The Role of Bcl-xL in Synergistic Induction of Apoptosis by Mapatumumab and Oxaliplatin in Combination with Hyperthermia on Human Colon Cancer

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Abstract

Colorectal cancer is the third leading cause of cancer-related mortality in the world. The main cause of death because of colorectal cancer is hepatic metastasis, which can be treated using isolated hepatic perfusion (IHP), allowing treatment of colorectal metastasis with various methods. In this study, we present a novel potent multimodality strategy comprising humanized death receptor 4 (DR4) antibody mapatumumab in combination with oxaliplatin and hyperthermia to treat human colon cancer cells. Oxaliplatin and hyperthermia sensitized colon cancer cells to mapatumumab in the mitochondrial-dependent apoptotic pathway and increased reactive oxygen species (ROS) production, leading to Bcl-xL phosphorylation at serine 62 in a c-jun-NH2-kinase (JNK)-dependent manner. Overexpression of Bcl-xL reduced the efficacy of the multimodality treatment, whereas phosphorylation of Bcl-xL decreased its antiapoptotic activity. The multimodality treatment dissociated Bcl-xL from Bax, allowing Bax oligomerization to induce cytochrome c release from mitochondria. In addition, the multimodality treatment significantly inhibited colorectal cancer xenografts' tumor growth. The successful outcome of this study will support the application of multimodality strategy to colorectal hepatic metastases. Mol Cancer Res; 10(12); 1567–79. ©2012 AACR.

Introduction

Molecular-targeted therapies such as antibodies and small-molecule inhibitors have emerged as an important breakthrough in human cancer therapeutics. One such agent, TRAIL, is believed to selectively induce apoptosis, but controversy about the use of TRAIL has centered on its potential hepatotoxicity, especially when combined with other drugs (1). This potential problem may be circumvented by the use of specific humanized anti-TRAIL receptor monoclonal antibodies (1). Such agonistic antibodies can induce cell death while avoiding decoy receptor-mediated neutralization of the signal. Moreover, the agonistic antibody may also activate Fc-mediated antibody effector functions, such as antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity (2). In this study, we used the death receptor (DR4) agonistic antibody mapatumumab as one of options for the treatment of colon cancer.

Mapatumumab is a fully human immunoglobulin G1 (IgG1) agonistic monoclonal antibody, which exclusively targets and activates DR4 with very high specificity and affinity. Apoptosis-inducing mechanisms of mapatumumab are thought to be similar to apoptosis mediated by TRAIL (3). TRAIL-induced cell death is triggered by the interaction of the ligand with receptors to assemble the death-inducing signaling complex. This complex triggers association of the intracellular adaptor, Fas-associated death domain (FADD). FADD then recruits procaspase-8, which undergoes spontaneous autoactivation. Following the extrinsic pathway, activated caspase-8 activates the effector caspases-3, -6, and -7, which cleave cellular substrates to execute cell death (4). Previous data suggest the existence of cross-talk between the extrinsic and intrinsic death signaling pathways. Caspase-8, which can proteolytically activate the BH3 only family member Bid, induces Bax- and Bak-mediated release of cytochrome c and Smac/Direct binding protein of the inhibitor of apoptosis proteins with low isoelectric point from mitochondria, and triggers intrinsic apoptosis (5). However, substantial number of cancer cells are resistant to mapatumumab. This resistance can occur at different points in the signaling pathways, such as dysfunctions of the death receptors, DR4 and DR5, defects in FADD, overexpression of antiapoptotic proteins, loss of proapoptotic proteins, etc. (6). It is therefore critical to develop applicable strategies to overcome this resistance.

We previously reported that hyperthermia (41°C–42°C) has a synergistic effect with mapatumumab in causing cytotoxicity in CX-1 human colorectal cancer through the mitochondrial-dependent pathway (7). Hyperthermia, a treatment...
often used with isolated hepatic perfusion (IHP), maximizes the tumor damage while preserving the surrounding normal tissue. In this study, we developed a multimodality treatment using mapatumumab concurrently with hyperthermia and oxaliplatin to treat human colon cancer. Oxaliplatin, a common chemotherapeutic agent for colon cancer, is thought to trigger cell death mainly by inducing platinum–DNA adduct (8). We report here that the multimodality treatment of mapatumumab concurrent with oxaliplatin and hyperthermia induces Bcl-xL phosphorylation at the serine 62 (S62) residue in a c-jun-NH2-kinase (JNK)-dependent manner and leads to the oligomerization of Bax. This then allows the release of cytochrome c from the mitochondria and induces a synergistic effect in vitro and in vivo.

Materials and Methods

Cell cultures

Human colorectal carcinoma CX-1 cells, which were obtained from Dr. J.M. Jessup (Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health), were cultured in RPMI-1640 medium (Gibco BRL) containing 10% FBS (HyClone). The human colorectal carcinoma HCT116 Bax-containing (Bax+/+), Bax-deficient (Bax−/−), Puma-containing (Puma+/+), and Puma-deficient (Puma−/−) cell lines kindly provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD) were cultured in McCoy’s 5A medium (Gibco-BRL) containing 10% FBS. Mycoplasma test was conducted routinely for all cell lines.

Figure 1. Effect of oxaliplatin and hyperthermia on mapatumumab-induced cytotoxicity in CX-1 cells. A, CX-1 cells were treated with oxaliplatin for 20 hours and exposed to normothermic or hyperthermic (42°C) conditions for 1 hour in the presence/absence of mapatumumab and oxaliplatin, and then incubated for 3 hours at 37°C in the presence/absence of mapatumumab and oxaliplatin. Morphologic features were analyzed with a phase contrast microscope. B and C, survival was analyzed by the Trypan blue dye exclusion assay (B) or colony formation assay (C). Error bars represent SD from triplicate experiments. D, proliferation assay was conducted at day 0 to 4 after indicated treatment. Mapa, mapatumumab.
Reagents and antibodies

Oxaliplatin, N-acetylcysteine (NAC), apogossypol hexaacetate, hygromycin, and protease inhibitor cocktail were obtained from Sigma Chemical Co. Mapatumumab was from Human Genome Sciences. JNK inhibitor (SP600125) and G418 were from Calbiochem. Rabbit polyclonal anti-phosphorylated JNK, anti-caspase-8, anti-Bax, anti-Puma, anti-COX-IV, anti-Bcl-xL, and anti-human influenza hemagglutinin (HA) antibody were from Cell Signaling. Anti-p-Bcl-xL (S62) antibody was from Chemicon/Millipore and Abcam. Antinucleolin antibody was from Abcam. Anti-JNK and anti-caspase-3 antibodies were from Santa Cruz. Anti-caspase-9 antibody was from Upstate Biotechnology. Monoclonal antibodies included anti-PARP antibody from Biomol Research Laboratory, anticytochrome c antibody from PharMingen, and antiaction antibody from ICN Pharmaceuticals (Costa Mesa, CA).

Treatment

Cells were pretreated with oxaliplatin and exposed to hyperthermia in the presence/absence of mapatumumab and oxaliplatin. For hyperthermia, cells were sealed with parafilm and placed in a circulating water bath (Thomas Scientific), which was maintained within 0.02°C of the desired temperature.

Survival assay

For Trypan blue exclusion assay, trypsinized cells were pelleted and resuspended in 0.2 mL of medium, 0.5 mL of 0.4% Trypan blue solution, and 0.3 mL of PBS solution and incubated at room temperature for 15 minutes. At least 300 cells were counted under a light microscope for each survival determination. For colony formation assay, after treatment, cells were trypsinized, counted, and plated at appropriate dilutions (200 – 1 × 10⁶ cells/dish). The dishes were incubated at 37°C for 7 to 14 days to allow colony formation. Colonies were fixed by 0.5% crystal violet solution and counted. For every surviving fraction, the plating efficiency value was normalized.

Cell proliferation assay

For cell proliferation assay, 4 × 10⁵ cells were plated into 60-mm Petri dish. Cells were treated and counted various times after treatment and then results were plotted on a graph.

Figure 2. Effect of oxaliplatin and hyperthermia on mapatumumab-induced apoptosis in CX-1 cells. Cells were treated with oxaliplatin/mapatumumab/hyperthermia. A, after treatment, cells were stained with fluorescein isothiocyanate (FITC)-Annexin V and PI. Apoptosis was detected by the flow-cytometric assay. B, after treatment, the cleavage of caspase 8, 9, 3, or PARP was detected by immunoblotting. Actin was used to confirm the equal amount of proteins loaded in each lane. Mapa, mapatumumab.
Figure 3. Multimodality treatment–induced ROS production, JNK activation, and Bcl-xL phosphorylation in CX-1 cells. A, cells were treated with/without 10 mmol/L NAC for 30 minutes followed by oxaliplatin/mapatumumab/hyperthermia and incubated with CMH2DCFDA (25 μmol/L). Morphologic features and
Annexin V binding
Cells were harvested and stained with anti-human Annexin V antibody and propidium iodide (PI). The immunostaining was terminated by addition of binding buffer and cells were immediately analyzed by flow cytometry.

Cell-cycle analysis
Cells were harvested and fixed with 70% ethanol. Cells were stained with PI/RNase staining buffer (BD Pharmingen) for 15 minutes at room temperature and analyzed by flow cytometry.

Measurement of reactive oxygen species generation
The cells were stained with 20 mmol/L 2,7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes) for 30 minutes at 37°C, and the fluorescence was detected by a fluorescence microscope.

Stable transfection
Cells stably overexpressing HA-Bcl-xL wild-type (WT) or mutant types were prepared by transfecting CX-1 cells with human Bcl-xL tagged with HA epitope in pCDNA3.1 vector: HA-Bcl-xL-WT, HA-Bcl-xL-S62A (serine 62 alanine), and HA-Bcl-xL-S62D (serine 62 asparagine; a kind gift from Dr. Timothy C. Chambers, University of Arkansas for Medical Sciences) and maintained in 500 μg/mL G418. pSilencer-Bcl-xL or pSilencer control was transfected into CX-1 cells, and hygromycin B (250 μg/mL)-resistant cell clones were isolated.

Immunoprecipitation
Briefly, cells were lysed in CHAPS lysis buffer with protease inhibitor cocktail (Calbiochem). Cell lysates were clarified by centrifugation at 13,000 rpm for 15 minutes, and protein concentration was determined by BCA Protein Assay Reagent (Pierce). For immunoprecipitation, 0.5 to 1 mg of lysate was incubated with 1.5 μg of rabbit anti-Bax or anti-HA antibody or rabbit IgG (Santa Cruz) at 4°C overnight, followed by the addition of protein A-agarose beads (Santa Cruz) and rotation at room temperature for 2 hours. The beads were washed and resuspended in SDS sample buffer and followed by immunoblot analysis.

Confocal microscope
HCT116 Bax−/− cells were transfected with Lipofectamine 2000 (Invitrogen) with plasmids containing GFP fused fluorescent protein (pBax-GFP) and/or red fluorescent protein fused to Bcl-xL (pBcl-xL-RFP; a kind gift from Drs. Justin Cross and Ingram Iaccarino, Institute of Genetics and Biophysics). Twenty-four hours after transfection, cells were treated with oxaliplatin/mapatumumab/hyperthermia as described earlier. Mitochondria were stained with MitoTracker (Invitrogen). Cellular DNA was stained with DRAQ5 (Cell Signaling). Phosphorylated Bcl-xL was stained with anti-p-Bcl-xL antibody. HA-Bcl-xL-WT, HA-Bcl-xL-S62A, and HA-Bcl-xL-S62D were stained with anti-HA antibody. Nucleolin was stained with anti-nucleolin antibody. Slides were visualized using an inverted Leica TCSSL laser scanning confocal microscope. For digital image analysis, the software Adobe Photoshop 7.0 version was used.

Bax oligomerization
Cells were pelleted and resuspended in homogenization buffer. The cell suspension was homogenized, and spun at 1,000 × g for 15 minutes at 4°C. The supernatant was transferred and spun at 10,000 × g for 15 minutes at 4°C to pellet mitochondria. Aliquots of isolated mitochondrial fractions and cytosolic fractions were cross-linked with 1 mmol/L dithiobis (Pierce). Samples were subjected to SDS-PAGE under nondenaturing conditions followed by immunoblotting for Bax

JC-1 mitochondrial membrane potential assay
Cells were stained using JC-1 mitochondrial membrane potential detection kit (Cayman) and analyzed by flow cytometry. Fluorescence intensity was measured with the Accuri C6 Flow Cytometer (Accuri Cytometers). Results were analyzed with VenturiOne software (Applied Cytometry).

Immunoblot analysis
Cells were lysed with 1 × Laemmli lysis buffer and boiled for 10 minutes. Protein content was measured with BCA Protein Assay Reagent (Pierce). Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane, which was blocked for 1 hour. The membrane was incubated with primary antibody at room temperature for 1.5 hours. Horseradish peroxidase–conjugated IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (Amersham).

Animal model
Human colon adenocarcinoma CX-1 tumors were established by subcutaneously injecting 106 cells into the right hind leg of 6- to 8-week old male NU/NU mice (Charles River Labs). Before treatment with oxaliplatin/mapatumumab/hyperthermia, tumor size was measured 2 days.
to 3 times per week until the volume reached above 200 mm$^3$. Tumor volume was calculated as $W^2 \times L \times 0.52$ in which $L$ is the largest diameter and $W$ is the diameter perpendicular to $L$. After establishment of these tumor xenografts, mice were randomized into 8 groups of 5 mice per group. Oxaliplatin was administered by intraperitoneal injection. One hour later, mapatumumab was administered by intratumoral injection and then tumor-bearing legs were immersed in a water bath at 42°C for 1 hour. All procedures involving the mice were in accordance with the Guide for the Care and Use of Laboratory Animals and on a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Pittsburgh, PA).

TUNEL assay

The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method was conducted after the protocol of TACS 2 TdT-Fluor In Situ Apoptosis Detection Kit (Trevigen). Briefly, sections of formalin-fixed, paraffin-embedded tissues were deparaffinized, then washed with PBS, and permeabilized with Proteinase K. DNA strand breaks were then end-labeled with terminal transferase, and the labeled DNA was visualized by fluorescence microscopy (magnification ×200).

Statistical analysis

Statistical analysis was carried out using Graphpad InStat 3 software (GraphPad Software). Results were considered statistically significant at $P < 0.05$.

Results

Effect of oxaliplatin and hyperthermia on mapatumumab-induced cytotoxicity in CX-1 cells

To investigate the efficacy of oxaliplatin/mapatumumab/hyperthermia, cells were incubated with oxaliplatin for 20 hours and exposed to normothermic or hyperthermic conditions for 1 hour in the presence/absence of mapatumumab and oxaliplatin, and then incubated for 3 hours at 37°C in the presence/absence of mapatumumab and oxaliplatin. As the doses of mapatumumab or oxaliplatin increased, the cell surface began blebbing and apoptotic bodies were formed. In particular, there was a dramatic increase in the number of rounded cells and detached cells during the modality treatment oxaliplatin/mapatumumab/hyperthermia (Fig. 1A). Similar results were obtained for survival measured by the Trypan blue exclusion assay, which was conducted immediately after treatment and indicates physiologic death (Fig. 1B). Synergistic interactions between oxaliplatin/mapatumumab/hyperthermia were also observed by colony formation assay, which took 1 to 2 weeks after treatment and indicates reproductive death (Fig. 1C). Cell proliferation assay was conducted to detect long-term growth in each treatment. The multimodality treatment effectively inhibits cell proliferation (Fig. 1D). Collectively, mapatumumab, oxaliplatin, and hyperthermia induced synergistic cytotoxicity in a dose-dependent manner.

Effect of oxaliplatin and hyperthermia on mapatumumab-induced apoptosis in CX-1 cells

To clarify whether the cytotoxicity of the multimodality treatment of oxaliplatin/mapatumumab/hyperthermia is associated with apoptosis, we used the Annexin V assay (Fig. 2A) and observed that a dramatic synergistic effect was achieved during the multimodality treatment of oxaliplatin/mapatumumab/hyperthermia. Next, we examined whether the multimodality treatment promotes caspase pathways. Data from Fig. 2B show that treatment with mapatumumab resulted in activation of caspase-8 and -3 but not caspase-9. Interestingly, hyperthermia in combination with mapatumumab enhanced mapatumumab-induced activation of caspases 8 and 3 and activated caspase-9. Treatment with oxaliplatin activated only caspase-9 and -3. Combined mapatumumab and oxaliplatin treatment enhanced the activation of caspases 8, 9, and 3. Furthermore, a dramatic synergistic activation of caspases 8, 9, and 3 was observed during the multimodality treatment of oxaliplatin/mapatumumab/hyperthermia. These synergistic effects were also confirmed by determining the hallmark of apoptosis, PARP cleavage (Fig. 2B).

ROS-induced JNK activation in the multimodality treatment

Next, we attempted to investigate the mechanisms by which the multimodality treatment induced apoptosis. Figure 3A shows that there were significant fluorescence signals of reactive oxygen species (ROS) when mapatumumab was combined with hyperthermia or oxaliplatin. Of note, maximum signals were detected in the multimodality treatment. We also observed that an antioxidant NAC pretreatment significantly decreased the signals of ROS. Figure 3B shows oxaliplatin increased the JNK activation in a dose-dependent manner, and maximum synergistic activation was detected in oxaliplatin/mapatumumab/hyperthermia. We also observed that pretreatment with NAC significantly blocked the activation of JNK and suppressed the effect of oxaliplatin/mapatumumab/hyperthermia-induced PARP cleavage (Fig. 3C).

Role of Bcl-xL in the multimodality treatment–induced apoptosis

Bcl-xL is a key antiapoptotic protein that characteristically undergoes phosphorylation in response to treatment with apoptotic agents (9). In this study, we assessed the status of phosphorylation of Bcl-xL at S62 during multimodality treatment (Fig. 3D). No detectable amounts of phospho-Bcl-xL were observed in the control or single treatment. Phosphorylated Bcl-xL was detected during treatment with oxaliplatin and mapatumumab. Interestingly, a large amount of phospho-Bcl-xL was detected in the multimodality treatment of oxaliplatin/mapatumumab/hyperthermia. Pretreatment with SP600125 significantly reduced oxaliplatin/mapatumumab/hyperthermia–induced PARP cleavage in CX-1 cells, indicating that the JNK pathway was crucial for multimodality treatment–induced apoptosis (Fig. 3E). Noticeably, SP600125 highly reduced the level of Bcl-xL.
phosphorylation in CX-1 cells, which provides strong evidence that multimodality treatment–induced Bcl-xL phosphorylation requires JNK activation.

To evaluate the effect of Bcl-xL phosphorylation at S62 on its own antiapoptotic activity, we established CX-1–derived cell lines stably overexpressing wild-type Bcl-xL (Bcl-xL-WT), S62/Ala phospho-defective Bcl-xL mutant (Bcl-xL-S62A), S62/Asp phospho-mimic Bcl-xL mutant (Bcl-xL-S62D), or the corresponding empty vector (pcDNA; Fig. 3F). As expected, overexpression of Bcl-xL-WT prevented
A

Hyperthermia  

Mapa (ng/mL)  0 0 0 100 100 0 0 100 100 0 0 100 100 0 0 100 100

Oxaliplatin (µg/mL)  0 0 0 0 0 0 0 10 10 10 10 10 10 10 10 10 10

Bcl-xL

Bax

Bax

Input:

Actin

C

Hyperthermia  

Mapa (100 ng/mL)  0 0 0 100 100 0 0 100 100 0 0 100 100 0 0 100 100

Oxaliplatin (10 µg/mL)  0 0 0 0 0 0 0 10 10 10 10 10 10 10 10 10 10

Bax-GFP

Bcl-xL-RFP

DRAQ5

Overlay

D

Hyperthermia  

Mapa (ng/mL)  0 0 0 100 100 0 0 100 100 0 0 100 100 0 0 100 100

Oxaliplatin (µg/mL)  0 0 0 0 0 0 0 10 10 10 10 10 10 10 10 10 10

DMSO

Apogossypol hexaacetate

PARP

Actin

E

Hyperthermia  

Mapa (ng/mL)  0 0 0 100 100 0 0 100 100 0 0 100 100 0 0 100 100

Oxaliplatin (µg/mL)  0 0 0 0 0 0 0 10 10 10 10 10 10 10 10 10 10

pSilencer-control

pSilencer-Bcl-xL

PARP

Bcl-xL

Actin
oxaliplatin/mapatumumab/hyperthermia–induced PARP cleavage. Interestingly, overexpression of Bcl-xL-S62D enhanced PARP cleavage, whereas that of Bcl-xL-S62A inhibited PARP cleavage. These data suggest that the level of Bcl-xL and its phosphorylation at S62 play an important role in the multimodality-induced apoptosis.

**Multimodality treatment–induced Bcl-xL phosphorylation (S62), cell-cycle arrest, and translocation of Bcl-xL**

It is reported that phosphorylation of the S62 residue on Bcl-xL is detected after treatment with microtubule inhibitors or other compounds and its phosphorylation induces G2/mitotic arrest (9, 10). In this study, we examined whether multimodality treatment induces phosphorylation of Bcl-xL and affects the cell-cycle distribution. Data from the kinetics of Bcl-xL phosphorylation show that phosphorylation of Bcl-xL was detected during pretreatment with oxaliplatin as well as treatment with oxaliplatin/mapatumumab/hyperthermia (Fig. 4A). Interestingly, G2–M cell-cycle arrest was observed during pretreatment with oxaliplatin and an increase in G2–M arrest occurred during the multimodality treatment (Fig. 4B). Recent studies have shown that G2–M arrest is associated with accumulation of a pool of phosphorylated Bcl-xL in nuclear structures (10). This possibility was investigated by using confocal immunofluorescence microscopy, which was undertaken to monitor the location of phospho-Bcl-xL (S62). Control panel in Fig. 4C shows residual amounts of phosphorylated Bcl-xL (S62) in untreated cells. The level of phosphorylated Bcl-xL (S62) increased and a pool of phosphorylated Bcl-xL (S62) translocated from the cytoplasm to the nuclei and nucleoli after oxaliplatin/mapatumumab/hyperthermia treatment (Fig. 4C). To examine whether phosphorylation of the S62 residue on Bcl-xL is important for translocation of Bcl-xL, CX-1 cells were stably transfected with HA-Bcl-xL-WT, phospho-defective HA-Bcl-xL-S62A, or phospho-mimic HA-Bcl-xL-S62D. Figure 4D shows that HA-Bcl-xL-WT and HA-Bcl-xL-S62D, but not HA-Bcl-xL-S62A, translocated to the nuclei and nucleoli after oxaliplatin/mapatumumab/hyperthermia treatment. These results suggest that phosphorylation of Bcl-xL at S62 plays an important role in the translocation of Bcl-xL.

**The dissociation of Bcl-xL from Bax in the treatment of mapatumumab, oxaliplatin, and hyperthermia**

We next tested whether the multimodality treatments alter the interactions between Bcl-xL and Bax. An interaction between Bcl-xL and Bax was detected by immunoprecipitation assay in control and mapatumumab-treated cells (Fig. 5A). This interaction was slightly reduced after hyperthermia or oxaliplatin treatment. Notably, Bax was dissociated from Bcl-xL during treatment with oxaliplatin in combination with mapatumumab or the multimodality treatment oxaliplatin/mapatumumab/hyperthermia (Fig. 5A). To further study the role of Bcl-xL phosphorylation at S62 on the interaction between Bax and Bcl-xL in response to oxaliplatin/mapatumumab/hyperthermia treatment, immunoprecipitation assay was conducted on Bcl-xL–WT, Bcl-xL–S62A, or Bcl-xL–S62D–transfected cells. Figure 5B shows the weakest interaction between Bcl-xL and Bax occurred in untreated Bcl-xL–S62D–transfected cells, whereas dissociation of Bax from Bcl-xL was reduced in Bcl-xL–S62A–transfected cells during the multimodality treatment. These observations suggest that phosphorylation of Bcl-xL at S62 plays an important role in Bcl-xL–Bax interaction.

Furthermore, confocal immunofluorescence microscopy assay (Fig. 5C) shows overlapping signals (yellow color) of Bcl-xL–RFP and Bax–GFP. Of note, we observed a large amount of Bcl-xL dissociated from Bax in oxaliplatin/mapatumumab and oxaliplatin/mapatumumab/hyperthermia treatment. Previous studies have shown that gossypol, the levorotatory isomer of a natural product isolated from cottonseeds and roots, binds to the BH3 binding groove of Bcl-xL and Bcl-2 and subsequently inhibits the heterodimerization of Bcl-xL or Bcl-2 with proapoptotic proteins, such as Bax or Bad (11). In this study, pretreatment with apogossypol hexaacetate sensitized the apoptotic effect of mapatumumab, oxaliplatin, and hyperthermia (Fig. 5D). Moreover, the multimodality treatment–induced apoptosis was markedly enhanced by knockdown of Bcl-xL expression (Fig. 5E). These results suggest that dissociation of Bax from Bcl-xL enhances apoptosis.

**Bax oligomerization, localization to the mitochondria, and subsequent cytochrome c release in the treatment of oxaliplatin, mapatumumab, and hyperthermia**

To examine the involvement of Bax in multimodality treatment–induced apoptosis, we used human colon carcinoma parental HCT116 wild-type (HCT116 WT) and HCT116 Bax–/– cells. As shown in Fig. 6A and B, HCT116 Bax–/– cells were resistant to PARP cleavage in the multimodality treatment compared with HCT116 WT cells, which clearly indicates that the synergy of oxaliplatin/mapatumumab/hyperthermia–associated apoptosis is mediated by the interaction between Bcl-xL and Bax.
Figure 6. Bax oligomerization, localization to the mitochondria, and subsequent cytochrome c release in mapatumumab/oxaliplatin/hyperthermia. A. Human colon carcinoma parental HCT116 (HCT116 WT), Bax knockout HCT116 Bax+/−, and PUMA knockout HCT116 PUMA−/− cells were treated with oxaliplatin/mapatumumab/hyperthermia and immunoblotted with anti-PARP antibody. B. HCT116 Bax+/+, HCT116 Bax−/−, HCT116 PUMA+/+, and HCT116 PUMA−/− cells were treated with oxaliplatin/mapatumumab/hyperthermia and immunoblotted with anti-PARP, anti-Bax, or anti-PUMA antibody. C. CX-1 cells were treated with oxaliplatin/mapatumumab/hyperthermia. Mitochondrial and cytosolic fractions were isolated and were cross-linked with dithiobis and subjected to immunoblotting with anti-Bax antibody. Bax monomer (1×) and multimers (2×, 3×, and 4×) are indicated. Actin was used as a cytosolic marker and COX IV as a mitochondrial marker. D. CX-1 cells were transfected with pBax-GFP plasmid. After 24 hours incubation, cells were treated with oxaliplatin/mapatumumab/hyperthermia. Mitochondria were stained red with MitoTracker. Localization of Bax-GFP was examined by confocal microscope. E. CX-1 cells were treated with oxaliplatin/mapatumumab/hyperthermia and stained with JC-1 and analyzed by flow cytometry. F. CX-1 cells were treated with oxaliplatin/mapatumumab/hyperthermia. Cytochrome c release into cytosol was determined by immunoblotting for cytochrome c in the cytosolic fraction. Actin was used to confirm the equal amount of proteins loaded. Mapa, mapatumumab.
through Bax. Another important protein is the p53 upregulated modulator of apoptosis (PUMA). It is also a proapoptotic Bcl-2 protein and is involved in p53-dependent and -independent apoptosis induced by a variety of signals (12).

In contrast to HCT116 Bax−/− cells, PUMA-deficient cells were not resistant to PARP cleavage in the multimodality treatment oxaliplatin/mapatumumab/hyperthermia compared with HCT116 WT cells (Fig. 6A and B). These results clearly suggest that PUMA is not involved in oxaliplatin/mapatumumab/hyperthermia–induced apoptotic death.

Because Bax oligomerization plays an important role in apoptosis, we examined how the multimodality treatment affected Bax oligomerization (Fig. 6C). There was more multimeric Bax oligomerization in the treatment of oxaliplatin/mapatumumab/hyperthermia compared with the other treatments. We also observed in confocal assay that more Bax-GFP translocalized to the mitochondria in the multimodality treatment as compared with the other treatments (Fig. 6D). As shown in Fig. 6E, cells with intact mitochondrial membrane potential were detected in the upper right quadrant of the plots and those with impaired mitochondrial membrane potential were detected in the lower right quadrant of the plots. A shift to the lower right part of the quadrants (a loss of membrane potential) occurred in the multimodality treatment oxaliplatin/mapatumumab/hyperthermia. More importantly, Fig. 6F shows that more cytochrome c release occurred during multimodality treatment oxaliplatin/mapatumumab/hyperthermia.

**Effect of oxaliplatin, mapatumumab, and hyperthermia on the growth of CX-1 xenograft tumors**

Finally, in vivo studies were conducted to examine the effect of the multimodality treatment oxaliplatin/mapatumumab/hyperthermia on growth of xenograft tumors. Figure 7A shows that hyperthermia alone has no effect on tumor growth compared with the control group. The effect of oxaliplatin alone on tumor growth was observed 6 days after treatment compared with the control group; however, there was only a slight, not statistically significant difference 12 days after treatment (Fig. 7B). Mapatumumab alone caused a statistically significantly decrease of tumor growth (P < 0.05). Moreover, bitherapy of mapatumumab combined with hyperthermia or mapatumumab combined with oxaliplatin caused a significant decrease of CX-1 tumor growth compared with single treatment groups (P < 0.01). In particular, the multimodality treatment was significantly more effective at inhibiting xenograft tumor growth than the single treatments or any other bitherapy strategies. TUNEL assay confirmed many apoptotic deaths in xenograft tumor tissue at day 12 after oxaliplatin/mapatumumab/hyperthermia treatment in comparison with sham group (Fig. 7C).

**Discussion**

Previous phase II trials showed that no/little clinical activity of single-agent mapatumumab was observed in patients with advanced refractory colorectal cancer or non–small cell lung cancer (13). So, enhancing the effect of mapatumumab is required for clinical application of mapatumumab. Various chemotherapeutic agents such as oxaliplatin, cisplatin, 5-fluorouracil, and paclitaxel have been shown to enhance and sensitize the apoptosis-inducing capacity of targeted therapies (14). Most of the research focused on chemotherapeutic agents to increase the effect of
TRAIL-based therapy in a xenograft model system (15). To the best of our knowledge, we are the first to test the efficacy of combined mapatumumab + oxaliplatin + hyperthermia in colon cancer cells and mouse xenograft tumor model, suggesting that this multimodality approach will be applicable to improve the clinical efficacy of mapatumumab for treatment of colon cancer. Hyperthermia has been explored as an anticancer agent for many decades. While the treatment effects of hyperthermia as a single agent are limited, its ability to potentiate the effects of standard chemotherapies has generated lasting interest (16). Our laboratory has focused on identifying strategies for thermal sensitization in an attempt to improve the clinical efficacy of IHP. We previously reported that hyperthermia combined with TRAIL induces cytotoxicity by facilitating activation of caspases through mitochondrial-dependent cytochrome c release in colorectal cancer cells (7). In this study, we observed that hyperthermia in combination with mapatumumab and oxaliplatin elevates the intracellular level of ROS and activates the JNK-Bcl-xL-Bax signal transduction pathway. Data from the kinetics of Bcl-xL phosphorylation indicate that Bcl-xL phosphorylation is an early event and a cause of cell death through cytochrome c release (Fig. 3E and F, 4A, and 6). Several researchers have revealed that hyperthermia induces an increase in ROS and increased antioxidant enzyme levels result in protection of cells from oxidative stress (17). It is well known that ROS is generated through several intracellular sources including mitochondrial electron transport chain and peroxisomal cytochrome P-450 oxidases as well as endogenous enzyme systems (18). Previous studies suggest that disruption of the mitochondria electron transport chain is the main source of ROS generation during hyperthermia (17). Our data and literatures (19) show that oxaliplatin/TRAIL treatment elevates the intracellular level of ROS. Hyperthermia in combination with oxaliplatin/mapatumumab may facilitate the disruption of the mitochondrial electron transport chain and increase ROS generation. We previously observed that ROS can be sensed through thioredoxin (TRX) and glutaredoxin (GRX), resulting in activation of the ASK1 (apoptosis signal-regulating kinase 1)-MEK (mitogen-activated protein extracellular signal-regulated kinase)-MAPK (mitogen-activated protein kinase) signal transduction pathway (20). These sensor molecules may be converted to the intramolecular disulfide form of TRX-(S-S) and GRX-(S-S) during treatment of oxaliplatin/mapatumumab/hyperthermia. This oxidized form of TRX and GRX may dissociate from ASK1 and subsequently activate the ASK1-MEK-JNK-Bcl-xL-Bax signal transduction pathway (21).

Bcl-xL, a key antiapoptotic molecule, is a target for JNK signaling. Bcl-xL undergoes phosphorylation in response to microtubule inhibitors and other apoptotic stimuli including ionizing radiation or chemotherapy (9). Our study for the first time revealed that hyperthermia and oxaliplatin synergistically promoted Bcl-xL phosphorylation, and thus significantly sensitized mapatumumab-induced apoptosis. We also observed that Bcl-xL phosphorylation required activated JNK, which can recognize a proline residue on the carboxyl side of the phospho-acceptor (22). Some studies reported phosphorylation to occur on S62, whereas others reported it to occur on threonines 47 and 115 (23, 24). This study with site-directed mutagenesis at S62 showed that cells expressing a validated phospho-defective Bcl-xL mutant are resistant to the multimodality treatment-induced apoptosis, whereas cells expressing a phospho-mimic Bcl-xL are sensitive to the multimodality-induced apoptosis, indicating that phosphorylation at S62 is a key regulatory mechanism for antagonizing antiapoptotic function in the multimodality treatment.

Phospho-Bcl-xL (S62) has been reported to play a key role at DNA damage-induced G2–M arrest (10). In this study, we observed that phosphorylation of Bcl-xL at S62 after oxaliplatin/mapatumumab/hyperthermia treatment was associated with G2–M arrest (Fig. 4). This is probably due to translocation of Bcl-xL in the nucleus and nucleolus during the G2 checkpoint (10). Previous studies show that Bcl-xL’s function in cell-cycle arrest is distinct from its function in apoptosis (10).

It is reported that Bcl-xL inhibits and maintains Bax in the cytosol by constant retro-translocation of mitochondrial Bax, which undergoes a conformational shift under apoptotic signaling, and inserts into the outer mitochondrial membrane (25). We observed in this study that upon Bcl-xL phosphorylation, the interaction of Bax and Bcl-xL was impaired, resulting in Bax translocating to the mitochondria. We also observed that a weak interaction between Bax and phospho-mimic Bcl-xL-S62D and Bcl-xL/Bax interaction was less impaired in Bcl-xL-S62A-transfected cells indicating Bcl-xL phosphorylation at S62 plays an important role in Bcl-xL/Bax interaction. Disruption of the interaction of Bcl-xL and proapoptotic proteins by apogossypol hexaacetate or knockdown of Bcl-xL sensitizes the effect of the multimodality treatment, which confirms the involvement of Bcl-xL in the multimodality treatment.

We also observed that that Bax but not PUMA is involved in the synergistic effect of the multimodality treatment in this study. However, data from Fig. 6B imply that Bax is necessary but not entirely sufficient as there is still some PARP cleavage in Bax+/− cells, which suggest that multimodality treatment–induced apoptosis occurs through the mitochondrial-dependent pathway as well as the mitochondrial-dependent pathway. Obviously, our next step will be to investigate the involvement of the mitochondrial-independent pathway in the multimodality treatment–induced apoptosis.

Taken together, our results reveal that treatment of hyperthermia concurrently with a DNA-damaging agent, such as oxaliplatin, can synergize mapatumumab-induced apoptosis in vitro and in vivo. Inactivating Bcl-xL represents a good strategy to enhance sensitivity to apoptosis. Given the facts that hyperthermia has a favorable safety profile, oxaliplatin is a commonly used chemotherapeutic drug for colon cancers, and mapatumumab currently is undergoing clinical testing (26), this multimodality...
treatment has an excellent translational potential and should be considered for colorectal hepatic metastases treatment in clinics.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Y.J. Lee
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Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): X. Song, Y.J. Lee
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.J. Lee
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References
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