

Inhibition of *NANOG*/*NANOGP8* Downregulates MCL-1 in Colorectal Cancer Cells and Enhances the Therapeutic Efficacy of BH3 Mimetics

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Abstract

Purpose: High levels of BCL-2 family members in colorectal carcinoma cause resistance to treatment. Inhibition of *NANOG* or its paralog *NANOGP8* reduces the proliferation, stemness, and tumorigenicity of colorectal carcinoma cells. Our hypothesis was that inhibition of *NANOG*/*NANOGP8* enhances the cytotoxic effect of BH3 mimetics targeting BCL-2 family members in colorectal carcinoma cells through reducing expression of MCL-1, a prosurvival BCL-2 protein.

Experimental Design: Lentiviral vector (LV) shRNA to *NANOG* (shNG-1) or *NANOGP8* (shNp8-1) transduced colorectal carcinoma cells that were also exposed to the BH3 mimetics ABT-737 or ABT-199 *in vivo* in colorectal carcinoma xenografts and *in vitro* where proliferation, protein and gene expression, and apoptosis were measured.

Results: Clone A and CX-1 were sensitive to ABT-737 and ABT-199 at IC_{50s} of 2 to 9 μmol/L but LS174T was resistant with IC_{50s} of 18 to 30 μmol/L. Resistance was associated with high MCL-1 expression in LS174T. LVshNG-1 or LVshNp8-1 decreased MCL-1 expression, increased apoptosis, and decreased replating efficiency in colorectal carcinoma cells treated with either ABT-737 or ABT-199 compared with the effects of either BH3 mimetic alone. Inhibition or overexpression of MCL-1 alone replicated the effects of LVshNG-1 or LVshNp8-1 in increasing or decreasing the apoptosis caused with the BH3 mimetic. The combination therapy inhibited the growth of LS174T xenografts *in vivo* compared with untreated controls or treatment with only LV shRNA or ABT-737.

Conclusions: Inhibition of *NANOGP8* or *NANOG* enhances the cytotoxicity of BH3 mimetics that target BCL-2 family members. Gene therapy targeting the NANOGs may increase the efficacy of BH3 mimetics in colorectal carcinoma. *Clin Cancer Res*; 20(21); 5446–55. ©2014 AACR.

Introduction

Colorectal carcinoma is the second leading cause of cancer death in the United States without recent improvements in stage-specific death rates. Chemotherapy is used for the adjuvant therapy of stage II and stage III colorectal carcinoma because it causes programmed cell death or apoptosis (1). However, chemotherapy may not kill colorectal carcinoma that expresses high levels of prosurvival BCL2 proteins (2–4). This supports the development of new treatments to overcome the overexpression of these BCL-2 proteins (5, 6).

The BCL-2 family of proteins decides whether a cell continues to live or undergoes death through the intrinsic or mitochondrial apoptotic pathway. Multidomain BCL-2, BCL-xL, MCL-1, BCL-W, and BFL-1 are the prosurvival members of the BCL2 family, whereas BAX, BAK, and BOK are the proapoptotic members (7). Single domain BH3, only members of the family, include PUMA, NOXA, BIM, BID, BAD, and BIK that modulate the actions of the multidomain members (7). Various models explain how the BH3 only proteins affect the function of BCL-2 proteins regulating apoptosis (8, 9). This has led to the development of such BH3 mimetics as ABT-737 and ABT-199 that induce apoptosis in cancer cells. ABT-737 has high affinity to BCL-2, BCL-XL, and BCL-W (10), whereas ABT-199, a second generation BH3 mimetic, is a highly potent and specific inhibitor of BCL-2 (11). ABT-737 has shown good response in killing colorectal carcinoma cell lines as a single agent or in combination with chemotherapy (3, 12), whereas ABT-199 has shown strong activity against chronic lymphocytic leukemia, multiple myelomas, and estrogen receptor-positive breast cancers, either alone or in combination with other drugs. (13–15). However, neither molecule inhibits

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Translational Relevance

Colorectal carcinoma is the second leading cause of cancer death in the United States with recurrence occurring in 30% to 50% of stage II and stage III colorectal carcinoma patients after surgery and adjuvant therapy that is resistant to chemotherapy. Inhibition of the stem cell transcription factor *NANOG* or its retrogene *NANOGP8* decreases stemness and proliferation in colorectal carcinoma. Because high levels of BCL2 family proteins are expressed in colorectal carcinoma, we hypothesized that inhibition of *NANOG/NANOGP8* will decrease the levels of prosurvival protein MCL-1 to enhance cytotoxicity of BH3 mimetics that target BCL2 proteins. Combining shRNA against *NANOG/NANOGP8* with BH3 mimetics decreased MCL-1, increased caspase-dependent apoptosis of colorectal carcinoma *in vitro*, and inhibited colorectal carcinoma xenograft growth *in vivo* more than treatment with BH3 mimetics alone. Inhibition of *NANOG/NANOGP8* may reduce the addiction of colorectal carcinoma to MCL-1 and enhance the effect of BH3 mimetics.

the other important prosurvival protein MCL-1. Thus, when MCL-1 is highly expressed in cancer cells, ABT-737 has shown activity only when used in combination with molecules which neutralize MCL-1 (3, 9, 16–19). At this point, there seem to be little data on the efficiency of ABT-199 in presence of MCL-1.

NANOG is a key embryonic transcription factor that maintains pluripotency (20, 21) and is located on chromosome 12. *NANOGP8* is a retrogene located on chromosome 15 that is expressed in a wide variety of cancers (22–25). Our group showed that inhibition of *NANOG* and its retrogene *NANOGP8* ablates stemness in human colorectal carcinoma as measured by reduced spherogenicity, side population size, proliferation *in vitro*, and tumorigenicity, and metastatic potential in NOD/SCID mice (26). *NANOGP8* may replace *NANOG* in supporting characteristics of stemness such as proliferation (22) and spherogenicity (26). Moreover, it was recently reported that inhibiting *NANOG* expression decreases MCL-1 protein levels indirectly through a decrease in the phosphorylation of AKT (27).

We postulated that inhibition of *NANOG* or *NANOGP8* may inhibit MCL-1 expression in colorectal carcinoma and enhance the cytotoxicity of ABT-737 or ABT-199. Our approach was to test this *in vivo* in mice, *in vitro* in the WST-1 survival assay as well as to measure the effect of the agents upon caspase-3 and -7 activity as a direct measure of the induction of apoptosis. *NANOG* and *NANOGP8* are essentially identical proteins of 305 amino acids whose coding regions differ by only five nucleotides that create nonsynonymous changes in two amino acids. Our allele specific shRNAs target codon 759 of *NANOG* (shNG-1) or *NANOGP8* (shNp8-1; ref. 26) decreased MCL-1 expression and enhanced the cytotoxicity of the BH3 mimetics in the

three colorectal carcinoma cell lines Clone A, CX-1, and LS 174T.

Materials and Methods

ABT-737 and ABT-199 were purchased from Selleck Chemicals LLC. ABT-737 and ABT-199 and stocks in DMSO at 10 mmol/L were stored at -20°C . Lipofectamine 2000 for transfections was purchased from Invitrogen. Polybrene and protamine sulfate for Lentivirus transduction and propylene glycol and Tween-80 were purchased from Sigma-Aldrich Chemical Co. Precast NU-PAGE 4–12% Bis Tris gels, NU-PAGE MES SDS Running Buffer, and NU-PAGE transfer buffer were purchased from Invitrogen. Ninety-six-well white plates (ViewPlate-96 TC) for Caspase Glo assay were purchased from PerkinElmer life sciences. Caspase-3 inhibitor (Z-DEVD-FMK) was obtained from R&D systems. MCL-1 overexpression plasmid pTOPO-MCL1 (Plasmid No 21605) was purchased from Addgene.

Cell culture, cell transfection, lentivirus packaging, and cell transduction

Clone A is a subclone of the DLD-1 cell line. (26, 28) CX-1 is a highly metastatic variant of HT29 (28) and LS174T is a colorectal carcinoma cell line obtained from ATCC and used in our previous study (26). The cell lines were authenticated by the University of Arizona Genetics Core, Tuscon, AZ (Supplementary Table S2). All these cell lines were cultured in RPMI (Invitrogen) media supplemented with 10% FBS (Invitrogen) and 2 mmol/L L-glutamine (Invitrogen) at 37°C , 5% CO_2 incubator. The Lentivirus particles containing the allele specific shRNAs for *NANOG* (shNG-1), *NANOGP8* (shNp8-1), and negative control (shNEG) were produced by cotransfection of 293T cells with packaging and envelope plasmids using Lipofectamine 2000 (Invitrogen) as described (26). The transduction of the lentiviral particles at a multiplicity of infection of five to eight for all the experiments was done using polybrene or protamine sulfate as the transducing agent.

Tumors. Animal experiments were performed under the protocol LEC-052 approved by the NCI Animal Care and Use Committee. Three million viable untreated LS174T cells or LS174T cells transduced with shNEG, shNG-1, or shNp8-1 were injected subcutaneously into eight groups of 5- to 6-week-old NOD/SCID male mice (Fig. 1). ABT-737 was dissolved in 30% propylene glycol, 5% Tween-80, 3.3% D5W (pH 1.0), and 1% DMSO, sonicated, and pH adjusted to pH 4–5. When tumors reached approximately 100 mm^3 , ABT-737 (100 mg/kg) was injected intraperitoneally daily for 5 days. Tumor volumes were calculated by the formula perpendicular length \times width². Mice were sacrificed when the control tumor volume reached $2,000\text{ mm}^3$ 8 days later. Statistical analysis of the treated tumors relative to control was done using one-way ANOVA with Holm–Sidak multiple comparisons correction test in GraphPad Prism 6.

Assays. WST-1 (Roche) and Caspase-Glo were used according to the directions supplied by the manufacturers. For the clonogenic or regrowth assay, the three cell lines were seeded with 10,000 cells in a 48-well plate (Costar/

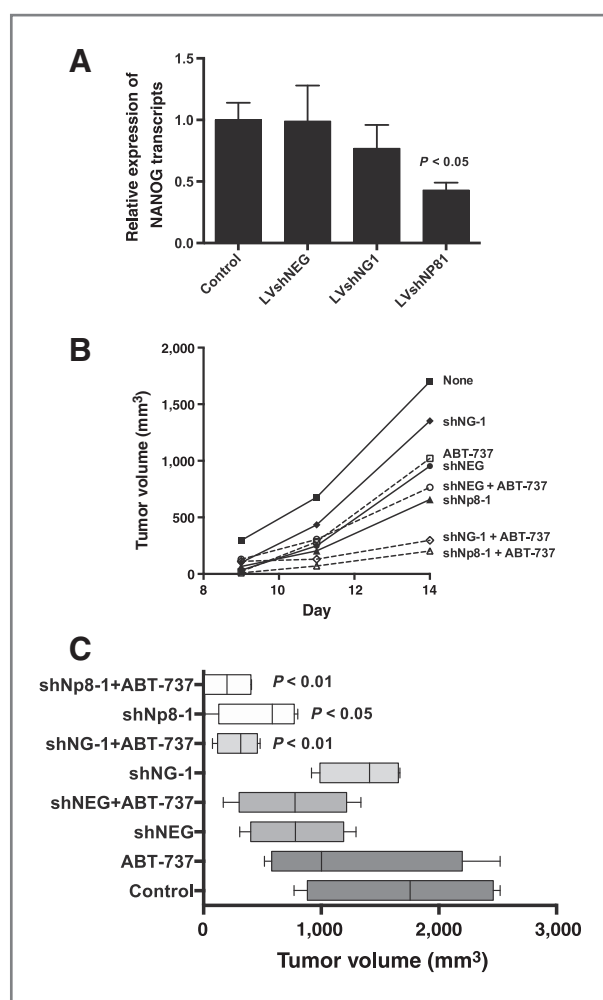


Figure 1. Antitumor effects of LVshNG1/LVshNP81-ABT-737 combinations. Three million parental LS174T cells or LS174T cells transduced with LV shNEG, shNG-1, or shNP8-1 were injected subcutaneously into groups of 8 NOD/SCID male mice. At day 8, ABT-737 (100 mg/mL) was injected daily intraperitoneally for 5 days. A, three mice from each group were sacrificed on the 8th day before ABT-737 treatment, tumors collected, and qRT-PCR performed to determine the relative expression of total transcripts of the *NANOGs*. Mean \pm SD of the expression of the *NANOGs* normalized by *GAPDH*. B, mean growth curves of each group of mice. C, box and whisker plots for each group of mice with mean and 5%–95% confidence intervals are presented by the error bars. The *P* values were determined by one-way ANOVA and compared with the control (untreated) group.

Corning) in RPMI-1640 media with supplements. After 16 hours, cells were treated with lentiviral vector (LV) shNEG, LVshNG-1, and LVshNP8-1 or ABT-737 alone for 8 days or with one of the three shRNAs for 5 days followed by ABT-737 (2 μ mol/L) for 3 more days. The supernatant was removed from the wells after 8 days and remaining adherent cells were trypsinized and an equal number of cells as described in the text were plated in 60 mmol/L dishes with complete RPMI media, in duplicates for each treatment. The cells were incubated for 14 days and fixed and stained with 0.05% Crystal violet in 10% neutral-buffered formalin

(37% vol), methanol (1%), and 0.15 mol/L PBS (62% vol). The plates were washed with water and the colonies counted. For assessment of caspase function, caspase-3 inhibitor (Z-DEVD-FMK) was added 4 hours after adding LV and ABT-737 at 10 μ mol/L and then the cultures were analyzed by WST-1 assay as explained above.

Western blot analysis. Colorectal carcinoma cells were washed with PBS and then solubilized with RIPA buffer containing both protease and phosphatase inhibitors. Pre-cast NU-PAGE 4% to 12% gels were used to separate cell lysates. Lysates transferred to Nitrocellulose membranes were probed with rabbit anti-MCL-1 (Cell Signaling Technology, Cat# 4572, Cat#sc819), BCL-2 (Cat#2876), BCL-XL (Cat#2762), NANOG (Cat#4903), AKT (Cat#9272), AKT-Ser473 (Cat#4060), BCL-W (Cat#2724), and BIM (Cat#2819), and β -actin (Cat#4967) to monitor changes in the level of these proteins. The primary antibody was detected with goat anti-rabbit-HRP (Jackson Immunoresearch).

Statistical analysis. ANOVA was performed for statistical analysis of multiple comparisons using GraphPad Prism 6. Data in graphs are presented as mean \pm SD except where indicated in the text. For the analyses, $P < 0.05$ was considered to be statistically significant. All experiments were repeated at least twice independently.

Results

Combination treatment of ABT-737 and LVshNG-1/LVshNP8-1 in mice bearing LS174T xenografts

To test the combination of ABT-737 with LVshNG-1 and LVshNP8-1 *in vivo*, we injected NOD/SCID male mice with 3×10^6 LS174T cells transduced with LV shNG-1 or shNP8-1 or the control shNEG or left untreated. The ABT-737 treatment was started at day 8 when the tumors were approximately 100 mm³. The mice were sacrificed at day 17 when the tumors of the control groups of mice reached around 2,000 mm³. When tumors were analyzed at day 8 before the start of ABT-737 treatment, the levels of total NANOG transcripts in tumors initiated with LV shNG-1 or LV shNP8-1 were two-thirds or one-half, respectively, of the transcript levels in the control tumors (Fig. 1A). The volume of tumors of mice treated with the single agents of LV shNEG, LV shNG-1, LV shNP8-1, or ABT-737 or the control combination of LV shNEG + ABT-737 was not significantly reduced compared with the size of the untreated controls (Fig. 1B and C). However, the mean volume of tumors in mice treated with combined LV shNG-1 + ABT-737 ($P < 0.01$) or LV shNP8-1 + ABT-737 ($P < 0.01$) was one-third that of the mean of the untreated controls (Fig. 1C). These results support the postulate that inhibition of NANOG/NANOGP8 enhances the efficacy of ABT-737 in colorectal carcinoma xenografts. Further studies were performed *in vitro* to elucidate mechanism.

Colorectal cancer cell lines are variably sensitive to ABT-737

We began these studies by determining the IC₅₀ of ABT-737 on three cell lines (Clone A, CX-1, and LS174T). IC₅₀

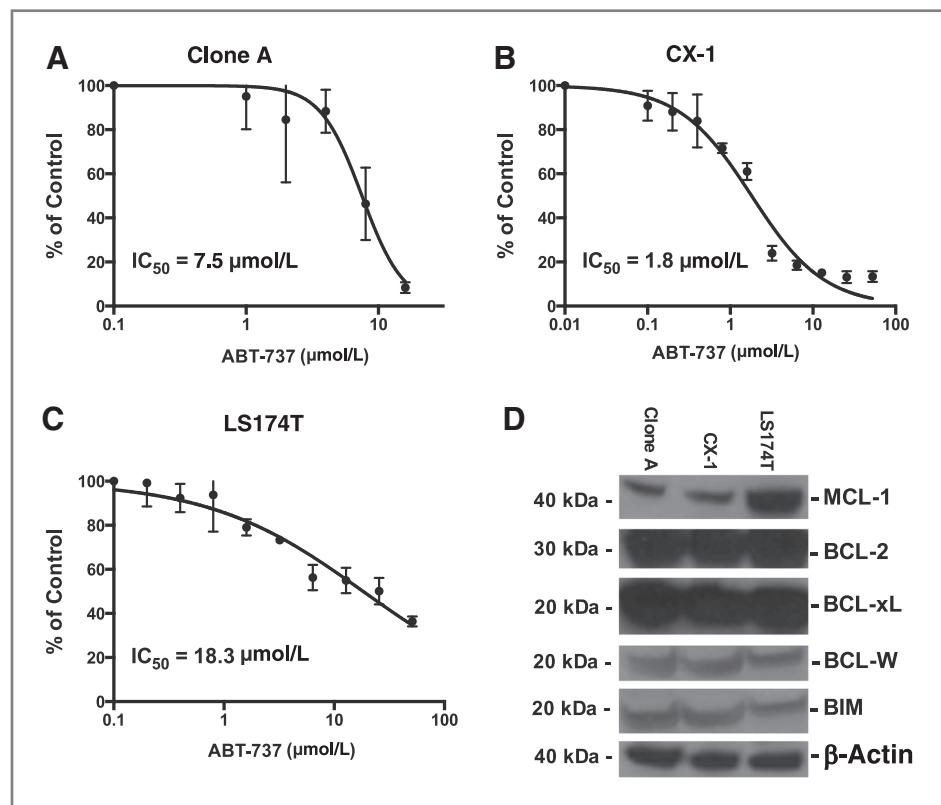
values were 7.5 $\mu\text{mol/L}$ for Clone A cells, 1.8 $\mu\text{mol/L}$ for CX-1, and high 18.3 $\mu\text{mol/L}$ for LS174T cells in a 48-hour viability treatment (Fig. 2A–C). The protein levels of the prosurvival proteins BCL-2 (BCL2), BCL-xL (BCL2L1), MCL-1 (MCL1), (BCL-W BCL2L2), and BIM (BCL2L11) were also analyzed (Fig. 2D). LS174T cells express nearly three times the amount of MCL-1 as Clone A and CX-1, but the other prosurvival Bcl-2 family proteins are expressed similarly by the three cell lines (Fig. 2D).

Enhanced killing via combinations of ABT-737 and LVshNG-1 and LVshNp8-1 in colorectal carcinoma cell lines

Prior studies have demonstrated that inhibition of NANOG decreases cell proliferation, causes cell-cycle arrest, induces apoptosis, and inhibits stemness in a variety of cancer cell lines (26, 29). Conditional knockout of NANOG in mice induces apoptotic cell death in murine migrating primordial germ cells (30). We tested the combination of ABT-737 with LV shRNAs in these colorectal carcinoma cell lines at a concentration of 1 $\mu\text{mol/L}$ ABT-737. This concentration of ABT-737 by itself produced little or no cytotoxicity in Clone A, CX-1, and LS174T (Fig. 3). The combination experiment was done to test also the timing of ABT-737 and shRNA on the response of colorectal carcinoma cells to the combination where colorectal carcinoma cells were exposed ABT-737 (1 $\mu\text{mol/L}$) for at least 3 days with exposure to LV shRNA for at least 5 days (Fig. 3). In "ABT First" experiments, cells were treated first with ABT-

737 (1 $\mu\text{mol/L}$) for 3 days followed by treatment with LV shRNAs (LV shNEG, LV shNG-1, and LV shNp8-1) for 5 days. In "ABT Second" studies, the three cell lines were treated with LV shRNAs for 5 days first followed by treatment with ABT-737 (1 $\mu\text{mol/L}$) for 3 days. "ABT Continuous" studies represent the cell lines treated with ABT-737 and LV shRNAs simultaneously for 8 days. All combination treatments lasted 8 days. Cell survival was determined by metabolism of WST-1 and results presented as the mean \pm SD of the % of control values of the untreated colorectal carcinoma cells after 8 days: fewer cells are associated with a lower amount of WST-1 metabolized (Fig. 3). In Clone A, the combination treatment (LV shNp8-1 + ABT-737) decreased cell survival by as much as by 40% relative to the lentiviral treatment control (LV shNEG + ABT-737) in all the combination therapy groups (Fig. 3A), and LS174T cells had a similar decrease in survival relative to the lentiviral control shNEG but only when the cells were transduced with lentiviral shRNA first or concurrently with ABT-737 addition (Fig. 3C). Interestingly, the CX-1 cells were only inhibited when the lentiviral shRNA was transduced first (Fig. 3B). Because CX-1 is sensitive to ABT-737, the BH3 mimetic effect may mask the potential effect from inhibiting NANOG and/or NANOGP8. As a result, a dose-response experiment with ABT-737 was performed with CX-1 cells treated with LV shNp8-1. The combination enhances the growth inhibition of CX-1 cells by reducing the IC_{50} of 2.8 $\mu\text{mol/L}$ for ABT-737 alone by more than 50% to 1.31 $\mu\text{mol/L}$ for the combination (Supplementary

Figure 2. Dose response of colorectal carcinoma cell lines to the BH3 mimetic ABT-737. A–C, Clone A, CX-1, or LS174T cells were treated with ABT-737 (0.2–60 $\mu\text{mol/L}$) for 72 hours. Cell survival was determined using the WST-1 reagent. The viability of cells (% of control) is presented as % absorbance of treated cells at 450 nm/% absorbance of untreated cells at 450 nm expressed as a percentage of the untreated parental cells. IC_{50} s were calculated with GraphPad Prism 6 using the nonlinear regression subprogram. D, immunoblot analysis for detection of MCL-1, BCL-2, BCL-xL, BCL-W, and BIM levels in Clone A, CX-1 and LS174T.



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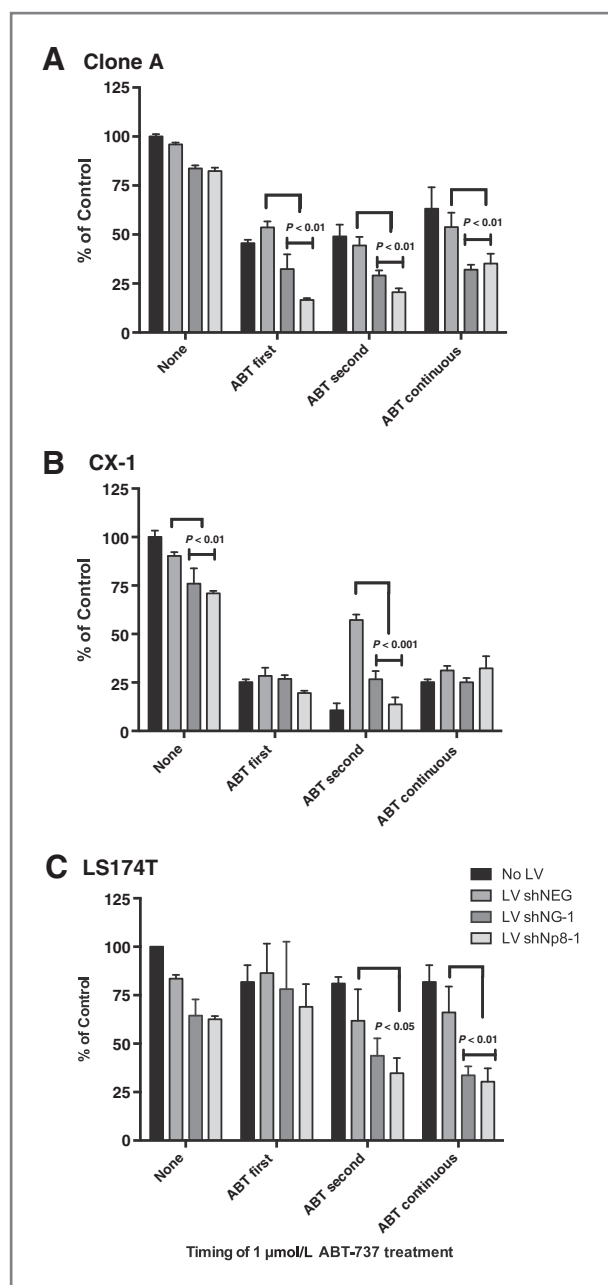


Figure 3. ABT-737 and LV shRNA combination therapy increases inhibition of colorectal carcinoma growth. Five thousand colorectal carcinoma cells were cultured in monolayer culture in individual wells of a 96-well microtiter plate in complete medium and LV shRNA or ABT-737 or both added as indicated for a total of 8 days. Cells were treated with either LV shRNA for 5 days or 1 $\mu\text{mol/L}$ ABT-737 for 3 days or with both from the beginning of the culture. The "None" cells did not receive any ABT-737, whereas the "ABT First," "ABT Second," or "ABT Continuous" represent cells exposed to ABT-737 first and then LV, or "ABT Second" with cells exposed to LV shRNA first for 5 days and then ABT-737 for 3 days or "ABT Continuous" where both agents were added at the beginning of the 8 day culture. Results are presented as means with SD. *P* values are the significance of the indicated LV shNG-1 and/or shNp8-1 compared with the LV shNEG in the same treatment group determined by two-way ANOVA. Where there is a horizontal cupped bracket spanning across shNG-1 and shNp8-1, the *P* value of each compared with the corresponding shNEG control is the indicated value.

Fig. S1, $P < 0.0001$). Also in each cell line, shNp8-1 transduction was more active than shNG-1 in inhibiting growth (Fig. 3A–C).

Killing of cells is associated with loss of MCL-1 and increase in caspase-3/7 activity

Inhibition of *NANOG* in cancer cell lines has been associated with loss of AKT phosphorylation and a decrease in MCL-1 levels (27). To elucidate whether enhanced killing associated with combination of LV shRNA against *NANOG* or *NANOGP8* and ABT-737 is associated with loss of AKT phosphorylation and decrease in MCL-1 levels due to inhibition of *NANOG*, we treated the three colorectal carcinoma cell lines with the LV-delivered shRNA. The inhibition of either *NANOG* or *NANOGP8* in Clone A cells resulted in a decrease in the levels of *NANOG*, phosphorylation of AKT at Ser-473, and in MCL-1 (Supplementary Fig. S2). Inhibition of *NANOG* and *NANOGP8* gene expression by shRNA did not decrease relative MCL-1 transcript levels (Supplementary Fig. S2A and S2B) but did decrease MCL-1 protein expression by at least 50% in all three colorectal carcinoma cell lines, whereas treatment with the control LVshNEG did not (Fig. 4A). Caspase-3/7 activity was induced in Clone A, CX-1, and LS174T cell lines when colorectal carcinoma cells were cultured with 1 $\mu\text{mol/L}$ ABT-737 after first being pretreated with LV shRNAs (Fig. 4B). Transduction with LV shNEG, LV shNG-1, or LV shNp8-1 alone did not increase caspase-3/7 activity (Fig. 4B), whereas ABT-737 alone increased caspase-3/7 activity significantly in CX-1 cells and to a lesser extent in Clone A treated for 7 days (Fig. 4B). The combination of LV shNEG and ABT-737 increased caspase-3/7 activity moderately 2- to 4-fold in the three cell lines (Fig. 4B). However, in all three cell lines, the combination treatment with LV shNG-1 or LV shNp8-1 increased caspase-3/7 activity by 5.5- to 7.5-fold compared with untreated cells and more than that caused by LV shNEG and ABT-737 (Fig. 4B). Inhibition of colorectal carcinoma growth induced by the combination therapy of shRNA and ABT-737 is associated with apoptosis as reflected by the activity of the executioner caspases. The inhibition of cell survival by combination therapy is caused by caspase-dependent cell death because addition of a caspase-3 inhibitor peptide blocked the cytotoxic effect of LV shNp8-1 and ABT-737 (Fig. 4C).

ABT-199 and its activity in colorectal carcinoma cell lines

The IC_{50} s for Clone A, CX-1, and LS174T treated with ABT-199 are 9.8 $\mu\text{mol/L}$, 6.7 $\mu\text{mol/L}$, and 29.5 $\mu\text{mol/L}$, respectively (Fig. 5A). Clone A showed similar sensitivity to ABT-737 and ABT-199 (Supplementary Table S1). In contrast, CX-1 and LS174T were more sensitive to ABT-737 than ABT-199 (Supplementary Table S1). These patterns of differing sensitivity have also been observed in other cancer cell lines (14). To test the activity of ABT-199 in combination with LV shNG-1 or shNp8-1 in these three colorectal carcinoma cell lines, the combination treatment was done in a similar manner as explained for ABT-737 above

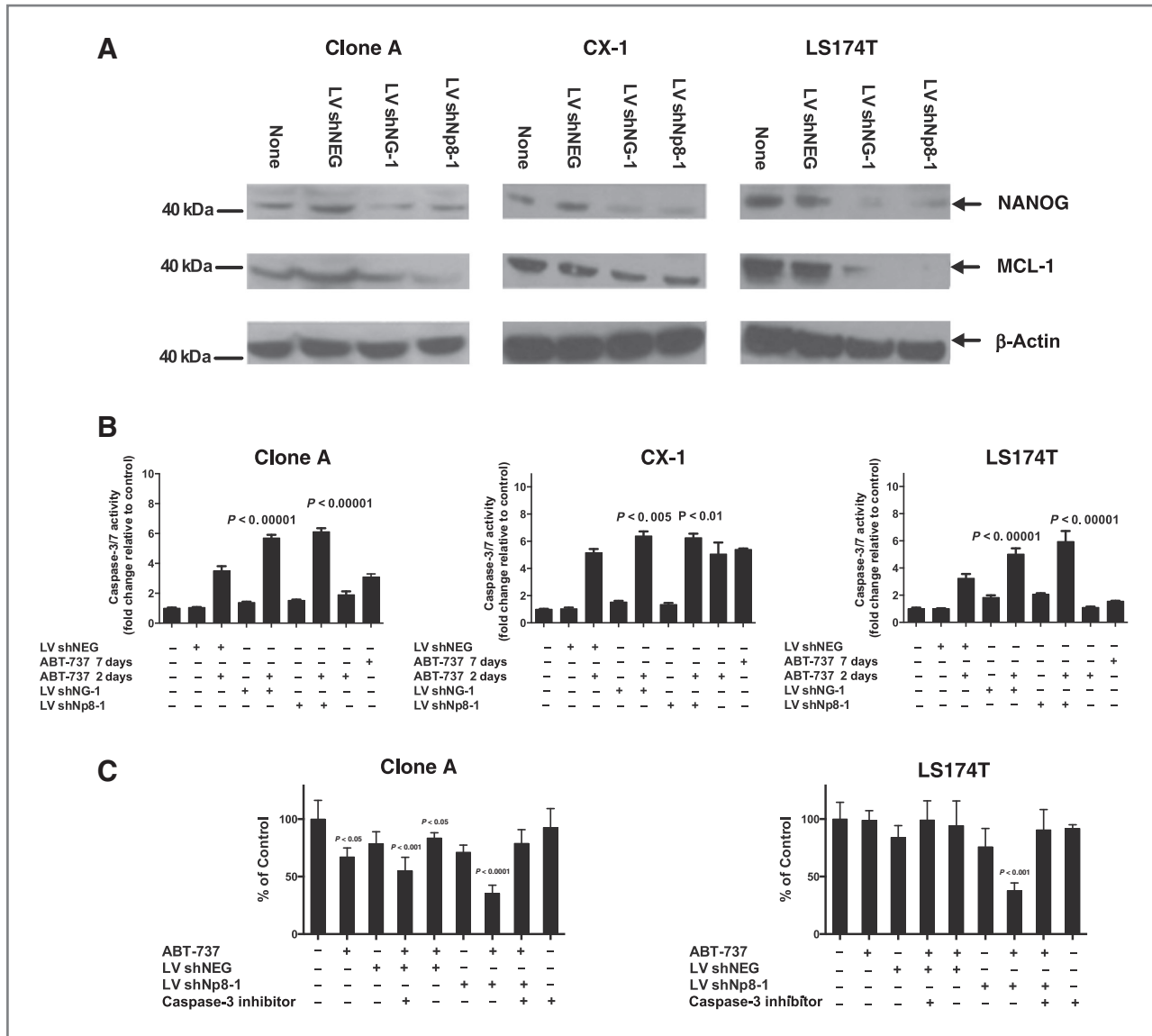


Figure 4. LV shRNA to *Nanog* or *NanogP8* inhibits MCL-1 and increases caspase-3/7 activity. **A**, Clone A, CX-1, or LS174T cells were treated with the indicated LV shRNA for 5 days in monolayer culture or left untreated. Lysates were blotted and probed for NANOG and MCL-1 protein expression. **B**, the three cell lines were treated with LV shRNA alone for 7 days or LVshRNA for 5 days followed by ABT-737 (1 μ mol/L) for 2 days in triplicate in complete medium. Caspase-3/7 activity was determined in the wells using the Promega caspase-3/7-Glo kit according to the manufacturer's protocol. The results are mean + SD of the activity normalized to the untreated control cells within each experiment. *P* values are the significance of the indicated caspase-3/7 fold increase in LV shNG-1+ABT-737(1 μ mol/L) and shNp8-1 + ABT-737 (1 μ mol/L) compared with the LV shNEG + ABT-737(1 μ mol/L) for each cell line determined by one-way ANOVA with the Holm-Sidak multiple comparisons test. **C**, Clone A or LS174T cells were cultured in complete medium for 16 hours and then treated with LV shNEG or shNp8-1, ABT-737 (2 μ mol/L), or the combination. Four hours later, 10 μ mol/L caspase-3 inhibitor (Z-DEVD-FMK) was added. Cell viability was determined 5 days later. Results are presented as the mean + SD normalized to the untreated control cells. *P* values are determined by one-way ANOVA with means compared with the untreated cells with the Holm-Sidak multiple comparisons test.

(Fig. 5B). The concentration of ABT-199 used for this experiment was 2 μ mol/L. In Clone A and LS174T cells, the combination treatment (LV shNp8-1 + ABT-199) resulted in the inhibition of cell growth by as much as 60% relative to LV control (LV shNEG + ABT-199; Fig. 5B). CX-1 survival was inhibited by 30% when treated with the combination (LV shNp8-1 + ABT-199) relative to control (LV shNEG + ABT-199). Inhibition of BCL-2 alone by a low concentration of ABT-199 enhances the inhibition of Clone

A and LS 174T cells treated with LV shNG-1 or LV shNp8-1, especially if the ABT-199 is given after or continuously with the LV shRNA (Fig. 5B).

Inhibition of MCL-1 by siRNA increases caspase-3/7 activity in colorectal carcinoma cell lines when used in combination with ABT-737/ABT-199

We tested whether the effect of inhibiting *NANOG*/*NANOGP8* on augmenting the cytotoxicity of the BH3

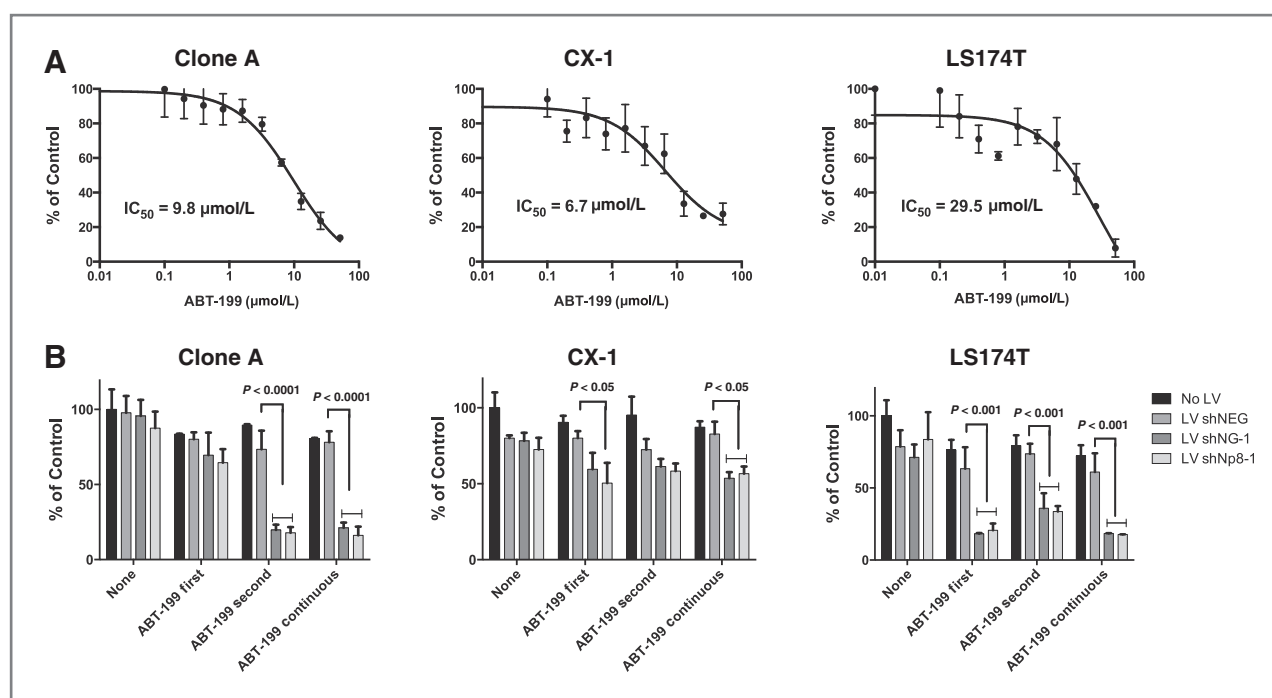


Figure 5. Activity of ABT-199 in colorectal carcinoma cells alone or in combination with shRNA against *Nanog* and *NanogP8*. A, Clone A, CX-1, and LS174T cells were treated with ABT-199 for 72 hours and viability determined with IC_{50} calculated for each cell line as described in Fig. 2. B, five thousand colorectal carcinoma cells were cultured in complete medium and LV or ABT-199 or both added as indicated for a total of 8 days. The scheme of the experiment was the same as described for Fig. 3. Results are mean \pm SD normalized to the untreated colorectal carcinoma cells. *P* values are the significance of the indicated LV shNG-1 and/or shNp8-1 compared with the LV shNEG in the same treatment group as determined by two-way ANOVA with the Holm–Sidak multiple comparisons test.

mimetics depended on the reduction of MCL-1 protein expression by directly modulating the expression of MCL-1 and assessing sensitivity to the BH3 mimetics. Colorectal carcinoma cells were transfected with siRNA *MCL-1* (100 nmol/L) alone or in combination with ABT-737/ABT-199 treatment. Transfection of the three colorectal carcinoma cell lines decreased MCL-1 by 3- to 6-fold (Fig. 6A). Transfection of LS174T cells with only siRNA to *MCL-1* increased 2-fold the caspase-3/7 activity, whereas such transfection did not increase caspase-3/7 activity in Clone A or CX-1 (Fig. 6B). Treatment with ABT-737 induced caspase-3/7 activity similar to what occurred earlier with inhibition of *NANOG/NANOGP8* (Fig. 6B). ABT-199 alone induced caspase-3/7 activity that was similar to ABT-737 in Clone A and LS174T cells (Fig. 5B). In CX-1 cells, ABT-737 induced more Caspase 3/7 activity than ABT-199 alone (Fig. 6). The combination of siRNA to MCL-1 with either BH3 peptide further increased caspase-3/7 activity in each cell line (Fig. 6B). In contrast, overexpression of *MCL-1* in LS174T cells rescued the growth of LS174T treated with the combination of LVshNp8-1 + ABT-737 (Fig. 6C) while increasing the level of MCL-1 protein in all cells transfected with the MCL-1 (Fig. 6D). Thus, direct modulation of MCL-1 expression mimics the effects of inhibition of *NANOG/NANOGP8* on BH3 mimetics on caspase activity and survival.

Clonogenic regrowth assay

The ability of lentiviral shRNA combined with ABT-737 to induce a long lasting inhibition of growth in the three colorectal carcinoma cell lines was determined by a colony-forming assay (ref. 31; Supplementary Fig. S3). Colorectal carcinoma cells were treated with ABT-737, LV shRNA, or the combination for 8 days and surviving adherent cells were collected and replated in fresh complete medium. In each experiment, 500 viable cells were plated for each condition and then after 14 days stained and colonies counted. The combination of LV shNp8-1 and ABT-737 significantly decreased regrowth colony efficiency compared with the combination of ABT-737 and LVshNEG1 by 50% and 80% (Supplementary Fig. S3). The combination of ABT-737 and LV shNG-1 had a lesser effect. These data suggest that even those cells that survive to the end of original incubation period have a residual persistent growth inhibition from the combination therapy.

Discussion

The inhibition of MCL-1 achieved through inhibition of *NANOGP8* or *NANOG* increases the growth inhibitory effects of the BH3 mimetics ABT-737 and ABT-199. ABT-737 has potent activity against leukemia and lymphoma cancer cell lines as a single agent and is also effective against multiple myeloma, glioma, and small cell lung cancers

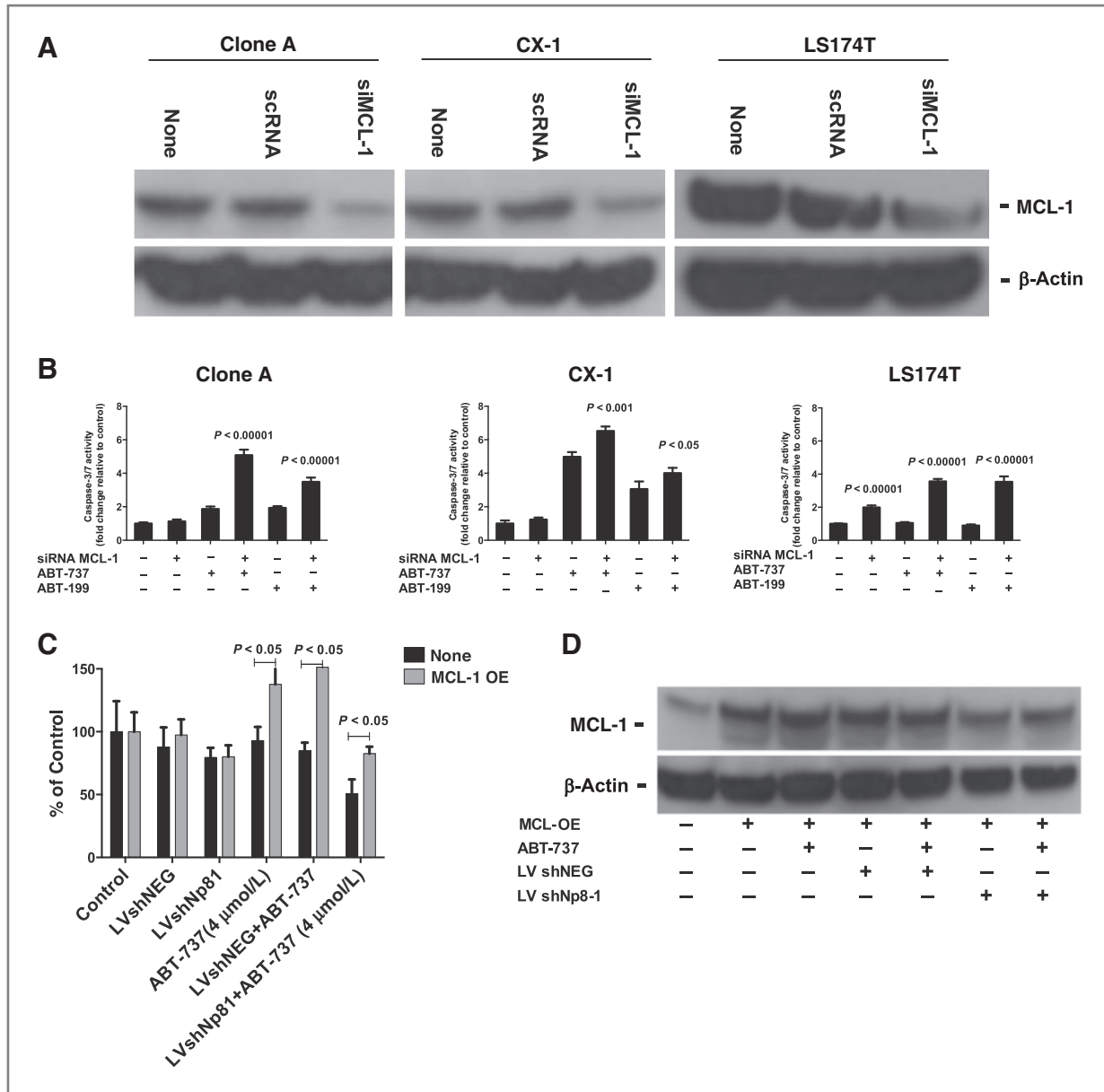


Figure 6. Inhibition of *Mcl-1* expression is similar to the effect of LV shNG-1 or shNp8-1 on caspase-3/7 activity when combined with BH3 mimetics. A, Clone A, CX-1, or LS174T cells were transfected with siRNA to *Mcl-1* (siMCL-1, 100 nmol/L) or scrambled RNA (scrRNA, 100 nmol/L) and lysed and immunoblotted for MCL-1 and β -actin after 72 hours. B, the three cell lines were transfected with siRNA *Mcl-1* (100 nmol/L) and 3 days later ABT-737 (2 μ mol/L) or ABT-199 (2 μ mol/L) was added as indicated. Caspase-3/7 activity was measured at a total of 5 days by Promegaspase 3/7 –Glo kit according to the manufacturer’s protocol. The results are presented as mean + SD of the activity normalized to the untreated control cells within each experiment. *P* values were determined by one-way ANOVA with Holm–Sidak multiple comparisons correction test. C, five thousand LS174T cells were seeded in individual wells of a 96-well plate in triplicate and next day treated with LVshNEG and LVshNP81. After 3 days, p-TOPO-MCL-1 was transfected using Lipofectamine. After 48 hours, 4 μ mol/L ABT-737 was added and cell viability was measured 3 days later by WST-1 metabolism. The viability of cells is represented as mean + SD of the % absorbance of cells at 450 nm/L compared with the controls. *P* values were calculated by one-way ANOVA. D, lysates of the combinations of ABT-737 and LV shRNAs on the MCL-1 and β -actin protein expression in LS174T cells treated as in C were probed and demonstrate that MCL-1 is overexpressed in all of the p-TOPO MCL-1-transfected cells.

(SCLC; ref. 32). In colorectal carcinoma cell lines, ABT-737 has shown poor efficacy as a single agent but the growth inhibition increases when ABT-737 is used in combination with other therapies (3, 5, 6, 12). In this study, the colorectal carcinoma cell lines exhibited low to moderate sensitivity

when treated with ABT-737 or ABT-199 as single agents with CX-1 being the most sensitive cell line (Supplementary Table S1). LS174T cell line exhibited the greatest resistance ($IC_{50} > 12 \mu$ mol/L; Supplementary Table S1) toward both ABT-737 and ABT-199 associated with the higher expression

of MCL-1. Our study demonstrates that the treatment of three colorectal carcinoma cell lines with the combination of LVshNG-1 or LVshNp8-1 and BH3 mimetics enhances the growth inhibitory effect in these cell lines. Earlier studies have also demonstrated that when SCLC cell lines are treated with a combination of ABT-737 and agents which decrease MCL-1 levels, ABT-737-resistant SCLC cell lines demonstrate enhanced killing compared with ABT-737-sensitive SCLC cell lines which show only moderate increase in cell killing when treated with the combination. (16).

Furthermore, we also show that the inhibition of *NANOG*/*NANOGP8* alone decreases the levels of MCL-1 protein. Our finding extends the study of Noh and colleagues (27) who demonstrated that *NANOG* promotes a stem-like and immune-resistant phenotype in multiple types of cancer cell lines, including the HCT-116 colorectal carcinoma cell line. They elucidated that *NANOG* acts through *TCL1A*-mediated AKT regulation of MCL-1 with knockdown of *NANOG* decreasing the levels of pAKT and MCL-1. Although Boyer and colleagues (33) demonstrated that *NANOG* binds to the MCL-1 promoter, we have confirmed that inhibition of *NANOG* or *NANOGP8* does not change the levels of MCL-1 transcripts (Supplementary Fig. S2A and S2B). However, inhibition of the *NANOGs* decreases pAKT (Supplementary Fig. S2C) and MCL-1 expression (Fig. 4A). These results suggest that regulation of MCL-1 is a posttranslational event. Moreover, when we treated the three colorectal carcinoma cell lines with the combination of LV shNG-1 or LV shNp8-1 and ABT-737, it increased the caspase-3/7 activity. Caspase-3 inhibition blocked the enhanced growth inhibitory effect of the combination (Fig. 4C). We also demonstrate that the combination of siRNA *MCL-1* and ABT-737/ABT-199 increased the caspase-3/7 activity in these colorectal carcinoma cell lines, whereas overexpression of MCL-1 neutralized the growth inhibitory effect of the shRNA-ABT combination (Fig. 6C). These findings further strengthen the finding that enhanced caspase-3/7 or growth inhibitory effect by combination of LVshNG-1/shNp8-1 and ABT-737/ABT-199 is the consequence of decrease in the levels of MCL-1. Thus, the combination of inhibition of *NANOG*/*NANOGP8* and the BH3 mimetic combination increased caspase-dependent apoptosis.

ABT-199 combined with LVshNG-1 and/or LVshNp8-1 treatment was approximately 20% more active than the combination with ABT-737 in Clone A and LS174T. Recent studies (11, 14, 15) also support this finding that ABT-199 is more potent than ABT-737 when used alone or in combination with other drugs. Furthermore, the combination of LV shRNA to *NANOG* or *NANOGP8* with ABT-199 reveals that the two agents administered together at the start of the experiment are more potent than when the treatments are administered sequentially (Fig. 5). These findings suggest that the combination of inhibition of the *NANOGs* could be administered on the same day in the clinic rather than on separate days. This would simplify preclinical testing of this combination. The proof of the principle was validated *in vivo* with the demonstration that transduction of a colorec-

tal carcinoma xenograft with either LVshNG-1 or shNp8-1 enhanced systemic therapy with a BH3 mimetic (Fig. 1).

The clonogenic regrowth experiment performed in this study demonstrates that treating the colorectal carcinoma cell lines with ABT-737 alone (in Clone A and LS174T cells) or the combination of LVshNG-1 or LVshNp8-1 and ABT-737 (in all 3 cell lines) decreased the ability of the treated cells to regrow when replated in complete culture media. This experiment indicates that the colorectal carcinoma cells treated with these agents have reduced capacity to form colonies (clonogenicity) in normal media. Clonogenicity is associated with the stem cell nature of colorectal carcinoma (34), and the decrease in clonogenicity has been used as an indicator of a decrease in stem cell nature or self-renewal potential of different cancers (35–37). Inhibition of *NANOG* or *NANOGP8* decreases the proliferation and self-renewal capacity of colorectal carcinoma and other cancers (26, 29, 38). Inhibition of antiapoptotic BCL-2 family members by other BH3 inhibitors, ABT-263 and sabutoclax, selectively killed stem cells in leukemias (39, 40). Taken together these findings suggest that the combination of shRNA against *NANOG* or *NANOG P8* and BH3 mimetics may target cancer stem cells and decrease the self-renewal capacity of these colorectal carcinoma cell lines.

In summary, inhibition of *NANOG* and *NANOGP8* by gene therapy combined with a BH3 mimetic may provide a rationale for new therapy regimen for colorectal cancers and target the stem cell properties of colorectal cancer cells, which may be essential in treatment and prevention of relapse of this resistant cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The opinions expressed in this article are those of the authors and do not necessarily represent those of the National Cancer Institute, the NIH, the Department of Health and Human Services, or the Department of the Army.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.R. Mattoo, J. Zhang, L.A. Espinoza, J.M. Jessup

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