000 nmol/L. Evaluation of the antiproliferative dose response (Fig. 4B, left) for HCT-116, A375, and MIA PaCa-2 cell lines yielded EC50 values of 167, 58, and 74 nmol/L, respectively. In contrast, an EC50 of approximately 5,000 nmol/L was observed for the BJ-hTert normal cell line (nontransformed hTERT-immortalized human foreskin fibroblasts). The lesser sensitivity of the normal cells was not due to a reduced uptake of CX-5461, as the IC50 for rRNA synthesis for the BJ-hTert normal cell line of 74 nmol/L correlated well with the IC50 values of the solid tumor cell lines (IC50 = 142, 113, and 54 nmol/L, respectively; see Fig. 4B, right). These findings illustrate that CX-5461 can...
CX-5461 exhibits broad antiproliferative potency in a panel of cancer cell lines in \( p53 \)-independent manner, but has minimal effect on viability of nontransformed 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 \( p53 \) wt and \( p53 \) mutant solid cancer cell lines.
equivalently inhibit Pol I transcription in cancer and normal cells, but the normal cells can tolerate reductions in rRNA 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 wild-type (wt) p53 A375 cells. As shown in Figure 4C, whereas CX-5461 slightly increased p53 levels, it did so at concentrations 3- to 10-fold higher than its IC50 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 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 wt p53 cell lines (median IC50 = 144 nmol/L) as it does in cell lines with mutant p53 (median IC50 = 138 nmol/L), P = 0.56 (Fig. 4D). Interestingly, the 3 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 wt p53 (3 cell lines, median IC50 = 5 nmol/L) and those with mutant p53 (8 cell lines, median IC50 = 94 nmol/L). Although the sample size is too limited to make strong conclusions, it does hint at an intriguing possibility that nucleolar stress signaling in hematologic cancers is more dependent on p53 function and is worthy of future investigation.

**CX-5461 induces autophagy and senescence in solid tumor 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 analyzed 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-hour treatment with 1 μmol/L of CX-5461 in Mia PaCa-2 (Fig. 5A). These data indicated that apoptosis is not the primary pathway through which CX-5461 affected 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 nmol/L 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 cells (Fig. 5B). In addition, CX-5461 promoted a concentration-dependent increase in acidic vesicular organelles (AVO) including autophagosomes, another marker of autophagic response (ref.s. 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). Although 24-hour 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 nmol/L and 24%–27% reduction at 1 μmol/L). 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 nmol/L 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 antitumor 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 3 days. Untreated animals received vehicle orally on the equivalent schedule, whereas the positive control gemcitabine was administered intraperitoneally once every 3 days at 120 mg/kg. CX-5461 demonstrated significant Mia PaCa-2 TGI with TGI equal to 69% on day 31 (Fig. 7A), comparable to that of gemcitabine (63% TGI). Likewise, CX-5461 demonstrated significant A375 TGI (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.
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 nmol/L 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 untreated control (UTC) are marked by asterisks (unpaired two-tailed t-test; *, P < 0.05; **, P < 0.01; ***, P < 0.001).
Discussion

Deregulated rRNA synthesis plays a fundamental role in tumorigenesis (reviewed in refs. 1–5), and Pol I is the key regulatory enzyme required for production of rRNA (reviewed in refs. 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 multiprotein 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; refs. 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 (e.g., unfolded protein response; reviewed in ref. 39), and this may prime them to become more susceptible to certain types of metabolic stress such as inhibition of RNA 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 ref. 41). Likewise,
Figure 7. CX-5461 demonstrates in vivo anticancer activity. MIA PaCa-2 human pancreatic cancer (A) and A375 human melanoma (B) 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 (*, \( P < 0.05 \); **, \( P < 0.01 \)).
this might illustrate how CX-5461 takes advantage of the unique qualities of cancer cells and elicits potent and selective antitumor activity, whereas having minimal effect on nontransformed cells.

CX-5461 exhibited a broad-spectrum antiproliferative activity of cancer cells in vitro, whereas having a lesser effect on the viability of nontransformed cells, indicating that certain cancer cells are significantly more susceptible to inhibition of rRNA synthesis than nontransformed 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.

Disclosure of Potential Conflicts of Interest


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