Characterization of the Cytotoxic Activities of Novel Analógues of the Antitumor Agent, Lavendamycin¹

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Abstract

Lavendamycin is a bacterially derived quinolinedione that displays significant antimicrobial and antitumor activities. However, preclinical development of lavendamycin as an anticancer agent was halted due to the poor aqueous solubility and relatively nonspecific cytotoxic activity of this compound. In this report, we have examined the cytotoxic activities of a series of novel lavendamycin analogues. The cytotoxic activities of these compounds were evaluated in clonogenic survival assays with A549 lung carcinoma cells. Compounds bearing an amide or amine substituent at the R² position were the most potent inhibitors of colony formation. MB-97, the most active member of this subgroup, decreased clonogenic outgrowth by 70% at a concentration of 10 nm. Treatment of A549 cells with MB-97 led to an increase in p53 protein expression and phosphorylation and a concomitant increase in the expression of the p53 target gene, p21. Exposure of p53-positive cells to MB-97 triggered cell cycle arrest in G₁ and G₂ phases but induced a selective G₂-phase arrest in p53-negative cells. MB-97 treatment also induced a higher level of apoptosis in p53-null cells relative to their p53-positive counterparts. Finally, MB-97 showed significant cytotoxic activity in the National Cancer Institute’s panel of 60 cancer cell lines and antitumor activity in vivo in hollow fiber tumorigenesis assays.

Introduction

Lavendamycin is a bacterially derived antibiotic that was isolated from Streptomyces lavendulae in 1981 (1). In initial studies, lavendamycin displayed modest antimicrobial activity and significant antiproliferative effects against various cancer cell lines in vitro (2, 3). At micromolar concentrations, lavendamycin strongly inhibited the proliferation of P388 murine leukemia cells, MKN45 gastric carcinoma cells, and WiDr colon carcinoma cells (3). Lavendamycin also displayed antitumor activity against P-388J leukemia cells implanted into BDF1 mice (2). Although the cytotoxic mechanism has not been defined, structure-activity studies have shown that the 7-aminoquinolinedione moiety is essential for this activity (4). Despite the interest in lavendamycin as an anticancer agent, the preclinical development of this drug was halted due to its poor aqueous solubility and high level of nonspecific cytotoxicity toward nontransformed cells.

Efforts to synthesize lavendamycin derivatives with improved physicochemical properties and therapeutic indices proved more challenging than anticipated. However, Behforouz et al. (5–7) developed a facile, robust synthetic strategy that allowed the preparation of more than 100 analogues based on the lavendamycin ring system as the chemical scaffold. Initial screens for antiproliferative activity against cancer cell lines revealed 35 compounds with efficacies comparable to or greater than that of the parent compound, lavendamycin.

In the present study, we have further analyzed the cytotoxic activities of the 10 lavendamycin derivatives that displayed the most promising combination of aqueous solubility and cell growth inhibition in the preliminary screens. Our results indicate that specific substituents on the lavendamycin ring system make important contributions to the cytotoxic activities of these compounds. One compound, termed MB-97, was selected for further evaluations based on its potency as an inhibitor of human A549 lung carcinoma cell survival in clonogenic assays. Our results indicate that MB-97 exposure triggers the activation of p53 in A549 cells, which suggests that this compound inhibits cell growth through the induction of DNA damage and cell cycle checkpoint activation. Accordingly, MB-97-treated A549 cells accumulated in the G₁ and G₂ phases of the cell cycle, which is consistent with the activation of both the G₁ and G₂ checkpoints due to genotoxic stress. In contrast, treatment of a p53-deficient A549 subline with MB-97 triggered a selective arrest at the G₂ checkpoint and a moderate increase in apoptotic cell death over that observed in the p53-positive, parental A549 cell line. Finally, we summarize the activity profile for MB-97 in the NCI’s³ 60-cell line screen for in vitro

¹ The abbreviations used are: NCI, National Cancer Institute; FBS, fetal bovine serum; IR, ionizing radiation; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DTP, Developmental Therapeutics Program, NCI; GC₅₀, 50% growth-inhibitory concentration; TGI, concentration that resulted in total growth inhibition; LC₅₀, concentration that resulted in 50% cytotoxicity; PI, propidium iodide.

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cytotoxic activity, as well as the results of initial assays for in vivo antitumor activity.

Materials and Methods

Synthesis of Lavendamycin Analogues. Compounds MB-21, MB-47, and MB-51 were synthesized as described by Behforouz et al. (5–7). Methods of synthesis for compounds MB-97, MB-119, MB-121, MB-311, MB-333, MB-323, and MB-331 will be published elsewhere and are available upon request. These derivatives were purified by recrystallization and/or chromatographic techniques. Chemical structures and purity were confirmed by infrared spectroscopy, nuclear magnetic resonance spectroscopy, and high-resolution mass spectrometry techniques. For experiments, MB-333 was dissolved in H2O. All other compounds were dissolved in DMSO at a final concentration of 1 mM, and DMSO stock solutions were stored at 4°C.

Cell Lines and Antibodies. All cell lines tested were of human origin and, unless indicated otherwise, were obtained from the American Type Culture Collection (Manassas, VA). A549 lung carcinoma cells and MG-63 osteosarcoma cells were grown in DMEM supplemented with 10% FBS. The prostate cancer cell lines, DU-145 and PC-3, were cultured in RPMI 1640 supplemented with 10% FBS. MCF-7 breast cancer cells were grown in MEM supplemented with 10% FBS, 1 mM pyruvate, and 0.1 mM nonessential amino acids. The A549-LXSN (p53-positive) and A549-E6 (p53-deficient) cell lines were kindly provided by Dr. Denise Galloway (University of Washington, Seattle, WA) and cultured in DMEM supplemented with 10% FBS. Unless indicated otherwise, cells were plated 1 day before drug treatments. Antibodies specific for p53 (catalogue number OP43) and the phosphorylated serine-15 (pSer15) residue in p53 (catalogue number PC386) were purchased from Oncogene (Boston, MA). Polyclonal α-p21 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-histone H3-specific antibodies were obtained from Upstate Biotechnology, Inc. (Waltham, MA).

Clonogenic Assays. Cells were harvested from log-phase growing cultures and plated at densities of 500–1000 cells/60-mm dish. After 24 h, the cells were treated with lavendamycin analogues or drug vehicle. After 10–14 days in culture, the medium was removed, and adherent colonies were fixed and stained with methanol containing 0.1% (w/v) Coomassie Blue dye. The plates were photographed with a digital camera, and staining intensities were quantified with the Image-Pro Plus software program (Media Cybernetics, Silver Spring, MD).

For replating assays, 5 × 10⁵ cells were transferred into a 100-mm tissue culture dish. After 24 h, cells were exposed to the indicated concentrations of MB-97 or the DMSO vehicle. After 48 h, cells were harvested by trypsinization, counted, and replated at 500 or 1000 cells/60-mm dish. Colonies were stained after 12 days and quantified by digital imaging as described above.

Immunoblotting. One million A549-LXSN or A549-E6 cells were treated with IR from a ¹³⁷Cs source or with MB-97 or DMSO only. IR-treated cells were harvested at 4 h postirradiation, and drug-treated cells were harvested after an additional 24 h in culture. The adherent cells were harvested by scraping into 0.5 ml of lysis buffer [50 mM HEPES (pH 7.4), 300 mM sodium chloride, 0.5% NP40, 1 mM magnesium chloride, 1.5 mM EGTA, 1 mM DTT, 0.1 mM sodium orthovanadate, 10 ng/ml microcystin, 5 µg/ml aprotinin, and 5 µg/ml leupeptin]. After centrifugation to remove insoluble material, the cellular extract (100 µg of protein) was resolved by SDS-PAGE, and the proteins were blotted onto a polyvinylidene difluoride membrane (Millipore). The protein blots were probed sequentially with α-p53, α-pSer15 p53, and α-p21 antibodies. The α-p53 blots were reacted with horse-radish peroxidase-labeled sheep α-mouse antibody, and the α-pSer15 p53 and α-p21 blots were reacted with horseradish peroxidase-labeled protein A (Amersham Life Science, Piscataway, NJ). The protein blots were then developed with Renaissance chemiluminescence reagent (NEN Life Science Products, Boston, MA), and immunoreactive bands were visualized by exposure to Kodak Biomax ML film (NEN Life Science Products).

Cell Cycle Analysis. Cells were exposed for 24, 48, or 72 h to either MB-97 or drug vehicle (DMSO) only and then detached by trypsinization. After washing in PBS, the cells were fixed in ice-cold 70% ethanol in PBS. The fixed cells were washed with PBS and then resuspended in PBS containing 20 µg/ml PI and 50 µg/ml RNase A. After incubation for 30 min at 37°C in the dark, samples were analyzed by flow cytometry (FACS-Calibur; Becton Dickinson, Franklin Lakes, NJ).

Apoptosis Assays. After treatment with MB-97 or DMSO vehicle for various times, cells were harvested by trypsinization, washed in PBS, and stained with Annexin V-FITC conjugate and PI according to the manufacturer’s protocol (PharMingen, San Diego, CA). Cells were then analyzed by flow cytometry as described above. Annexin V-positive/PI-negative cells are in the early stages of apoptosis, whereas the annexin V-positive/PI-positive subpopulation marks late-stage apoptotic cells.

Mitotic Indices. Cells were treated with the indicated concentrations of MB-97 or DMSO vehicle for 24 h. After 30 min, nocodazole (500 ng/ml) was added to trap cycling cells in M phase. Cells were harvested after 24 h, washed in PBS, and fixed in 95% ethanol/5% acetic acid. After washing with PBS, samples were resuspended in blocking solution (8% BSA) and incubated for 1 h. Samples were subsequently incubated with 5 µg/ml α-phospho-histone H3 antibody in 1% BSA overnight at 4°C. Samples were then washed and incubated for 1 h at room temperature with FITC-conjugated goat antirabbit IgG antibody diluted 1:100 in 1% BSA. After washing with PBS and incubating with 20 µg/ml PI and 50 µg/ml RNase A in PBS at 37°C for 30 min in the dark, samples were analyzed by flow cytometry. Mitotic index was calculated as the percentage of post-S-phase cells (i.e., cells with 4N DNA content) that stained positive for phospho-histone H3.

NCI Cancer Cell Line Screen and in Vivo Tumorigenesis Assays. More than 40 of the newly synthesized lavendamycin analogues were screened by the NCI’s Cancer Drug Discovery and Development Program. Compound MB-97 was evaluated for in vitro growth-inhibitory activity against
The NCI’s panel of 60 human cancer cell lines (8). Each compound was tested over a 5-log concentration range. Exponentially growing cells were exposed to drug for 48 h, and then total cell mass was estimated with a sulforhodamine B protein assay (9). The hollow fiber-based in vivo tumor-igenesis assay was performed as described previously (10). Briefly, human tumor cells were sealed into polyvinylidene hollow fibers and then implanted into the peritoneal or s.c. cavity of 5–6-week-old NCr nu/nu mice. The mice were treated with drug daily for 4 days. The fibers were recovered from the animal, and the viable cells were determined with a MTT-based colorimetric assay.

Results

Effects of Lavendamycin Ring Substituents on Cytotoxic Activity. For the present studies, we selected 10 lavendamycin analogues from a total of more than 100 novel derivatives synthesized by Behforouz et al. (5–7). The compounds were numbered based on the order of synthesis, and the selection for further testing was based on a combination of favorable aqueous solubility and cytotoxic activity in preliminary screens. The selected compounds contained the pentacyclic (ABCDE) ring structure found in lavendamycin, with various substituents at positions R1 through R4 of the ABCDE nucleus (Table 1 and Fig. 1). For the selected compounds, the R1 substituents were NH2, CH3CONH, or C3H7CONH; the R2 substituents were H, OCH3, or Cl; the R3 substituents were CONH2, CON(CH2)4 (pyrrolidine ring), CO2CH3, CO2Na, CO2(CH2)7CH3, or CONHC4H9; and the R4 groups were either H or CH3.

The cytotoxic activities of the selected compounds were examined in clonogenic survival assays with A549 human lung carcinoma cells as the test cell line. These cells are p53 positive and undergo a p53-dependent G1 cell cycle arrest after exposure to genotoxic stress (11). The cells were plated and treated with varying concentrations (1–1000 nm) of the lavendamycin analogues and DMSO vehicle. For presentation purposes, the compounds were divided into three groups based on activity in the clonogenic assays. Analogues MB-331 and MB-333 (group I) showed no cytotoxic activity at concentrations of <100 nm, and analogue MB-51 (group I) failed to affect clonogenic survival at concentrations of <1000 nm (Fig. 2). Compounds MB-47, MB-311, MB-21, and MB-121 (group II) caused clear suppression of clonogenic survival at drug concentrations of 100–349 nm (Fig. 2). The most active member of this group was MB-121, which reduced colony outgrowth by 70% at a concentration of 100 nm, with complete inhibition of colony formation observed at a drug concentration of 1000 nm. The group III compounds (MB-119, MB-323, and MB-97) were the most potent inhibitors of A549 cell colony formation. Compound MB-323 reduced colony outgrowth by 90% at a concentration of 100 nm and virtually abrogated clonogenic activity at a concentration of 1000 nm. MB-97 was the most potent analogue of this series, reducing colony outgrowth by approximately 70% and 100% at concentrations of 10 and 100 nm, respectively.

Cytotoxic Activities against Other Cancer Cell Lines. To extend the results to other human tumor cell lines, we tested the two most potent compounds, MB-323 and MB-97, from the above screen against several distinct human cancer cell lines, including PC-3 and DU-145 (prostate cancer), MCF-7 (breast cancer), and MG-63 (osteosarcoma). Interestingly, PC-3 cells were particularly sensitive to the cytotoxic activities of MB-323 and MB-97, despite the fact that these cells are relatively resistant to many established anticancer agents (12, 13). For example, treatment with 10 nm MB-323 decreased PC-3 cell clonogenicity by >80%, whereas exposure to this drug concentration caused little or no reduction in colony outgrowth with MCF-7, MG-63, and DU-145 as the test cell lines (Fig. 3A). In contrast, the clonogenic survival of MG-63, DU-145, and PC-3 cells was uniformly sensitive to MB-97, with all three cell lines showing a >70% decrease in colony outgrowth after treatment with 10 nm MB-97. The same concentration of MB-97 reduced the

<table>
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<th>Abbreviation</th>
<th>R1</th>
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<td>H</td>
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Fig. 1. Pentacyclic (ABCDE) ring structure found in lavendamycin.
clonogenic survival of MCF-7 cells by only 20%, indicating that MCF-7 cells were relatively resistant to this lavendamycin analogue. However, this drug sensitivity difference largely disappeared when the cancer cell lines were exposed to a 1-log higher concentration (100 nM) of MB-97. Of note, MG-63, DU-145, and PC-3 cells harbor mutant p53 alleles (14, 15), whereas the MCF-7 breast cancer cell line expresses functional p53 protein in response to genotoxic stress (16). These results suggested that p53 activation partially protects cells against the cytotoxic effects of MB-97.

Lavendamycin Analogue MB-97 Activates the p53 Tumor Suppressor. The p53 tumor suppressor protein is activated during cellular stress induced by a diverse array of stimuli (17). To determine whether exposure to lavendamycin-related compounds triggers p53 activation, we analyzed the expression level of p53, as well as the phosphorylation of this protein at Ser\(^{15}\), after cellular exposure to MB-97. Previous studies have shown that p53 is phosphorylated at Ser\(^{15}\) in response to genotoxic stress and that this modification is mediated by the DNA damage-responsive checkpoint kinases, ATM and ATR (18–20). Although the exact function of Ser\(^{15}\) phosphorylation remains unclear, this modification may promote both the stabilization and transactivating functions of p53 in damaged cells (21). To examine the role of p53 activation in cellular responses to MB-97, we took advantage of an isogenic model system based on the A549 lung cancer cell line (22). As indicated above, the parental cell line expresses functional p53 when exposed to DNA-damaging agents. These cells were infected with a recombinant amphotrophic retrovirus (LXSN) encoding the human papilloma virus E6 protein, which continuously targets p53 for degradation by the ubiquitin-proteasome pathway. The resulting A549-E6 subline is therefore functionally deficient for p53 function. To generate a control (p53-positive) cell line, A549 cells were infected with nonrecombinant virus to produce the A549-LXSN subline. These isogenically matched A549 sublines were then comparatively evaluated for their responses to MB-97.

For the biochemical assays, logarithmically growing cultures of A549-LXSN cells and A549-E6 cells were treated for 24 h with 1 \(\mu\)M MB-97, and detergent extracts were immunoblotted with p53-specific antibodies. Exposure of A549-LXSN cells to MB-97 led to the accumulation of p53 and stimulated the phosphorylation of this protein at Ser\(^{15}\) (Fig. 4). The induction of p53 by MB-97 was dependent on the drug concentration, with readily detectable increases in this protein observed after treatment with 10 nM MB-97 (data not shown), and the maximal response was obtained at the 1 \(\mu\)M drug concentration shown in Fig. 4. The drug-treated cells also expressed elevated levels of the cyclin-dependent kinase inhibitor p21, which is the product of a known p53 target gene (23). Thus, exposure of A549-LXSN cells to MB-97 triggered the appearance of the Ser\(^{15}\)-phosphorylated, transcriptionally active form of p53 in A549-LXSN cells. Similar results were obtained with cells that had been treated...
with 25 Gy of IR, which suggests that MB-97, like IR, triggers a DNA damage response program leading to p53 activation in A549-LXSN cells. In contrast, the A549-E6 cells showed lower basal and drug-inducible levels of p53 and no detectable induction of Ser15 phosphorylation or p21 expression (Fig. 4). The residual p53 expression observed in A549-E6 cells indicates that the rate of p53 protein synthesis exceeds the rate of HPV-E6-mediated p53 degradation. Thus, we cannot rule out the possibility that these cells retain a low level of p53 function, despite the complete block of p21 expression. Regardless, these results show that exposure to a cytotoxic concentration of MB-97 activates the p53 stress response program in A549 cells. These results suggest that MB-97 exposure, like IR treatment, induces DNA damage and genotoxic stress responses in human cancer cell lines.

Effect of MB-97 on Cell Cycle Progression. The stimulatory effects of MB-97 on p53 and p21 protein expression suggested that the drug activates a stress-induced cell cycle checkpoint. Because p53 and p21 play critical roles in establishment of the G1 checkpoint (17), we hypothesized that cells expressing p53 should accumulate in G1 in response to lavendamycin treatment, whereas cells lacking p53 should not. To test this hypothesis, A549-LXSN and A549-E6 cells were treated with 1 \( \mu M \) MB-97 for 24, 48, or 72 h, and cell cycle distributions were analyzed by PI staining and flow cytometry. As shown in Fig. 5, A549-LXSN cells accumulated in G1 and G2-M phases of the cell cycle after 24 h of MB-97 exposure, and this cell cycle arrest profile was maintained for at least 72 h of continuous drug exposure. We observed no increase in hypodiploid cells in the drug-treated cultures, suggesting that MB-97 did not trigger apoptotic death of A549-LXSN cells over the course of these assays. In contrast, A549-E6 cells accumulated primarily in S and G2-M phases at 24 h after treatment with MB-97. After 72 h of drug exposure, the reappearance of a G1 peak suggested that, unlike their p53-proficient counterparts, the p53-deficient A549 cells had passed through the G2-M checkpoint and had resumed cycling (Fig. 5). Notably, these cell cycle responses were accompanied by the emergence of hypodiploid cells, which is consistent with intra- or postmitotic cell death due to apoptosis (see below).

Lavendamycin Analogue MB-97 Prevents Both p53-positive and p53-negative Cells from Entering Mitosis at 24 h. Although we had observed that MB-97-treated A549-E6 cells accumulated with 4N DNA content, it was unclear whether these post-S-phase cells resided in G2 or M phase of the cell cycle. We therefore assayed the effect of MB-97 on the mitotic indices of A549-LXSN and A549-E6 in log phase cultures. Cells were treated for 24 h with varying concentrations of MB-97 and then stained with a-phospho-histone H3 antibody to mark mitotic cells (24). Phosphohistone H3-positive cells were enumerated by flow cytometry. The microtubule poison nocodazole was included in the treatment with MB-97 to trap cycling cells in mitosis. Cells treated with nocodazole only served as the positive control. As shown in Fig. 6, both A549-LXSN and A549-E6 cells displayed a drug concentration-dependent reduction in the percentages of mitotic cells after exposure to MB-97. These results indicate that MB-97 treatment induces a block to mitotic entry, which is consistent with the activation of the G2-phase DNA damage checkpoint in both the p53-positive and p53-deficient A549 sublines.

Activation of Apoptosis by the Lavendamycin Analogue MB-97. The relative sensitivities of A549-LXSN and A549-E6 cells to MB-97-induced apoptosis were further examined by flow cytometric determination of Annexin V-positive cells at 48 h and 72 h after drug exposure. An increase in cellular staining with FITC-conjugated Annexin V serves as an early marker for apoptosis (25). The cells were simultaneously stained with PI to score for overt loss of cell membrane integrity. The double staining procedure distinguishes early-stage apoptotic cells (Annexin V positive, PI negative) from late-stage apoptotic cells (annexin V positive, PI positive). As shown in Fig. 7, treatment of the A549-LXSN cells with 1 \( \mu M \) MB-97 triggered moderate increases in the percentages of early-stage apoptotic cells after either 48 or 72 h. However, a substantially greater percentage of the A549-E6 population became annexin V positive after MB-97 exposure, particularly at the earlier (48 h) time point (Fig. 7). Thus, these results confirm that loss of p53 function sensitizes A549 cells to the proapoptotic effects of MB-97.

Clonogenic Survival of p53-positive and -deficient Cells after Transient MB-97 Exposure. To further characterize the relative cytotoxic activities of MB-97 toward p53-positive versus p53-deficient cells, we treated the A549-LXSN and A549-E6 cell lines with 0.1 or 1 \( \mu M \) MB-97. After 48 h, the cells were harvested and replated at low density in drug-free medium, and colony formation was assayed after 12 days (Fig. 8). Under these drug treatment conditions, the A549-E6 cells were more sensitive to killing by 100 \( \mu M \) MB-97 than were their p53-positive A549-LXSN cell counterparts. At the higher drug concentration (1 \( \mu M \)), MB-97 dramatically reduced colony outgrowth in both the A549-E6 and A549-LXSN cell populations, although the increased drug sensitivity of the A549-E6 cell line was still evident in these samples. These results indicate that transient exposure to MB-97 is sufficient to commit A549 cells to irreversible cell cycle.
arrest and/or apoptosis and that p53-deficient cells are more sensitive to the persistent cytotoxic activity of MB-97.

Assessment of Lavendamycin Analogue MB-97 Efficacy in NCI in Vitro and in Vivo Screening. A sample of MB-97 was submitted to the DTP of the NCI for further evaluation. The DTP has established a three-tiered test structure for candidate drug evaluation that includes (a) in vitro testing against a panel of 60 human cancer cell lines (tier 1), (b) in vivo testing in mice with a hollow fiber implant assay (tier 2), and (c) in vivo testing against tumor xenografts in immunocompromised mice (tier 3). From the tier 1 assays, we obtained valid antiproliferative data for MB-97 against 50 of the cancer cell lines in the NCI’s 60-cell line panel.

For the sake of brevity, we present the data obtained from renal, ovarian, and colon cancer cell lines representing three of the seven adult tumor types tested in the assay. We have organized the data in the “mean graph profile” format, which is a graphic presentation tool developed by the DTP to provide convenient view of the sensitivity of each cell line to a test drug, relative to the mean sensitivity of all cell lines tested in that experiment. As shown in Fig. 9, three response parameters were calculated for each cell line: (a) GI_{50}; (b) TGI; and (c) LC_{50}. The data collected for each of these parameters is represented by a separate bar graph. The arithmetic mean for each of the response parameters tested is indicated at the top of the graph and is symbolized by a drop-down vertical bar. The sensitivity of each cell line tested, compared with the mean, is represented by a horizontal bar to the left or the right of the mean graph midpoint. Bars extending to the left of the mean graph midpoint indicate that the cell line was more resistant to the effects of MB-97 relative to the mean, whereas bars extending to the right indicate an increased sensitivity of the cell line to MB-97 compared with the mean.

As observed in the earlier studies, MB-97 showed significant activity at submicromolar concentrations, with a mean GI_{50} of 229 nM. Of the renal cell lines tested, the UO-31, SN12C, RXF 393, CAKI-1, ACHN, and 786- were particularly sensitive to MB-97 (in at least one of the three growth parameters), whereas the GI_{50} values for the TK-10 and A498 cell lines were higher than the average, indicating relative resistance to this drug. The ovarian cancer cell lines OVCAR-8, OVCAR-3, and IGROV1 displayed higher than average sensitivity to MB-97, whereas the remaining cell lines in this subset were relatively resistant to drug exposure. Finally, among the colon carcinoma lines, the SW-620 and HT-29 cell lines were relatively sensitive to MB-97, and the remaining five cell lines were relatively drug-resistant. Thus, different cancer cell lines display widely varying sensitivities to MB-97, which indicates that genetic or epigenetic variables among the different cell lines play determinant roles in either the response to this drug, or the recovery of the cells from MB-97-induced stress.

Based on the promising results obtained in the 60-cell line screen, MB-97 was moved forward to tier 2, the hollow fiber tumorigenesis assay with 12 human cancer cell lines. Groups of animals were treated with two different doses (100 or 150 mg/kg) of MB-97 by i.p. or s.c. injection on a daily basis, for 4 consecutive days. One day after the final drug treatment, the fibers were collected from the mice, and the resident tumor cells were assayed for metabolically active cells with a MTT assay. A reduction of 50% or greater in the MTT assay, relative to the non-drug-treated control samples, was as-
Fig. 7. Induction of apoptotic cell death by MB-97 in p53-positive versus p53-deficient A549 cells. A549-LXSN and A549-E6 cells were treated for 48 or 72 h with 1 μM MB-97 or DMSO vehicle only (Control). Cells were harvested, costained with Annexin V-FITC and PI, and analyzed by flow cytometry. Vi-able cells score as Annexin V negative and PI negative (bottom left quadrant), whereas early-stage apoptotic cells are Annexin V positive and PI negative (bottom right quadrant). Late-stage apoptotic and necrotic cells are positive for both Annexin V and PI (top right quadrant). The percentage of early-stage apoptotic cells in each sample is shown in the figure.

Discussion

In this study, we examined the cytotoxic and antiproliferative activities of several novel analogues of lavendamycin, a compound that bears structural similarity to streptonigrin, a chemotherapeutic agent that was used in humans until the 1970s but was withdrawn from clinical application because of its high toxicity (2, 26, 27). Although lavendamycin itself shows weak cytotoxic activity, this drug exhibits poor aqueous solubility and selectivity for transformed versus normal cells. Our studies of a small subset of the more than 100 lavendamycin analogues prepared by Behforouz et al. (5–7) indicate that the chemical modifications imposed on the pentacyclic lavendamycin ring structure generated compounds with significantly greater potency and higher selectivity toward p53-deficient cancer cells than was previously observed with the parent drug, lavendamycin.

Clonogenic survival experiments provided some clues regarding the contributions of certain substituents on the core pentacyclic ring structure to the cytotoxic activities of the lavendamycin analogues. For example, a comparison of the ring substituents of group I compounds with low cytotoxic activities versus the group III compounds, which were highly potent cytotoxic agents, indicates that the R2 position of ring C (see Table 1 and Fig. 1) plays a determinant role in the pharmacological activities of these compounds. Group I compounds bear an amide group at R1 and an ester, salt, or substituted amide at R3. Group III compounds have an amino or an amide linkage at R1 and an amide or a substituted amide at R3. Both subsets of lavendamycin analogues contain no substituent group at R2 and R4, which eliminates these sites as sources for the variation in pharmacological activity between group I and group III. The importance of the R3 substituent is highlighted by comparison of the group I compound MB-331 with the highly active group III analogue MB-323. MB-331 contains a butyl amide group at R3, whereas the corresponding substituent of MB-323 at R3 is an unsubstituted amide. Hence, a relatively modest change in carbon chain length at R3 translates into a dramatic difference in the activities of MB-331 and MB-323 as inhibitors of A549 cell clonogenic survival.

A further insight into the structure-activity relationship comes from a comparison of group II and III compounds. The highly active group III analogues bear amide functions at R3; in contrast, the less active group II compounds contain esters at this position. Thus, the presence of an amide group at R3 confers increased anticolonogenic activity against A549 cells. However, this conclusion is qualified by the observation that R3 amide substituents were not uniformly su-
ior to R^3 ester groups in the NCI’s 60-cell line assay. Moreover, the NCI screen revealed that the R^1 position also contributes to antiproliferative activity, with NH_2, CH_3CONH, or C_3H_7CONH groups present in the most active compounds. The most obvious explanation for these apparent discrepancies rests with important differences in assay methodology, i.e., the short-term cell growth inhibition assay used by the NCI versus the long-term clonogenic survival assay used to evaluate activity in our laboratories.

Our screen of 10 selected lavendamycin derivatives revealed that MB-97 was the most potent inhibitor of the series, with significant activities observed at submicromolar drug concentrations in both the long-term A549 clonogenic assays and the NCI 60-cell line proliferation assays. Continuous exposure to 10 nM MB-97 suppressed A549 cell colony outgrowth by >70%, and treatment with 100 nM MB-97 under the same conditions completely ablated clonogenic activity in this long-term assay. Higher concentrations of MB-97 were generally required for inhibition of cell growth in the short-term assay performed by the NCI (mean GI_{50}, 229 nM). The potency difference in the two assays likely reflects also the continuous degradation of newly synthesized protein by the ubiquitin-proteasome pathway (28). During cellular stress, both MDM2, the protein that targets p53 for proteolysis, and p53 itself undergo a complex series of posttranslational modifications that increase the stability and transactivating functions of p53. An extraordinarily broad range of stress-inducing stimuli, including DNA damage, reactive oxygen species, and oncogene activation, trigger the p53 response program in mammalian cells. Our findings with MB-97 are consistent with the conclusion that this drug provokes DNA damage responses in cancer cells. First, we noted that MB-97 exposure caused a rapid increase in the phosphorylation of p53 at Ser^15. This modification is tightly linked to the DNA damage response and is mediated by the checkpoint kinases ATM and ATR (29).

The conclusion that MB-97 exposure leads to DNA damage is also consistent with earlier results obtained with the structurally related antitumor agents streptonigrin, anthracyclines, and mitomycins (4, 30–36). These compounds share the benzoquinone pharmacophore with the lavendamycin analogues, and both streptonigrin and the anthracyclines have been characterized as DNA-damaging agents in mammalian cells.

Cell cycle distribution studies revealed a prominent role for p53-mediated checkpoint activation in the cancer cell lines that express a functional version of this protein. After 48 h of MB-97 exposure, p53-positive A549 cells accumulated primarily in G_2 and G_2-M phase, accompanied by a reduction in the numbers of S-phase cells. The accumulation of damaged cells in G_2 phase is contingent upon the activation of p53 and the subsequent expression of the cyclin-dependent kinase inhibitor p21 (23). A concomitant increase in G_2-M-phase cells was observed in both p53-positive and -deficient cell lines after MB-97 treatment. The latter response was attributable to a drug-induced blockade of G_2-to-M-phase progression. The activation of this G_2 checkpoint-mediated arrest presumably protects the damaged cells from entering into a potentially lethal mitosis. In IR-damaged cells, ATM and ATR, as well as p53, are required for the initiation and maintenance of the G_2 checkpoint (29, 37, 38). In summary, our cell cycle data suggest that MB-97 triggers cell cycle

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4 M. Behforouz and NCI, unpublished results.
arrest through the induction of the $G_1$ and $G_2$ DNA damage checkpoints in p53-positive A549 cells.

Loss of p53 expression and/or function occurs in the majority of human cancer cells (17). Previous results suggest that p53 status is centrally involved in the therapeutic response to DNA-damaging antitumor agents (39). Loss of cytotoxic p53-dependent checkpoint functions confers genetic instability and renders cells prone to the lethal consequences of cell cycle progression in the presence of extensive, unrepaired DNA damage (39). Our results with MB-97 were consistent with this model and lend further support to the idea that MB-97 acts, at least in part, as a DNA-damaging agent. We compared the sensitivities of isogenic p53-positive and p53-deficient A549 cells to MB-97 and found that the p53-deficient cells were less able to recover from a transient exposure to this drug than were their p53-proficient counterparts. At higher drug concentrations, we observed that the p53-deficient cells displayed significantly higher levels of apoptotic cell death than the p53-positive A549 cells. Thus, it appears that the cytotoxic activity of MB-97 shows a certain degree of selectivity for cells that lack p53, a favorable characteristic, should MB-97 or related compounds move forward to clinical testing.

The mechanism whereby MB-97 triggers genotoxic stress responses in A549 cells remains unclear. The quinolinedione nucleus could undergo cyclic reduction-oxidation reactions that lead to the generation of DNA-damaging free radicals, as appears to be the case for the related compound, streptonigrin (33, 35). However, preliminary evidence indicates that cytotoxic activity is poorly correlated with the reduction potentials of the quinolinedione moiety among a series of lavendamycin analogues (6). Furthermore, the lavendamycin analogues tested in vivo at the NCI showed significantly lower animal toxicity than streptonigrin and other quinolinedione-containing compounds. For example, streptonigrin has been reported to cause significant lethality in mice at single doses as low as 0.4 mg/kg and causes severe bone marrow depression in humans at daily doses of 0.4 μg/kg (26, 27, 40, 41). In contrast, toxicity studies at the NCI indicated that the maximal tolerated dose of MB-97 in mice was 400 mg/kg. In a separate study, the antitumor activity of the lavendamycin analogue MB-51 was tested in immunodeficient mice bearing established tumor xenografts (K-Ras-transformed normal rat kidney cells). At a daily dosage regimen of 300 mg/kg over 10 days, tumor mass was reduced by an average of 80% ($n = 7$ mice), with no drug-related weight loss or lethality (6). The substantially lower levels of toxicity observed with the lavendamycin-like compounds may be related to the presence of the $\beta$-carboline moiety, which is absent in other cytotoxic quinolinedione compounds, including streptonigrin.

The mechanism whereby MB-97 and other lavendamycin analogues trigger genotoxic stress responses in cycling cancer cells remains unclear. In recent studies, we have shown that cell culture under hypoxic conditions has little effect on the cytotoxic potency of MB-97, suggesting that, unlike IR, MB-97-induced cell damage does not depend on the accumulation of reactive oxygen species. A potentially relevant observation is that the COMPARE analysis of MB-97 activity against the NCI 60-cell line panel indicates that MB-97 is most similar to platinum-based analogues, although the level of correlation did not reach statistical significance (42). Platinum-based agents induce intrastrand DNA cross-links, which strongly interfere with replication fork progression during S phase and evoke an ATR-dependent checkpoint response (43). Additional studies are clearly needed to define the mechanism by which MB-97 damages DNA and the type of genetic lesion induced by this compound. The preferential cytotoxic activity of MB-97 toward p53-deficient cancer cells provides not only a starting point for further mechanistic studies but may also serve as a guide for in vivo efficacy predictions regarding the evaluation of new lavendamycin analogues with improved aqueous solubility characteristics. Given the high frequency of p53 mutations in human tumors, further optimization of the cytotoxicity ratio toward p53-deficient versus p53-positive cells could render these newer derivatives promising candidates for clinical development as antitumor agents against a broad spectrum of human cancers.

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Cytotoxic Mechanism of Lavendamycin Analogues


