A novel small molecule with potent anticancer activity inhibits cell growth by modulating intracellular labile zinc homeostasis

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Abstract
ML-133 is a novel small molecule with potent antiproliferative activity, as shown in cancer cell lines and in a human colon tumor xenograft model. ML-133 reduces the concentration of intracellular labile zinc in HT-29 colon cancer cells, leading to induction of the Krüppel-like factor 4 transcription factor. Krüppel-like factor 4 displaces the positive regulator SP1 from the cyclin D1 promoter, thereby negatively regulating the expression of cyclin D1 and promoting the G1-S phase arrest of cell proliferation. The antiproliferative and antitumor activity of ML-133 described in the present study suggests modulation of intracellular zinc homeostasis as a potential strategy for the treatment of several cancer types, and ML-133 represents a promising new class of antitumor agents that deserves further development. [Mol Cancer Ther 2009;8(9):2586–96]

Introduction
Zinc has regulatory and structural functions in a large number of enzymes and transcription factors. The structural functions involve a highly stable association of zinc to folded protein domains, whereas a more dynamic, exchangeable labile zinc pool is involved in the regulatory functions (1). Intracellular zinc homeostasis is regulated by sensor proteins, such as the metal-responsive transcription factor 1, which regulates the transcription of zinc-sensitive genes, including membrane transporter proteins, involved in the cellular and vesicular influx and efflux of zinc, and metallothionein and thionein, which play an important role in the storage and distribution of intracellular zinc (2). Although labile zinc has been shown to be involved in several cellular pathways related to the regulation of cell fate, these mechanisms are not well characterized (3). Reduction of intracellular labile zinc has been associated with the induction of apoptosis, decreased cell proliferation, and altered cell cycle progression in a number of cancer cell types, including mammary adenocarcinoma (4), melanoma (5), colon adenocarcinoma (6), and lymphocytic leukemia (7–10).

Studies of zinc-responsive gene regulation induced by intracellular labile zinc depletion in colon carcinoma HT-29 cells identified Krüppel-like factor 4 (KLF4, also known as GKLF) as one of the genes whose expression is most significantly changed (up-regulated) among >10,000 target genes tested (6). KLFs are members of the SP/XKLF family of transcription factors defined by an amino acid binding domain at the C termini that comprises three C2H2-type zinc fingers with similarity to the developmental gene Krüppel of Drosophila melanogaster (11). KLFs play an important role in mammalian morphogenesis by controlling the proliferation and/or differentiation of distinct cell lineages (12). The expression and function of KLFs are relatively tissue restricted (11), with KLF4 mainly expressed in epithelial cells of the gastrointestinal tract, lung, testis, and skin, with a functional role in skin barrier and gastric epithelial homeostasis (13), and development (14).

KLF4 mRNA is significantly reduced in colorectal cancer compared with normal matched tissues (15), and induction of KLF4 expression in a colorectal cancer cell line results in diminished tumorigenicity (16). Furthermore, overexpression of KLF4 causes cell cycle arrest at G1-S transition in RKO human colon carcinoma cells (7). In addition, KLF4 is down-regulated in adenomas from the APCmin mice model of colorectal cancer, and crossing APCmin mice with KLF4+/- heterozygotes resulted in significantly more adenomas than in APCmin mice alone (17). Taken together, these results indicate a role of KLF4 as tumor suppressor factor in colon cancer. A similar function for KLF4 has also been reported in bladder cancer (18), gastric cancer (19), esophageal cancer (20), pancreatic cancer (21), and adult T-cell leukemia (22).

Here we present the characterization of the anticancer activity of the compound ML-133, selected from a novel series of 2-indolyl imidazo[4,5-d] phenanthroline derivatives with metal chelation activity that exhibits a potent and selective antitumor activity against multiple cancer cell types (23). ML-133 reduces the concentration of intracellular labile zinc in HT-29 colon cancer cells, leading to the induction of KLF4 expression. KLF4 displaces the positive regulator SP1 from the cyclin D1 promoter, thereby negatively regulating the expression of cyclin D1 and promoting the arrest of cell proliferation.
Materials and Methods

Chemical Synthesis
ML-133 was synthesized as described elsewhere (23).

In vitro Cell Line Cancer Screen
To evaluate the potential antitumor activity of ML-133 and to prioritize the selective activity on particular types of tumor cell lines, the antiproliferative activity of ML-133 was tested by the in vitro cell-line cancer screen at the National Cancer Institute (NCI; ref. 24). The detailed method is described at the NCI Developmental Therapeutics Program website.1

In vivo Hollow Fiber Assay
To assess the initial drug efficacy of ML-133 in vivo, the activity of ML-133 was tested by NCI hollow fiber assay (25) on a panel of 12 tumor cell lines; (breast: MDA-MB-231, MDA-MB-435; glioma: U251, SF-295; ovarian: OVCAR-3, OVCAR-5; colon: COLO-205, SW-620; lung: NCI-H23, NCI-H522). The detailed method is described at the NCI Developmental Therapeutics Program website.2

Cell Culture Maintenance
HT-29 colon carcinoma cell line was purchased from ATCC (Manassas, VA) and maintained in McCoy’s 5A modified 1× medium (Sigma, Oakville, Ontario, Canada), supplemented with 2 mmol/L l-glutamine (Gibco, Grand Island, NY), 10% fetal bovine serum (Multicell, Wisent Inc., St-Bruno, Quebec, Canada), and antibiotic-antimycotic solution (Multicell) at 37°C in a 5% CO2-humidified incubator.

Cell Proliferation Inhibition Assay
Cells (2 × 10^5/well) in 100 μL of growth medium were seeded in 96-well cell culture plates and incubated overnight at 37°C. The medium was removed and replaced with a total volume of 100 μL growth medium containing indicated concentrations of ML-133 or metal supplements, or 0.1% DMSO vehicle control, as described in respective experiments. After incubation of the cells at 37°C for 5 d, cell viability was quantitated with the use of sodium 3’-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) colorimetric assay (Roche Applied Science, Penzberg, Germany). XTT labeling reagent (1 mg/mL) was mixed with electron-coupling reagent, following the manufacturer’s instructions, and 50 μL of the mixture was added directly to the cells. The plates were further incubated at 37°C for 4 h, and the absorbance of each well was measured at 490 nm with a multiwell spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT) following the manufacturer’s instructions and absorbance was measured at 450 nm. Results are expressed as percentage SOD activity relative to DMSO control.

RNA Preparation and Real-time PCR
Total RNA from HT-29 cells or HT-29 tumor xenografts was extracted with the use of TRIzol (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer’s instructions. First-strand cDNA was synthesized from 200 ng total RNA in a Biometra Tpersonal Thermal Cycler (Abgene, Epsom, United Kingdom), with the use of pd(N) 6 random hexamer (Amersham Biosciences, Piscataway, NJ) and the SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer’s protocol. Real-time PCR was done with the ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc., Foster City, CA) with the use of 5 μL of cDNA synthesized by the abovementioned procedure and with respective human TaqMan Gene Expression Assays (ActB; KLF4, Hs00358836_m1; cyclin D1, Hs00277039_m1; transferrin receptor C, Hs00174609; Sp1, Hs00412720_m1) by following the ABITaqMan Universal PCR Master Mix protocol. Alteration in the respective gene expression was normalized with β-actin gene expression in the same sample with the use of the comparative cycle threshold method. Fold changes in the respective genes were expressed relative to the corresponding gene level of the indicated control, as described in respective experiments.

Cell Cycle Analysis by Flow Cytometry
HT-29 cells (1 × 10^6) in 10 mL of growth medium were seeded in 100-mm dishes and incubated overnight at 37°C. Cells were treated with the indicated concentrations of ML-133 or 0.1% DMSO vehicle control, followed by incubation with zinquin (Biotium Inc., Hayward, CA) at 30 μmol/L final concentration at room temperature for 30 min before the measurement of zinquin-Zn complex fluorescence. The cell suspensions in triplicates were transferred to a 96-well plate (Corning #3603), and the fluorescence count was measured in a Fluoroskan Ascent luminescence spectrophotometer (Thermo Electron Corporation, Vantaa, Finland) at 355 nm excitation and 485 nm emission wavelengths.

Cu/Zn Superoxide Dismutase Assay
HT-29 cells were seeded in 6-well dishes (2.5 × 10^5 cells per well) and incubated overnight. The culture medium was removed and replaced with growth medium containing the indicated concentrations of ML-133, the copper-specific chelator 2,3,2-tetramine, or 0.1% DMSO vehicle control. After 24 h, cells were lysed in 200 μL of cold lysis buffer (20 mmol/L HEPES, pH 7.2, containing 1 mmol/L EGTA, 210 mmol/L mannitol, 70 mmol/L sucrose) and centrifuged at 1, 500 × g for 5 min at 4°C. The supernatant was then centrifuged at 10,000 × g for 15 min at 4°C to separate Cu/Zn superoxide dismutase (SOD; cytosolic SOD) from Mn SOD (mitochondrial SOD). Protein concentration was determined by Bradford assay (Bio-Rad), and samples were diluted to 10 μL with sample buffer from the SOD assay kit (Cayman Chemical, Ann Arbor, MI). Assay was done according to the manufacturer’s instructions, and absorbance was measured at 450 nm. Results are expressed as percentage SOD activity relative to DMSO control.

Intracellular Zinc Measurement by Zinquin Fluorescence Assay
HT-29 cells were harvested by trypsinization, and 8.0 × 10^6 cells in 1 mL volume of PBS were aliquoted into Eppendorf tubes. The cells were incubated at room temperature for 30 min with indicated concentrations of ML-133 or 0.1% DMSO vehicle control, followed by incubation with zinquin (Biotium Inc., Hayward, CA) at 30 μmol/L final concentration at room temperature for 30 min before the measurement of zinquin-Zn complex fluorescence. The cell suspensions in triplicates were transferred to a 96-well plate (Corning #3603), and the fluorescence count was measured in a Fluoroskan Ascent luminescence spectrophotometer (Thermo Electron Corporation, Vantaa, Finland) at 355 nm excitation and 485 nm emission wavelengths.

1 http://dtp.nci.nih.gov
2 http://dtp.nci.nih.gov
collected by centrifugation at 1,000 g for 4 min, washed once with PBS, and fixed in 70% ethanol at -20°C for 4 h. The fixed cells were centrifuged at 800 g for 3 min, washed once with cold PBS containing 2% fetal bovine serum, and treated with 3 mg/mL ribonuclease (Sigma) and 50 μg/mL propidium iodide (Sigma) for 30 min at 37°C. The fluorescence counts of the stained cells were analyzed with the use of a FACScan flow cytometer and the CellQuest program (BD Biosciences, San Jose, CA). Data were analyzed with the use of Modfit software (Verity Software House, Topsham, ME).

**SDS-PAGE and Western Blot Analysis**

Whole cell protein extract was prepared from HT-29 cells (5.5 × 10^5 cells in 35-mm culture dishes) in lysis buffer (50 mmol/L HEPES, pH 8.0, 0.5% Triton X-100, 150 mmol/L NaCl, 10% glycerol, 2 mmol/L EGTA, 1.5 mmol/L MgCl_2). Extracted proteins (10 μg/lane) were resolved on 12% SDS-PAGE and transferred to nitrocellulose membranes. The following antibodies were used: anti-cyclin D1 rabbit monoclonal antibody (Lab Vision, Fremont, CA; 1:1,000), anti-KLF4 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:500), anti-Sp1 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:500), and anti-glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibody (Biosign International, Saco, ME; 1:10,000), followed by a 1:2,000 dilution of donkey anti-rabbit or 1:20,000 dilution of goat anti-mouse horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences, Arlington Heights, IL), respectively, and visualized with the use of the ECL Plus Western blotting detection system (Amersham Biosciences).

**Chromatin Immunoprecipitation**

Cell lysates from HT-29 cells grown in three 15-cm culture plates were prepared at the end of the indicated experiments, and chromatin immunoprecipitation assays were done with the use of anti-Sp1 or anti-KLF4 antibodies (Santa Cruz Biotechnology Inc.) and a ChIP-IT kit (Active Motif, Carlsbad, CA) by following the manufacturer’s instructions. The primers were synthesized at Invitrogen and encompass the -231 to -92 region of the cyclin D1 promoter: 5′-CGGACTACAGGGGCAA-3′ and 3′ primer (5′-GCTCAGGACGTITCCA-3′).

**Small Interfering RNA Transfection**

Predesigned KLF4 siRNA (ID #115492) was from Ambion (Austin, TX), whereas the nonspecific double-stranded RNA [5′r(CUAGGGUAGACGAUGAG)d(TT)3′] and [3′d(TT) r(GAUCCCAUGCGCUACUCU)5′] were synthesized at Qagen (Cambridge, MA) based on the sequence of an unrelated gene. HT-29 cells were transfected with indicated concentrations of small interfering RNA with the use of Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. At the end of the incubation period, the transfection medium was supplemented with a complete growth medium, and the cells were incubated at 37°C for 24 h before the indicated experiments. For the measurement of gene expression by real-time PCR, the cells (3 × 10^5 cells in 35-mm culture dishes) were transfected with 100 nmol/L small interfering RNA for 6 h, and RNA was extracted at the end of 24-h incubation in a complete medium. For the cell proliferation experiment, ML-133 or DMSO vehicle control was added to the cells (2 × 10^3 cells per well in 96-well plates) at the end of 24-h incubation in a complete medium, following 6-h transfection with 100 nmol/L small interfering RNA. The experiment was stopped 3 d later.

**KLF4 Overexpression**

HT-29 cells were transfected with either KLF4 expression plasmid (Origene, Rockville, MD) or empty vector (0.8 μg/1 × 10^4 cells) with the use of Lipofectamine (Gibco) according to the manufacturer’s instructions. Twenty-four hours post transfection, cells were either lysed for Western blot, or treated with 1 μmol/L ML-133 or DMSO for 4 d, followed by cell proliferation inhibition assay (measured by XTT assay).

**In vivo Antitumor Activity in HT-29 Xenograft Mouse Model**

CD-1 athymic nude mice (four per group) were injected s.c. with HT-29 cells (3 × 10^5 cells in 0.1 mL PBS). At 5 d after tumor cell inoculation, the mice were treated i.p. or p.o. with 200 μL vehicle control or 100 mg/kg ML-133 for 5 days, followed by a 10-d interval, for two cycles (days 5 to 9 as the first cycle and days 20 to 24 as the second cycle). The tumor size was measured with the use of calipers during the course of treatment. The mice were sacrificed at 34 d after tumor cell inoculation. The tumor tissues were excised, frozen immediately, and stored at -80°C until RNA extraction. Gene expression studies in xenografted tumors were done after 5-d treatment with ML-133.

**In vitro 4-(2-Pyridylazo)Resorcinol Zinc Binding Assay**

ZnCl_2 or CuCl_2 (final concentration of 10 μmol/L) was incubated with the indicated concentrations of ML-133, EDTA, EGTA, N,N,N’N’-tetrais[2-pyridylmethyl]-ethylene-diamine, or 2,3,2-tetramine in a total volume of 100 μl for 15 min in 0.2 mol/L Tris-HCl (pH 7.5), followed by the addition of 4-(2-pyridylazo)resorcinol (Sigma; 100 μmol/L final concentration). The color development of 4-(2-pyridylazo)resorcinol–metal ion complex was measured at 500 nm with the use of a multiwell spectrophotometer (Bio-Tek Instruments Inc.; ref. 26).

**Statistical Analysis**

All data in quantitative assays represent the mean ± SD from triplicate samples. The data are representatives of at least three independent experiments, and were analyzed by two-tailed Student’s t tests. Differences were considered statistically significant at P < 0.05.

**Results**

**Compound ML-133 Exhibits In vitro and In vivo Tumor Growth Inhibition**

1H-imidazol [4,5-f][1,10] phenanthroline, 2-(2-methyl-1H-indol-3-yl) (ML-133; Fig. 1A) was selected from a library of novel compounds based on its potent and selective antiproliferative effects against particular cancer types (23). ML-133 showed consistent growth inhibition of colon, leukemia, non–small cell lung, renal, and prostate cancer cell lines in a screening test of 50 cell lines done at the NCI Developmental
Therapeutic Program, with average growth inhibition by 50% values of 0.33, 0.75, 0.74, 0.93, and 0.13 μmol/L, respectively (Fig. 1B). The anticancer activity of ML-133 was also shown by the NCI hollow fiber assay, a solid tumor efficacy model based on the cell growth of 12 human tumor cell lines encased in biocompatible hollow fibers implanted in mice (25). This method was statistically validated with the use of a “training set” of standard anticancer compounds to represent the score achieved by clinically used anticancer agents. ML-133 produced a total growth inhibition score of 32 (compounds with a total score of ≥20 are considered significantly active), also showing a positive cytocidal effect. Furthermore, ML-133 showed in vivo antitumor activity in a human colon carcinoma (HT-29) xenograft model (Fig. 1C) when it was given p.o. or i.p. into athymic nude mice, with 71% (P = 0.0032) and 69% (P = 0.007) tumor growth inhibition, respectively.

ML-133 Preferentially Chelates Labile Zinc In vivo

As with other 1,10-phenanthroline–containing compounds (27), 2-indolyl imidazol [1,10] phenanthroline derivatives exhibit metal chelation properties. To assess whether metal chelation plays a role in ML-133–mediated...
cell growth inhibition, HT-29 cells were incubated with ML-133 in the presence or absence of metal ions (100 μmol/L). ML-133-mediated cell growth inhibition was completely blocked by Zn\(^{2+}\) or Cu\(^{2+}\) supplementation, partially blocked by Fe\(^{2+}\), and was not affected by addition of Fe\(^{3+}\), Mg\(^{2+}\), or Ca\(^{2+}\) (Fig. 2A). However, the results obtained from adding supplemental metals to cells merely indicate whether a metal is capable of blocking the active site of ML-133 through chelation and do not represent a physiologic cellular environment. To assess whether ML-133 affects the endogenous levels of metals in vivo, metal-specific assays were undertaken.

Zinquin, a zinc-specific fluorophore, has been used to detect the intracellular changes in zinc available for cellular reactions (28). ML-133 decreased the fluorescence produced by zinquin-Zn\(^{2+}\) complex formation in a dose-dependent manner (Fig. 2B), indicating that ML-133 does reduce the concentration of endogenous intracellular labile zinc in HT-29 cells.

The activity of copper-dependent enzymes is commonly used to assess the copper status of animal tissues and cells. Copper functions as the active center of the cuproenzyme Cu/Zn SOD, which protects cells from the effects of superoxide anions (29). Cu/Zn SOD activity was decreased in a dose-response manner in HT-29 cells treated with the copper-specific chelator 2,3,2-tetramine. In contrast, HT-29 cells treated with ML-133 showed no significant dose-dependent changes in Cu/Zn SOD activity (Fig. 2C), indicating that chelation of intracellular copper by ML-133 does not occur significantly in vivo.

Expression of the iron-sensitive transferrin receptor 1 gene (30) was not significantly altered by ML-133 treatment, in contrast to the iron chelator desferoxamine, which up-regulated the expression of this gene after 16 hours (Fig. 2D), indicating that chelation of intracellular iron by ML-133 does not occur significantly in vivo.

Overall, these results indicate that ML-133–mediated HT-29 cell growth inhibition is mainly associated with the reduction of intracellular zinc levels.

**ML-133–Mediated Cell Cycle Arrest Involves Cyclin D1 Repression**

Cell cycle analysis by flow cytometry of HT-29 cells treated with ML-133 showed a dose-dependent increase in the percentage of cells in the G\(_1\) phase of their cell cycle,

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**Figure 2.** Reduction of intracellular zinc levels is associated with ML-133–mediated cell proliferation inhibition of HT-29 carcinoma cells. A, ZnCl\(_2\), CuCl\(_2\), FeCl\(_3\), FeCl\(_2\), Mgl\(_2\), MgCl\(_2\), or CaCl\(_2\) were added (100 μmol/L) to cells simultaneously with 2.5 μmol/L ML-133 or DMSO, and reversal of ML-133–induced cell growth inhibition was measured after 5 d by XTT assay. Results are presented as percentage cell growth inhibition relative to cells treated with DMSO and metal supplements. B, ML-133 dose-dependent decrease in endogenous intracellular labile zinc pool measured by zinquin assay in HT-29 treated cells. C, comparison of intracellular copper levels in HT-29 cells treated with the copper chelator 2,3,2-tetramine and ML-133 measured by Cu/Zn SOD activity. Results, percentage of SOD activity relative to DMSO-treated control. D, comparison of intracellular iron levels in HT-29 cells treated with the iron chelator desferoxamine (DFO) and ML-133, determined by the expression level of the iron-sensitive gene transferrin receptor 1. Fold change in gene expression is relative to 0.1% DMSO vehicle control at the same time point.
results are representative of three replicate experiments. C, decreased gene expression of cyclin D1 determined by real-time PCR after treatment with 1 μmol/L ML-133 for 16 h and 25 μmol/L ZnCl2. (*, P < 0.05 versus ML-133).

Figure 3. ML-133-mediated cell cycle arrest involves cyclin D1 repression. A, HT-29 cells were treated for 24 h with the indicated concentrations of ML-133; a dose-dependent G1-S phase cell cycle arrest was observed as measured by flow cytometry (*, P < 0.05 at 1 and 5 μmol/L ML-133 compared with control). B, decreased protein expression of cyclin D1 detected by Western blotting at the indicated time points post treatment with 1 μmol/L ML-133. Densitometric analysis was done to compare cyclin D1 protein levels to glyceraldehyde-3-phosphate dehydrogenase protein levels to normalize the results. These results are representative of three replicate experiments. C, decreased gene expression of cyclin D1 determined by real-time PCR after treatment with 1 μmol/L ML-133 for 16 h and 25 μmol/L ZnCl2. (*, P < 0.05 versus ML-133).

ML-133–Mediated Up-Regulation of the KLF4 Transcription Factor

Studies of zinc-regulated gene expression in HT-29 colon carcinoma cells indicate that KLF4 shows the most pronounced change in expression among other zinc finger–containing transcription factors under reduced zinc level conditions (6). Because KLF4 is known to inhibit cell proliferation by blocking G1-S progression of the cell cycle through transcriptional repression of cyclin D1 (7, 31), we addressed the question of whether KLF4 is involved in the cell growth inhibition mechanism of ML-133. Increased expression of the KLF4 gene was detected in HT-29 cells after 4-hour treatment with ML-133, with a peak at 16 hours (Fig. 4A), and this effect was partially reversed upon zinc supplementation (Fig. 4B), which supports the role of ML-133–mediated zinc level reduction in the induction of KLF4 gene expression. KLF4 protein was also increased after treatment of HT-29 cells with ML-133 for 16 hours (Fig. 4C). Moreover, ML-133 induced KLF4 gene expression in various cancer cell types, including colon, lung, prostate, breast, leukemia, and melanoma (Table 1).

KLF4 has been shown to repress the constitutive expression of the cyclin D1 gene through competition with the activator SP1 for binding to transcriptional control sequences in the cyclin D1 promoter (31). Therefore, we examined whether this mechanism was involved in the repression of cyclin D1 by ML-133 in HT-29 colon cancer cells with the use of the chromatin immunoprecipitation assay. ML-133 treatment produced increased binding of KLF4 to the cyclin D1 promoter and displacement of SP1, as shown by decreased SP1 binding (Fig. 4D), indicating that induction of KLF4 by ML-133 represses Sp1-dependent constitutive cyclin D1 transcription in vivo. ML-133 had no significant effect on the mRNA or protein expression of SP1 (Supplementary Fig. S1A and B). These results provide a molecular link between reduction of intracellular zinc levels, cyclin D1 down-regulation, and cell growth inhibition produced by ML-133.

KLF4 Up-Regulation Contributes to ML-133–Mediated Growth Inhibition

In an effort to evaluate the biological significance of KLF4 up-regulation by ML-133, we transiently transfected HT-29 cells with a KLF4 expression vector (Fig. 5A) and examined the impact of KLF4 overexpression on ML-133–mediated cell growth inhibition (Fig. 5B). KLF4–expressing cells were growth inhibited relative to vector-transfected cells in the absence of ML-133 (P < 0.05). Importantly, the effect of ML-133 on growth inhibition was enhanced as a result of KLF4 overexpression (P = 0.01). As a flip side to the overexpression of KLF4, we examined the effect of decreased KLF4 expression with the use of KLF4–targeted small interfering RNA (Fig. 5C). In the absence of ML-133, cell growth was not significantly altered by KLF4 knockdown, probably due to the relatively low basal level of KLF4 in the cells. Importantly, ML-133–mediated cell growth inhibition was significantly muted as a result of KLF4 knockdown (P = 0.01; Fig. 5D). Together, these results support the role of KLF4 in mediating the effect of ML-133 on the growth inhibition of cancer cells.
To validate the proposed molecular mechanism for ML-133–mediated cell growth inhibition in vivo, the levels of gene expression of KLF4 and cyclin D1 were determined by real-time PCR in HT-29 tumors. These studies showed a consistent increase in KLF4 gene expression and decreased cyclin D1 expression in tumors grown in ML-133–treated CD-1 nude mice compared with tumors from mice treated with the vehicle control (Fig. 5E). Taken together, these results indicate that ML-133 treatment reduces cyclin D1 expression through zinc-dependent up-regulation of the transcription repressor KLF4, ultimately leading to cell cycle arrest.

**Discussion**

The present study shows the anticancer properties of the novel small molecule ML-133. We have shown that chelation of labile intracellular zinc is a key factor in the molecular events that lead to cell growth inhibition. The concentration of zinc in cells is controlled through a complex zinc homeostatic system. Total cellular zinc consists of a large pool of tightly bound zinc, and a small but measurable pool of "free" zinc ions involved in regulatory functions. At least three factors control "free" zinc and the amplitudes of its fluctuations: total zinc, zinc buffering, and redox buffering capacity (32). Zinc buffering is determined by changes in

**Table 1. Effects of ML-133 on growth of cancer cell lines and KLF4 gene expression**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type of cancer</th>
<th>Growth inhibition IC50 (μmol/L)</th>
<th>KLF4 induction (fold increase)</th>
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<tr>
<td>HT-29</td>
<td>Colon</td>
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<td>HCT-116</td>
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<td>Prostate</td>
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<td>SK-MEL-2</td>
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</table>

NOTE: Growth inhibition (IC50 values in μmol/L) and KLF4 induction after 16-h treatment (gene expression measured by real-time PCR relative to DMSO-treated control) in various cancer cell lines treated with ML-133.

*KLF4 induction cannot be accurately determined due to the low basal levels of KLF4 in these cell lines.
the metallothionein-to-thionein ratio (33). In cells, metallothionein is a dynamic protein with species constantly changing due to Zn(II) transfer to apo-metalloproteins and re-equilibration when thionein expression is induced.

We propose that ML-133 chelates zinc from the labile pool of zinc, mainly from MTF1 and metallothionein. In agreement, after inducing the synthesis of metallothionein-Zn²⁺ in TE671 cells with zinc, the addition of 25 μmol/L of the zinc chelator TPEN for 30 minutes markedly reduced the zinc content of the metallothionein pool without clearly affecting the high–molecular weight zinc pool, which includes the majority of zinc-containing metalloproteins. This

Figure 5.  KLF4 up-regulation contributes to ML-133–mediated growth inhibition. A, Western blot of KLF4 expression in vector-transfected and KLF4-transfected HT-29 cells. B, effect of KLF4 overexpression on ML-133–mediated cell growth inhibition of HT-29 cells as detected by XTT assay. The results are from three separate experiments (*, P < 0.05, statistically significant versus DMSO-treated vector-transfected cells; **, P = 0.01, statistically significant versus ML-133-treated vector-transfected cells). C, KLF4 gene expression after transfection of HT-29 cells with KLF4-targeted small interfering RNA (siRNA) or nonspecific control siRNA treated with 1 μmol/L ML-133 or DMSO, determined by real-time PCR analysis. D, ML-133–mediated inhibition of cell proliferation is impaired in HT-29 cells transfected with KLF4-targeted siRNA but not with nonspecific control siRNA (*, P = 0.01). E, KLF4 and cyclin D1 gene expression determined by real-time PCR analysis in individual HT-29 tumors grown in athymic CD-1 nude mice treated with ML-133 for 5 d (i.p. administration of 100 mg/kg ML-133). Gene expression is represented as fold change relative to the average expression in tumors obtained from four control mice injected with vehicle control.
suggests that metallothionein-Zn$^{2+}$ is particularly labile compared with the inertness of the high–molecular weight zinc pool (34). ML-133 is able to chelate Zn$^{2+}$ in vitro (with a similar affinity to EGTA), as shown by its ability to impair the formation of a colored 4-(2-pyridylazo)resorcinol–Zn$^{2+}$ complex (Supplementary Fig. S2A). However, ML-133 chelates zinc with a much lower affinity than TPEN, indicating that ML-133 is unlikely to access the pool of tightly bound zinc. A zinc-chelating drug, such as ML-133, may interfere with cellular zinc buffering, leading to perturbation of zinc homeostasis.

Exogenously added copper blocks ML-133–mediated cell growth inhibition (Fig. 2A), and ML-133 is able to chelate copper in vitro (with a similar affinity to EGTA), as shown by its ability to impair the formation of a colored 4-(2-pyridylazo)resorcinol–Cu$^{2+}$ complex (Supplementary Fig. S2B). However, we do not believe that ML-133 is a chelator of copper in vivo. The results obtained from adding supplemental metals to cells merely indicate whether a metal is capable of blocking the active site of ML-133 through chelation and do not represent a physiologic cellular environment. An excess of copper likely blocks the growth inhibitory activity of ML-133 by preventing ML-133 from accessing the labile zinc within the cell. In agreement, ML-133 had no effect on copper status in vivo, as assessed by Cu/Zn SOD activity (Fig. 2C). The activity of copper-dependent enzymes, such as Cu/Zn SOD, is commonly used to assess the copper status of animal tissues and cells. Copper is an essential, but potentially reactive and toxic ion, and the free ionic copper concentration is extremely low in cells, estimated at <1 ion per cell (35). Chaperone proteins safeguard copper ions and make them available for incorporation into specific cuproenzymes (36). In contrast to ML-133, the copper-specific chelator 2,3,2-tetramine impaired Cu/Zn SOD activity in a dose-dependent manner, correlating with its higher affinity for copper in vitro (Supplementary Fig. S2B). ML-133 does not have a high enough affinity for copper to access this tightly bound metal in vivo.

In this report, we provide evidence that KLF4 is critical in mediating the effect of ML-133 on growth inhibition of cancer cells. KLF4 is a stress-associated and differentiation-associated inhibitor of proliferation (37) with tumor-suppressive functions (18, 38, 39). Indeed, loss of KLF4 expression is a frequent occurrence in various human cancers (14, 15, 18, 40–42). We show here that KLF4 is significantly up-regulated in response to ML-133 in HT-29 cells (Fig. 4A), in multiple cancer cell types (Table 1), as well as in HT-29 xenograft tumor samples (Fig. 5E). Additionally, the growth inhibitory effect of ML-133 is enhanced when KLF4 is overexpressed (Fig. 5B) and suppressed when KLF4 is knocked down in HT-29 cells (Fig. 5D).

In response to ML-133, we have shown that KLF4 functions as a negative regulator of cyclin D1 through competition with Sp1 at Sp1-binding motifs on the cyclin D1 promoter (Fig. 4D). Competition between KLF4 and Sp1 at the cyclin D1 promoter has already been established (31), and indeed, this seems to be a common mechanism as KLF4 has been shown to repress transcription of other genes through competition with Sp1, including histidine de-carboxylase (43), Cyp1A1 (44), and ornithine decarboxylase (45). In addition to direct competition with Sp1 for binding to promoters, Ai et al. (43) have proposed that KLF4 could mediate transcriptional repression through several additional mechanisms. First, physical interaction between Sp1 and KLF4 has been shown (44), and this interaction might disrupt the recruitment of the transcriptional coactivator complex, resulting in transcriptional inhibition. Second, KLF4 might also interact directly with coactivator complexes, leading to failure of the recruitment of the complexes to the Sp1 binding site or to the inhibition of the activity of the coactivator complexes. However, all these possibilities are not mutually exclusive (43).

KLF4 is also known to act as a transcriptional repressor of other cell cycle promoters as well as a transcriptional activator of several genes encoding inhibitors of the cell cycle (46). Further studies are required to identify other ML-133–responsive targets of KLF4 and their role in ML-133–mediated cell growth inhibition. Overall, these results suggest that KLF4 is a molecular link between intracellular zinc depletion by ML-133, cyclin D1 down-regulation, G1-S phase arrest, and cell growth inhibition. Further studies are also required to determine the mechanism by which the expression of KLF4 is regulated in response to zinc depletion. An obvious candidate for regulation of KLF4 expression is the zinc-sensitive transcription factor MTF1. Indeed, in HT-29 cells transfected with MTF1 small interfering RNA, the ML-133–mediated induction of KLF4 expression is partially blocked (data not shown), indicating that MTF1 is involved in the regulation of KLF4 expression. MTF1 is a cellular zinc sensor that coordinates the expression of genes involved in zinc homeostasis, including metallothionein. MTF1 DNA-binding activity, nuclear translocation, and occupancy of metal response elements in the promoter regions of genes are responsive to intracellular zinc concentration (47). However, it is still unclear how genes are regulated in conditions of low zinc availability, as MTF1 is transcriptionally active in conditions of high zinc availability. Importantly, MTF1 interacts or cooperates with a diverse set of factors, including NF-κB, hypoxia-inducible factor 1α, USF, SP1, heat shock transcription factor 1, and ribosomal protein S1, in addition to forming a coactivator complex with p300/ CBP and SP1 in a zinc-dependent manner (48). These interactions may be altered by ML-133–mediated changes in labile zinc concentrations affecting the transcription of target genes, including KLF4. Moreover, heat shock transcription factor 1, a known transcriptional regulator of KLF4 in response to heat stress (49), can be negatively regulated by forming a complex with MTF1 (50). Interestingly, we have shown with the use of a second-generation derivative of ML-133 that heat shock transcription factor 1 had the highest activation level among 50 transcription factors tested in HT-29 colon cancer cells (data not shown), suggesting that heat shock transcription factor 1 may also be involved in the regulation of KLF4.
Modulation of intracellular zinc homeostasis by zinc-specific chelators represents a potential new strategy for the treatment of certain types of cancer. We have shown that ML-133 is a chelator of zinc and that it potently inhibits multiple cell types with growth inhibition by 50% values in the nanomolar range as determined by the in vitro cancer cell line screen of the NCI. Moreover, ML-133 efficiently impairs tumor growth in a HT-29 colon tumor xenograft mouse model. In conclusion, we have identified a new zinc chelator, ML-133, as a potential anticancer therapeutic drug.

Disclosure of Potential Conflicts of Interest

All authors are current or former employees of Lorus Therapeutics, Inc. No other potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

A novel small molecule with potent anticancer activity inhibits cell growth by modulating intracellular labile zinc homeostasis

Mario Huesca, Lisa S. Lock, Aye Aye Khine, et al.


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