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Autophagy inhibition enhances apoptosis triggered by BO-1051, an N-mustard derivative, and involves the ATM signaling pathway

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ABSTRACT

In a previous study, BO-1051, an N-mustard linked with a DNA-affinic molecule, was shown to target various types of cancer cell lines. In the present study, we aimed to investigate the cytotoxicity, as well as the underlying mechanism, of BO-1051. We found that BO-1051 simultaneously induced apoptosis and autophagy in hepatocellular carcinoma cell lines. DNA double strand breaks induced by BO-1051 activated the ATM signaling pathway and subsequently resulted in caspase-dependent apoptosis. When autophagy was inhibited in its early or late stages, apoptosis was significantly enhanced. This result indicated autophagy as a cytoprotective effect against BO-1051-induced cell death. We further inhibited ATM activation using an ATM kinase inhibitor or ATM-specific siRNA and found that while apoptosis was blocked, autophagy also diminished in response to BO-1051. We not only determined a signaling pathway induced by BO-1051 but also clarified the linkage between DNA damage-induced apoptosis and autophagy. We also showed that BO-1051-induced autophagy acts as a cytoprotective reaction and downstream target of the ATM-signaling pathway. This research revealed autophagy as a universal cytoprotective response against DNA damage-inducing chemotherapeutic agents, including BO-1051, cisplatin, and doxorubicin, in hepatocellular carcinoma cell lines. Autophagy contributes to the remarkable drug resistance ability of liver cancer.

1. Introduction

DNA bifunctional alkylating agents containing a mustard moiety belong to an important class of antitumor drugs. The mustard derivatives are capable of crosslinking DNA double strands but lack the affinity to bind DNA, which precludes them from being effective antitumor agents. This drawback has been improved by adding a DNA-affinic carrier to the original mustard derivatives. The newly synthesized molecules showed higher cytotoxicity and therapeutic efficacy as compared to the corresponding untargeted mustards of similar reactivity [1–3].

Klionsky et al. designed and synthesized a series of N-mustard derivatives of 9-anilinoacridine based on the evidence mentioned. In the previous study, BO-1051 (Fig. S1) showed remarkable ability to target a variety of cancer cell lines, including two drug-resistant cell lines [4]. In in vivo experiments, BO-1051 was demonstrated to have potent antitumor efficacy in nude mice bearing human breast MX-1 xenografts. BO-1051 could also effectively suppress human glioma U87MG xenografts in nude mice [4]. The underlying mechanism of cell death induced by BO-1051, however, was not determined.

Macroautophagy (henceforth referred to as autophagy) is considered as programmed cell death type II, which occurs in certain situations and results in cell death [5–7]. Nevertheless, more evidence has revealed that autophagy is a novel response of cancer cells against various types of stress [8–10]. Inhibition at different stages in the process of autophagy may also lead to different consequences [11]. Despite studies showing that genotoxic stress can activate autophagy [8,12], direct links between DNA damage and autophagy are still lacking.

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The aim of the present study was to determine the molecular mechanism of BO-1051 and the crosstalk between autophagy and apoptosis in BO-1051-induced cytotoxicity. We focused our attention on hepatocellular carcinoma (HCC)-derived cell lines due to the poor prognosis and lack of effective therapies in treating hepatocarcinoma, except liver transplantation. Our results indicate that BO-1051 induced autophagy in early stages and acted as a defense system against apoptosis. Inhibition of autophagy in its early or late stages resulted in an increase in the number of annexin V-positive cells. BO-1051-induced autophagy has a cytoprotective role and is connected to the ATM signaling pathway. This research revealed autophagy as a universal cytoprotective response against DNA damage-inducing chemotherapeutic agents, including BO-1051, cisplatin, and doxorubicin, in hepatocellular carcinoma cell lines. Therefore, autophagy contributes to the remarkable drug resistance ability of liver cancer.

2. Materials and methods

2.1. Materials

BO-1051 was a gift synthesized by Su [4]; the compound was numbered 24d in the previous literature. The chemical structure of BO-1051 is shown in Fig. S1. Acridine orange, E64d, pepstatin A, bafilomycin A1, chloroquine, methylpyruvate, doxorubicin, and cisplatin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Z-VAD-fmk was purchased from Promega (Madison, WI, USA). ATM kinase inhibitor, Chk1 inhibitor, and Chk2 inhibitor II were purchased from Merck (Darmstadt, Germany).

2.2. Cell lines and culture

HA22T/VGH and Mahlavu cells are both poorly differentiated human hepatoma cell lines. They were obtained from the Bioresource Collection and Research Center (BCRC) in the Food Industry Research and Development Institute (Hsinchu, Taiwan) and were cultured in Dulbecco's modified eagle medium (GIBCO, Grand Island, NY, USA), with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO) under standard culture conditions (37 °C, 95% humidified air and 5% CO_2).

2.3. MTT assay

Cells were seeded in 96-well (6000 cells per well) or 24-well plates (30,000 cells per well) in complete culture medium. After overnight culture, the medium was replaced with either solvent or chemicals at indicated concentrations in complete medium. The cells were cultured until the time indicated, and the MTT assay was then performed. In brief, cells were stained with 0.1 mg/ml MTT (Sigma) for 2–4 h and then dissolved in DMSO (Sigma). MTT values were measured at 570 nm using a microplate reader.

2.4. Detection of acidic vesicular organelles (AVO) with acridine orange

To quantify the development of AVOs in BO-1051-treated cells, cells were stained with acridine orange (Sigma), and the intensity of the red fluorescence was measured by flow cytometry. Green (510–530 nm) and red (>650 nm) fluorescence emission from 10,000 cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur from Becton Dickinson (San Jose, CA, USA) using CellQuest Software.

2.5. Immunofluorescence staining

Briefly, cells were sub-cultured in a 4-well Lab-Tek chambered coverglass system (Nalge Nunc, Rochester, NY) for 24 h. After overnight cultured, cells were treated with BO-1051 in complete culture medium for indicated times. Then, cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.1% Triton X-100 (Sigma), immunostained with indicated antibodies, and labelled with FITC-conjugated secondary antibodies that allowed for fluorescent imaging. The LC-3 antibody was purchased from Novus Biologicals (Littleton, CO, USA) and the γH2AX antibody was purchased from Millipore Corporation (Bedford, MA, USA).

2.6. Immunoblotting

Harvested cells were pelleted by centrifugation, washed with PBS, and lysed with RIPA buffer. Protein content was measured with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Fifty micrograms of total protein were separated by SDS/PAGE (10% or 12% gels) and transferred to nitrocellulose membranes (Pall Corporation, MI, USA) for immunological detection of proteins. The blots were probed using antibodies against LC3 (Novus Biologicals or Cell Signaling), ATG5 (Novus), Beclin 1 (Sigma), p62 (Progen biotechnik, Heidelberg, Germany), p-Chk1, p-Chk2, cleaved PARP, cleaved caspase-3, cleaved caspase-7 (Cell Signaling Technology, Beverly, MA, USA), tubulin (Abcam, Cambridge, MA, USA), p-Rad17 (Montgomery, TX, USA), p-ATM, γH2AX, and beta-actin (Millipore Corporation, Milford, MA, USA).

2.7. Apoptosis assays

Both FITC-conjugated annexin V and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays (Roche, Palo Alto, CA) were used to determine the presence of apoptosis. Cells were seeded in a 6-cm dish one day before BO-1051 treatment. After BO-1051 treatment for the indicated time, cells were harvested and stained with annexin V-FITC and PI (BD Falcon, Bedford, MA, USA) or labelled using the TUNEL assay (Roche) according to the manufacturer's instructions. Both annexin V and TUNEL staining were detected by flow cytometry.

2.8. shLuc and shBECN1 expression construct and lentiviral transduction

The stable ablation of Beclin1 in HCC cell lines was obtained using small hairpin RNA (shRNA) probes for the *Homo sapiens* gene beclin 1 (BECN1): TRCN0000033549 (shBECN1 A01) and TRCN0000033550 (shBECN1 B01). Control cells stably expressed shLuc (pLKO.1-shLuc). Cells were infected with shRNA lentiviruses generated using a three-plasmid-based lentivirus system (all plasmids are available from the RNAi Consortium [TRC]). Lentivirus production was performed by transfection of 293T cells at 5×10^6 cells per 10 cm plate using Lipofectamine 2000 (LF2000, Invitrogen Life Technologies, Carlsbad, CA, USA). Supernatants were collected 48 h after transfection and then were filtered. Subconfluent cells were infected with lentivirus in the presence of 8 μg/ml polybrene (Sigma). Infected cells were selected with puromycin (2 μg/ml) until control uninfected cells were completely dead. Immunoblotting was used to confirm the knockdown efficiency of shBECN1.

2.9. siRNA transfection

On-TARGETplus siRNA smart pools for nontargeting control, p62/SQSTM1 (NM_003900), and ATM (NM_138292) were purchased from Dharmacon Research (Lafayette, CO, USA). Transient transfection was carried out using INTERFERin™ siRNA transfection reagent (Polyplus Transfection, Huntingdon, UK) according to the manufacturer's guide. Two days after transfection, cells were treated with BO-1051 for further experiments.