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Mithramycin Inhibits Etoposide Resistance in Glucose-deprived HT-29 Human Colon Carcinoma Cells

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Abstract Physiological cell conditions such as glucose deprivation and hypoxia play roles in the development of drug resistance in solid tumors. These tumor-specific conditions cause decreased expression of DNA topoisomerase IIα, rendering cells resistant to topo II target drugs such as etoposide. Thus, targeting tumor-specific conditions such as a low glucose environment may be a novel strategy in the development of anticancer drugs. On this basis, we established a novel screening program for anticancer agents with preferential cytotoxic activity in cancer cells under glucose-deprived conditions. We recently isolated an active compound, AA-98, from *Streptomyces* sp. AA030098 that can prevent stress-induced etoposide resistance *in vitro*. Furthermore, LC-MS and various NMR spectroscopic methods identified AA-98 as mithramycin, which belongs to the aureolic acid group of antitumor compounds. We found that mithramycin prevents the etoposide resistance that is induced by glucose deprivation. The etoposide-chemosensitive action of mithramycin was just dependent on strict low glucose conditions, and resulted in the selective cell death of etoposide-resistant HT-29 human colon cancer cells.

Keywords: Glucose deprivation, solid tumor, anticancer, etoposide resistance, mithramycin

Solid tumors are especially difficult to treat because of their incomplete vascularizations [32]. *In vivo*, these cancer cells are exposed to stressful microenvironments, including low glucose levels, hypoxia, low pH, and other nutrient deprivations [11, 12, 18], which are not commonly observed in normal tissues [1, 4, 32]. Glucose deprivation, in particular, is a physiological cell condition associated with several human diseases such as tissue ischemia and cancer [28]. In addition, the aberrant microenvironment of solid tumors stems from their insufficient formation of blood vessels and is a source of drug resistance, since tumor cells that are distant from blood vessels are exposed to lower drug concentrations [21, 29, 31].

In these microenvironmental conditions, cancer cells, as well as cultured cell lines, develop resistance to antineoplastic drugs. Drug resistance tends to be correlated with decreases in topoisomerase IIα (topo IIα) levels, or with mutations in the protein that reduces enzymatic activity by altering the amount of the target enzyme from itself [23]. In addition, resistance to topo IIα inhibitors can develop from the overexpression of drug efflux pumps like P-glycoprotein [5, 6]. In a previous study, Ogiso *et al.* [24] found that stress-induced resistance to topo IIα-directed drugs was effectively prevented by lactacystin and other proteasome inhibitors. Furthermore, lactacystin significantly enhanced the antitumor activity of etoposide in a solid tumor model. It is widely recognized that hypoglycemia, hypoxia, and defects of apoptosis can generate resistance to anticancer agents, a principal problem in most cancer chemotherapies. This serious problem has meant that much effort has been spent on developing novel anticancer agents with few side effects and low cytotoxicity on normal tissues and cells [4, 16, 19, 22].

Based on this, we established a novel screening system to discover anticancer agents that can preferentially inhibit the chemoresistance of solid tumors under glucose-deprived conditions. Using this screening system, we recently isolated an active compound, AA-98, from the culture broth of *Streptomyces* sp. AA030098 that can inhibit the chemoresistance of solid tumors. By examining its structure and physicochemical properties through LC-MS analyses and various NMR spectroscopic methods, its chemical structure was identified as that of mithramycin (MM), which is from the aureolic acid group of antitumor compounds [2]. Despite its high toxicity, MM is useful for

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treating disseminated testicular carcinomas and Paget's disease [7, 8, 15]. The antitumor property of MM is probably associated with its inhibitory effects on the replication and transcription of tumor cells [10]. Recently, Tagashira *et al.* [30] reported that MM repressed the transcription of the multidrug resistance 1 (*MDR1*) gene in SBC-3/ADM cells, and that MM decreased P-glycoprotein (Pgp) in MDR tumor cells and sensitized the cells to adriamycin.

In this paper, we investigated the striking selective cytotoxicity that MM exhibits towards etoposide resistance under low glucose conditions. Furthermore, we have suggested that a combination of MM and etoposide may improve drug efficacy within the microenvironmental conditions of solid tumors.

MATERIALS AND METHODS

Materials

The AA030098 strain was obtained from the Functional Metabolomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was provided by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Etoposide was commercially available, and was purchased from Korea United Pharma Inc. (Korea). All organic solvents, which were used in isolated and purified forms, were purchased from Duksan Pure Chemical Co. (Korea) and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Microorganism and Culture Conditions

Strain AA030098 was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of modified Bennett's agar medium consisting of 1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% beef extract, and 1.5% agar, adjusted to pH 7.0 and incubated at 28°C for 5 days on a rotary shaker at 140 rpm.

Cell Culture and Treatments

HT-29 and SW620 human colon cancer cells were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). These cell lines were maintained in RPMI1640 medium (containing 2 mg of glucose/ml; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 μg/ml $NaHCO_3$ at 37°C in a humidified atmosphere containing 5% CO_2. 2-Deoxyglucose (2DG, Sigma) was added to the culture medium at the final concentration of 20 mM. Glucose deprivation was performed by substituting a glucose-free RPMI1640 (Gibco) supplemented with 10% heat-inactivated FBS as described previously [24, 25].

Morphological Analysis

HT-29 cells (1×10^5 cells/ml) were plated in 6-well plates, and after exposure to 2DG- and glucose deprivation-stressed conditions for 18 h, cells were treated with 20 μg/ml of etoposide as an anticancer agent. The cellular morphology was observed using a phase-contrast microscope (Nikon, Japan). Photographs were taken at a magnification of ×100.

MTT Reduction Assay

Cell viability was measured with blue formazan that was metabolized from MTT by mitochondrial dehydrogenase, which is active only in live cells [27]. HT-29 and SW620 cells were preincubated in 96-well plates at a density of 1×10^5 cells/ml for 24 h in a humidified atmosphere of 5% CO_2 in 37°C. Cells were pretreated with various concentrations of MM. After 30 min incubation, 2DG, a chemical stress, was added to the wells at a final concentration of 20 mM, and the plates were reincubated. After incubation for 24 h, MTT reagent (5 mg/ml) was added to each of the wells, and the plate was incubated for an additional 1 h at 37°C. The media were then removed, and the intracellular formazan product was dissolved in 100 μl of DMSO. The absorbency of each well was then measured at 540 nm using the ELISA reader (BioRad, Model 680, U.S.A.), and the percentage viability was calculated.

Colony Formation Assay

The sensitivity of HT-29 cells to etoposide was measured using the colony formation sensitivity assay, performed as follows: 1×10^5 cells/ml were seeded into 6-well plates. At 24 h after seeding, cells were treated with MM in the presence or absence of glucose and 20 mM of 2DG, and the plates were incubated for 18 h. For the colony formation assay, cells were treated with etoposide for 4 h. The cells were then diluted in fresh medium lacking MM, reseeded at 1×10^3 cells/well in 6-well plates, and cultured under normal growth conditions for 7–8 days to form colonies. Formed colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted [24, 25]. Cell survival (mean values with 95% confidence intervals from triplicate determinations) was calculated by setting the survival of control cells (*i.e.*, none treated with MM) as 100%. IC_{50} values (concentration required for 50% inhibition of colony formation) were determined from the dose-response curves of colony formation inhibition.

RESULTS

Acquirements of Chemoresistance to Etoposide Under Glucose-deprived HT-29 Cells

HT-29 cells have shown a strong resistance to etoposide under glucose-deprived conditions [26], and since HT-29

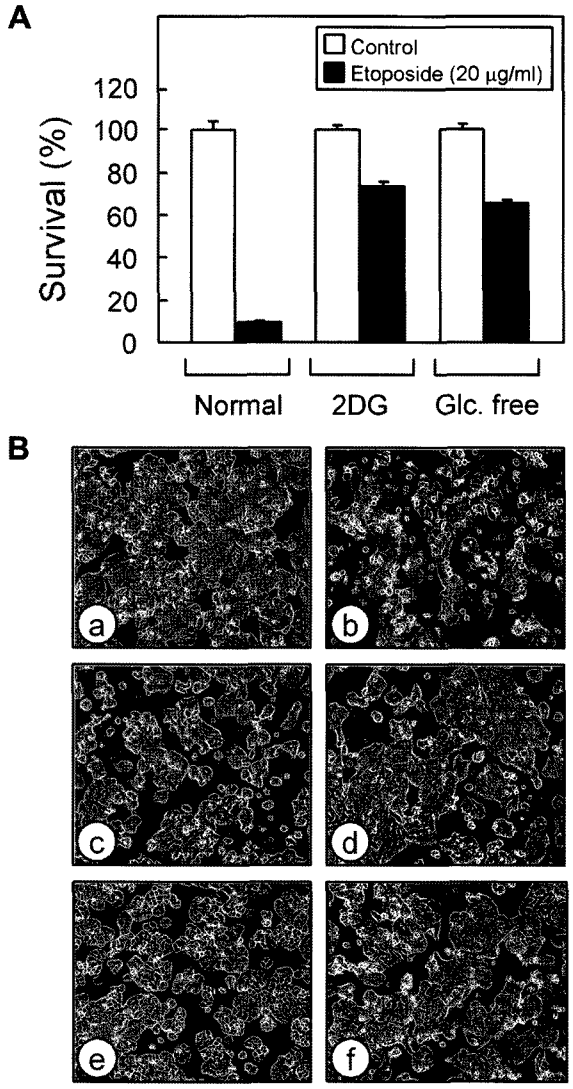

Fig. 1. Cellular sensitivity to etoposide on 2DG- or glucose deprivation-stressed HT-29 cells.

A. HT-29 cells were cultured for 24 h under normal and low glucose conditions. During the last 4 h of treatment, cells were exposed to 20 μg/ml of etoposide. After colony formation assay, survival factors (means±SD of triplicate determinations) were calculated by comparison with each of the control survival factors in the absence of etoposide. **B.** HT-29 cells were exposed to etoposide for 48 h under 2DG and glucose-deprived conditions. a, control; b, etoposide (20 μg/ml); c, 2DG (20 mM); d, 2DG/etoposide; e, glucose deprivation; f, glucose deprivation/etoposide. Photographs were taken at a magnification of ×100. The results are representative of three independent experiments.

cells were originally derived from human colon cancer cells, they have shown strong tolerance to glucose deprivation *in vitro* and *in vivo*, as found previously [24, 36]. To investigate etoposide chemoresistance, we performed a colony formation assay. HT-29 cells were cultured for 18 h under normal or low glucose (2DG and glucose-free medium, respectively) conditions. Etoposide was then added to each medium at a concentration of 20 μg/ml for 4 h. In the case of the low glucose conditions, the colony-forming ability of the etoposide-treated cells at the indicated concentration was approximately 70%, but was less that 10% under normal conditions (Fig. 1A). We then investigated the morphological changes of the etoposide-treated cells under the same conditions. As shown in Fig. 1B, after 48 h of incubation with 20 μg/ml of etoposide under normal growth conditions (*upper panel*), many of the cells exhibited cytoplasmic shrinkage, and either detached from each other or floated in the medium. In contrast, under the 2DG-stressed (20 mM; *middle panel*) and glucose-deprived (*lower panel*) conditions, etoposide exhibited no effect on microscopic cell morphology, indicating that the glucose-deprived HT-29 cells have obtained chemoresistance to etoposide.

Isolation and Purification of Mithramycin

Here, we used a culture broth of *Streptomyces* sp. AA030098 to carry out the purification of the active compound against etoposide-resistant HT-29 cells. This broth was obtained from 10 l of the culture and extracted with acetone. The extract was then concentrated *in vacuo* to eliminate acetone, and the active principle was extracted with ethyl acetate. The solvent layer was dried over Na_2SO_4, and concentrated to give an oily residue. The organic layer was concentrated and applied to a column of silica gel eluted with $CHCl_3$:MeOH (5:1), and the active fractions were collected and concentrated under reduced pressure to give a brown oil (87 mg). The active eluate was then chromatographed on a Sephadex LH-20 column eluted with 100% MeOH. The active fractions were concentrated to give a yellow powder (25 mg). Finally, the pure compound was obtained by HPLC (Shimadzu Co. Ltd, Japan) using a CLIPEUS ODS-A column (250×4.6 mm, Higgins Analytical, Inc., U.S.A.) eluted with 40% CH_3CN to give a yellow powder (3 mg).

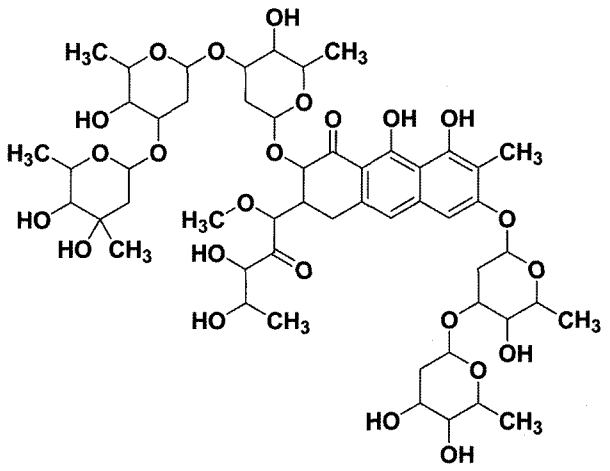

Fig. 2. Chemical structure of mithramycin (MM).

The active compound, AA-98, inhibited the drug resistance to etoposide that was induced by a variety of cellular stresses such as 2DG-treated and glucose-deprived conditions. Using LC-MS spectroscopy, the molecular formula of AA-98 was found to be $C_{52}H_{76}O_{24}$ (data not shown). The structure of AA-98 was determined by its physicochemical properties and by various NMR spectroscopic methods. Through a database and literature search, we found that AA-98 had the same structure as mithramycin, which was previously reported as a member of the aureolic acid group (Fig. 2) [2].

Selective Cytotoxicity of Mithramycin to Etoposide-resistant HT-29 Cells

To investigate the etoposide-chemosensitive activity of MM under glucose-deprived conditions, we examined the effects of MM on cell viability by the MTT reduction assay. HT-29 and SW620 cells were pretreated with the indicated concentrations of MM. These cells were originally derived from human colon cancer cells, and were chosen as representatives of a hypovascular tumor. Under normal and 2DG-stressed conditions, 24-h MM treatment of the HT-29 and SW620 cells had only a general effect on cell viability (Figs. 3A and 3B). Furthermore, MM has the same effect on HeLa (human cervical adenocarcinoma) and MCF-7 (human breast adenocarcinoma pleural effusion) cells (data not shown).

To evaluate whether the specific cytotoxic activity of MM could inhibit the development of etoposide resistance, we next examined the effects of MM on cell viability under etoposide-resistant conditions. The HT-29 cells, in the presence or absence of 2DG (20 mM), were exposed to MM at the indicated concentration for 18 h, and then etoposide (20 μg/ml) was added to the medium for 4 h. Cell survival was measured using colony formation assays.

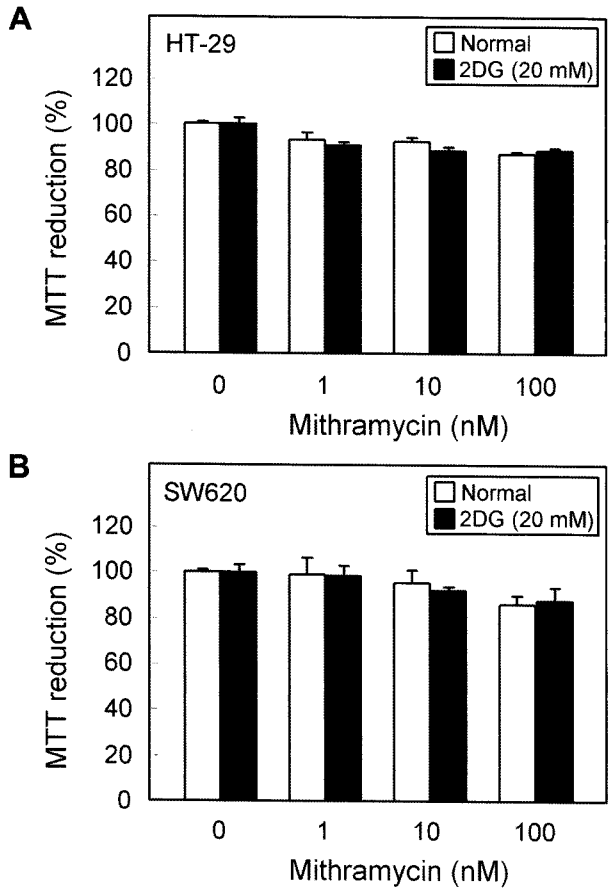

Fig. 3. Effects of mithramycin on cell viability under normal growth and 2DG-stress conditions.

Cell viability was measured with the MTT reduction rate in the HT-29 (**A**) and SW620 (**B**) human colon cancer cells, respectively. The cells were exposed to the indicated concentration of mithramycin for 24 h. After MTT assay, the MTT reduction rate was calculated by setting each of the control survivals. Data (means±SD of triplicate determinations) are representative of at least three independent experiments.

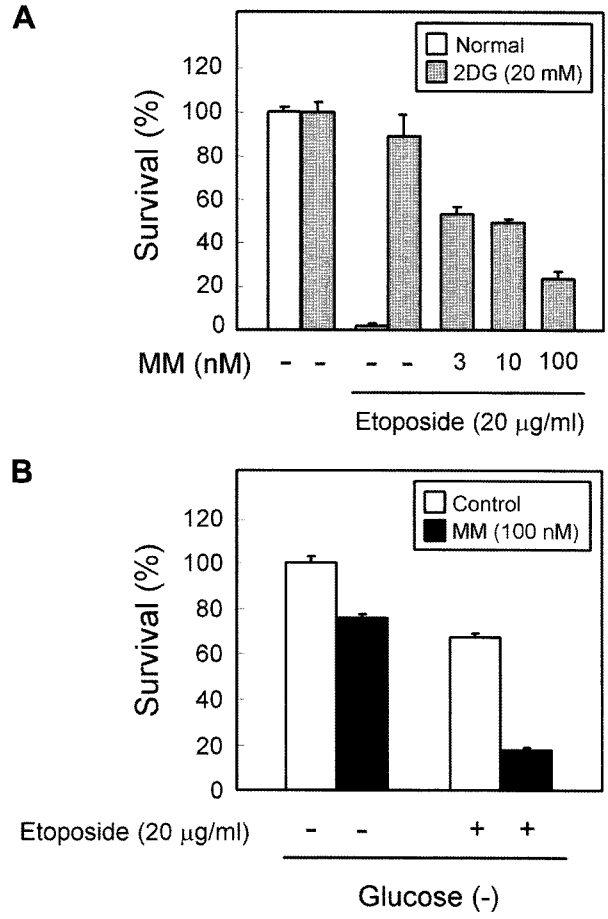

Fig. 4. Effect of mithramycin on etoposide resistance under low glucose conditions.

HT-29 cells were exposed to the indicated concentration of MM for 24 h in the presence or absence of 2DG (20 mM) (**A**) and glucose (**B**). During the last 4 h of treatment, cells were exposed 20 μg/ml of etoposide. After the colony formation assay, survival factors (means±SD of triplicate determinations) were calculated by setting each of the control survival rates as those under normal growth or stress conditions.

Under the 2DG-stressed condition, MM was highly toxic in the etoposide-resistant HT-29 cells, with the MM level required for 50% inhibition (IC_{50}) of colony formation being approximately 9.1 nM (Fig. 4A). In the case of the glucose-free medium, we found that a 100 nM MM treatment enhanced etoposide chemosensitivity in the HT-29 cells (Fig. 4B).

Therefore, a strong cytotoxic effect of MM occurred on etoposide-resistant HT-29 cells during glucose deprivation, indicating that MM could play a role as an anticancer agent in the combination cancer chemotherapy of etoposide-resistant solid tumors.

DISCUSSION

Resistance to topo II-directed drugs such as etoposide has been demonstrated in many tumor cell lines, and several different mechanisms have been suggested to account for this phenotype [23]. In particular, solid tumors are characterized by insufficient, as well as inappropriate, vascular supplies compared with normal cells [4, 32, 34]. In addition, microenvironmental stress conditions such as hypoglycemia and hypoxia may select human colon cancer cells that have decreased apoptotic potential through genetic alterations, thereby leading to apoptosis resistance [32, 33]. In order to chemotherapeutically treat colorectal cancer, etoposide and 5-fluorouracil (5-FU) have been used in combination with other agents, improving the overall and disease-free survival of patients [20]. Combinations with newer medicines such as irinotecan or oxaliplatin have improved the response rates for advanced colorectal cancers [3, 9]. Despite advances in therapy, the prognosis of advanced solid tumors remains poor owing to cancer cell resistance to conventional chemotherapeutic drugs.

Therefore, a hypoglycemia-targeted study against solid tumor-specific conditions may be an attractive tool for exploring the potential development of novel anticancer drugs. On that basis, we searched for a compound that prevents stress-induced etoposide resistance *in vitro*. In our current study, we demonstrated that MM on etoposide-resistant HT-29 cells induced selective cytotoxicity during 2DG or glucose-deprived conditions (Fig. 4). MM is a natural product, derived from *Streptomyces* sp. AA030098, which was initially recognized in a cell-based screen for agents that could be used in combination cancer chemotherapies with etoposide. Its mechanism of action involves a reversible interaction with double-stranded DNA with GC base specificity. Moreover, MM inhibits DNA-dependent RNA synthesis by forming complexes with DNA in the presence of Mg^{2+} [35]. Despite its high toxicity [13, 14], however, it is used clinically for the treatment of certain tumors, such as chronic myeloid leukemia and acute myeloid leukemia [17].

As shown in Fig. 3, although MM exhibited weak antiproliferative effects in both normal and glucose-deprived conditions, the strong cytotoxic effects of MM on etoposide-resistant HT-29 cells occurred during glucose deprivation (Fig. 4). Therefore, the molecular and biochemical mechanisms of the cytotoxicity of MM cannot be fully understood based on the results of this study. However, our findings suggest that a MM and etoposide combination would have good efficacy in the cancer chemotherapy of glucose-deprived HT-29 cells.

In conclusion, this study clearly indicated that MM selectively inhibited stress-induced etoposide resistance in HT-29 cells. Therefore, we provide strong new evidence for a novel therapeutic approach to induce selective cell death in solid tumor tissue cultures within the microenvironmental conditions of cancer cells.

Acknowledgments

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