Camptothecin and Fas receptor agonists synergistically induce medulloblastoma cell death: ROS-dependent mechanisms

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Medulloblastoma, a common malignant pediatric brain tumor, is highly resistant to death receptor-mediated apoptosis despite death receptor expression by tumor cells. Developing new strategies to overcome this resistance to death receptor activation could positively impact therapeutic outcomes. We explored the modulation of death receptor-induced medulloblastoma cell death by the topoisomerase I inhibitor camptothecin (CPT). CPT significantly increased the human medulloblastoma DAOY cell death response to agonistic anti-Fas antibody (CH-11). Cell death after CPT, CH-11, and CPT + CH-11 treatment was 9, 7, and 33%, respectively. Isobologram analysis showed that CH-11 and CPT act synergistically to induce cell death in DAOY cells. A similar pattern of synergism between CPT and CH-11 was found in ONS-76 medulloblastoma cells. Synergistic cell death was found to be predominantly apoptotic involving both extrinsic and intrinsic pathways as evidenced by annexin V staining, cleavage of caspases (3, 8, and 9), Bid and PARP, and cytoprotection by caspase inhibitors. Flow cytometric analyses showed that expression of cell surface Fas or Fas ligand did not change with drug treatment. Western blot analyses showed that the combination of CH-11 + CPT induced a significant decrease in XIAP levels. Furthermore, reactive oxygen species, especially O_2^-, were elevated after CPT treatment, and even more so by the CH-11 + CPT treatment. The antioxidants glutathione and *N*-acetyl-cysteine prevented cell death induced by CPT + CH-11. Moreover, the mitochondrial respiratory chain complex I inhibitor rotenone potentiated CH-11-induced apoptosis in DAOY cells. Taken together, these findings show that CPT synergizes with Fas activation to induce medulloblastoma apoptosis through a mechanism involving reactive oxygen species and oxidative stress pathways. *Anti-Cancer Drugs* 20:770–778 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Induction of apoptosis in cancer cells is a key mechanism for most antitumor therapies. Despite considerable advances in the treatment paradigms for solid malignancies, resistance to cytotoxic chemotherapy and ionizing radiation limit the potential of many therapeutic agents. Apoptosis can be triggered by the mitochondrial pathway (intrinsic) or the death receptor pathway (extrinsic) [1–3]. Death receptors are members of the tumor necrosis factor receptor superfamily [1,4,5] and include tumor necrosis factor-R1, Fas and the two tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 [1,4]. These death receptors are characterized by the presence of a well-preserved cytoplasmic death domain capable of inducing apoptosis upon stimulation by their cognate ligands. Binding of their cognate ligands results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD), which interacts with procaspase-8 and procaspase-10 to form a death-inducing signaling complex (DISC) [1,6]. DISCs then activate caspase-8/caspase-10 and induce apoptosis by direct cleavage of caspase-3.

Medulloblastoma, a malignant embryonal childhood tumor of the central nervous system, is thought to be derived from granule cell precursors in developing cerebellum [7]. They are highly invasive neoplasms with a tendency to disseminate throughout the subarachnoid space. Common molecular determinants of medulloblastoma include the amplification of MYCN oncogene activation of c-myc, and activation of the hedgehog pathway through *PTCH* mutations and other mechanisms. Treatment, which currently includes surgery, chemotherapy, and craniospinal radiation therapy, has substantially improved long-term survival, yet morbidity and mortality remain high. This is largely because of considerable defects in the apoptotic machinery in medulloblastoma cells. Therefore, the development of novel therapies remains essential.

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Medulloblastoma shows a high degree of insensitivity to death receptor-induced apoptosis [8]. Researchers have shown that subsets of ex-vivo pediatric brain tumors are highly resistant to Fas-mediated apoptosis and/or topoisomerase II-directed chemotherapy [9]. Furthermore, although medulloblastoma cell lines and pediatric brain tumor specimens express Fas and TRAIL receptor [9,10], medulloblastomas cells are typically resistant to death receptor activation-induced apoptosis [9,11,12]. Elucidating mechanisms by which tumor cells resist cytotoxic cell death and developing new strategies to enhance medulloblastoma apoptosis will increase our understanding of death receptor pathways and potentially impact therapeutic outcomes.

Targeting death receptors to trigger apoptosis in tumor cells is an attractive concept for cancer therapy, as death receptor-mediated cell death can be independent of common genetic mutations found in tumor cells [5]. Different laboratories have reported that chemotherapeutic drugs can sensitize cancer cells to apoptosis induced by death receptor ligands. Preexposure of tumor cells to various cytokines, chemotherapy drugs, and γ-radiation were shown to enhance Fas ligand (FasL) or TRAIL cytotoxicity in glioblastoma, prostate cancer, ovarian cancer cells, etc. [13–20]. However, relatively few address the sensitization of medulloblastoma cells to death receptor ligand. In this study, we show that chemotherapy drugs increase medulloblastoma cell sensitivity to death receptor activation-induced apoptosis. The cell death pathways and the mechanisms involved in the synergistic cell death response are also examined.

Materials and methods

Reagents

All reagents were purchased from Sigma (St. Louis, Missouri, USA) unless otherwise stated. Anti-Fas antibody (clone CH-11) was purchased from Upstate (Charlottesville, Virginia, USA). Camptothecin (CPT) was dissolved in 0.1 N NaOH and diluted in cell culture medium. Caspase-3, caspase-8, caspase-9 inhibitors were dissolved in dimethyl sulfoxide. Glutathione (GSH) and *N*-acetyl-cysteine (NAC) stock solutions were prepared using water and neutralized with 5 mol/l NaOH. All primary antibodies used for western blot were obtained from Cell Signaling Technology (Beverly, Massachusetts, USA) unless otherwise stated and the concentrations for western blotting followed the manufacturers' recommendations. All secondary antibodies conjugated with horseradish peroxidase were purchased from Jackson Immunoresearch Laboratories (West Grove, Pennsylvania, USA) and were used in Western blotting at 1 : 1000 dilution.

Cell culture

Two cell lines DAOY and ONS-76 were purchased from American Type Culture Collection (Rockville, Maryland, USA) and used in our study. DAOY has been generated from a desmoplastic medulloblastoma [21] and the ONS-76 cell line was derived from a case of classic medulloblastoma [22]. DAOY cells were grown in Zinc Optimum medium (Gibco, Rockville, Maryland, USA) supplemented with 10% fetal bovine serum (Cellgro, Washington, DC, USA) and 500 μg/ml penicillin–streptomycin (Gibco). ONS-76 cells were cultured in RPMI1640 medium (Gibco) supplemented with 10% fetal bovine serum and 500 μg/ml penicillin–streptomycin. Cells were grown in a 37°C humidified incubator with 5% CO_2.

Cell viability assay

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Cells were plated at 5000 cells/well in 24-well tissue culture plates and cultured for 48–72 h before treatment with different drugs. After 24 h treatment, MTT was added to each well at a final concentration of 150 μg/ml, and the cells were incubated for 1–2 h at 37°C. The medium was then removed, and the formazen reaction product was dissolved with dimethyl sulfoxide and quantified spectrophotometrically at 570 nm using a Spectra MAX 340pc plate reader (Molecular Devices, Sunnyvale, California, USA). The results are expressed as a percentage of absorbance measured in control cultures after subtracting the background absorbance from all values.

We used isobologram analysis to determine whether the drug combination is synergistic as described in Ref. [23]. In the isobologram equation $I_x = (a/A) + (b/B)$, A is the IC_{50} concentration of CH-11; B is the IC_{50} concentration of CPT; a and b are the concentrations of CH-11 and CPT, respectively, required to produce the same effect in combination with the other agent. The combination effect is synergistic if $I_x < 1$, additive if $I_x = 1$, and inhibitory if $I_x > 1$.

Annexin V and propidium iodide staining

Apoptosis was quantified by the Annexin V-FITC apoptosis kit (BD Biosciences, San Diego, California, USA) by following the manufacturer's instructions. Briefly, cells were trypsinized (Gibco), pelleted by centrifugation, and resuspended in annexin V binding buffer (150 mmol/l NaCl, 18 mmol/l $CaCl_2$, 10 mmol/l HEPES, 5 mmol/l KCl, 1 mmol/l $MgCl_2$). FITC-conjugated annexin V (1 μg/ml) and propidium iodide (50 μg/ml) were added to cells and incubated for 30 min at room temperature without light. Analyses were performed on a Becton-Dickinson FACScan (BD Biosciences). Data were analyzed with CellQuest software (BD Biosciences).

FACScan analysis of death receptor expression

Expression of Fas and FasL was measured by flow cytometry. DAOY cell monolayers were treated with

drugs for 24 h, and harvested by trypsinization and centrifugation. The suspended cells were incubated with 5% normal goat serum in PBS, and then with either control or anti-Fas antibodies (1 : 500; BD Biosciences) for 1 h at room temperature. For FasL staining, cells were permeabilized with 0.1% Triton X-100 for 5 min before incubation with anti-FasL antibody (1 : 500; BD Biosciences). After being washed twice in PBS, cells were incubated with FITC-conjugated secondary antibody at room temperature for 30 min, and then analyzed. Mouse IgG was used as the negative control. Analyses were performed on a Becton-Dickinson FACScan (BD Biosciences). Data were analyzed with CellQuest software (BD Biosciences).

Determination of reactive oxygen species

DAOY monolayers were treated with different agents as described and then incubated for 30 min with the oxidation-sensitive dyes dichlorodihydrofluorescein diacetate (DCFDA, 10 μmol/l; Molecular Probes, Eugene, Oregon, USA) or dihydroethidium (DHE, 10 μmol/l; Molecular Probes). Cells were collected by trypsinization and centrifugation, washed with PBS and subjected to FACScan analysis. Reactive oxygen species (ROS) were quantified based on DCFDA (FL1-H) or DHE (FL2-H) fluorescence intensity and presented as the percentage of intensity in comparison to control cells.

Western blot analysis

Cells grown in 10-cm-diameter tissue culture dishes were lysed with RIPA buffer (50 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 1% NP-40, 0.25% Na-deoxycholate) containing 1 × protease and phosphatase inhibitor cocktail (Calbiochem, San Diego, California, USA). After sonication for 15 s, the suspensions were centrifuged at 3000*g* for 10 min. Protein concentrations were determined using the Coomassie Protein Assay Reagent (Thermo Fisher Scientific, Rockford, Illinois). Thirty micrograms of protein was subjected to 4–20% SDS–polyacrylamide gel electrophoresis and was transferred to a nitrocellulose membrane for 1 h. The membrane was incubated in 5% non-fat dry milk (Carnation, Nestle Food Co., Glendale, California, USA) in Tris-buffered saline Tween-20 (TBST) for 1 h at room temperature, and then incubated overnight at 4°C in 5% non-fat dry milk or 5% BSA containing a primary antibody. Membranes were subsequently rinsed in TBST, and then incubated for 1 h at room temperature with secondary antibody conjugated with horseradish peroxidase. After incubation, the membranes were rinsed, and antibody binding was detected with the enhanced chemiluminescence system (GE Healthcare, Piscataway, New Jersey, USA).

Statistical analysis

A one-way analysis of variance followed by the Tukey's multiple comparison test using GraphPad Prism (GraphPad, San Diego, California, USA) was used for statistical analysis. All experiments reported here represent at least three independent replications. Data are represented as the mean value ± SE.

Results

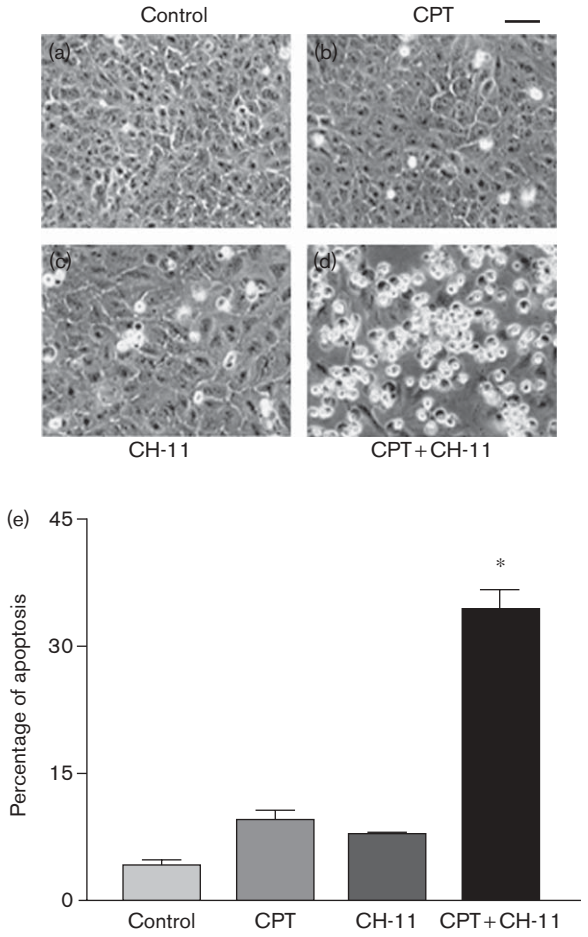
Death receptor ligand and topoisomerase inhibitor cooperatively induce cell death in medulloblastoma cells

The human medulloblastoma cell line DAOY was used to study cell death induced by the combination of FasL and the topoisomerase I inhibitor CPT. CPT (0.1 μmol/l) did not induce cell death in DAOY cells (Fig. 1b and e). The agonistic anti-Fas antibody (CH-11) at 50 ng/ml did not induce apoptosis by itself (Fig. 1c and e). However, coapplication of CPT and CH-11 for 24 h induced extensive cell death in DAOY cells (Fig. 1d and e). Trypan blue staining showed that the viability of cells treated with CPT (0.1 μmol/l) + CH-11 (50 ng/ml) for 24 h was approximately 61% compared with 86% in cells treated with CPT alone and 89% in cells treated with CH-11 alone, respectively. Cell death was also quantified by flow cytometry with annexin V-FITC staining. Annexin V-positive cells after CPT and CH-11 treatment were 9 and 7%, respectively; CH-11 + CPT induced approximately 33% cells to undergo apoptosis (Fig. 1e, $P < 0.001$). Thus, CPT and Fas activation-induced cell death cooperatively.

MTT assay was used to determine the concentration-dependent sensitivity of DAOY cells to CPT and CH-11. CH-11 at 500 ng/ml induced only 18% cell death. Thus, DAOY cells were minimally sensitive to CH-11 at high concentration. CPT alone induced DAOY cell death in a dose-dependent manner. At 0.1 μmol/l, CPT only caused a 5% decrease in cell viability (Fig. 2a). Simultaneous application of sublethal concentrations of CPT (0.1 μmol/l) with CH-11 induced synergistic cell death (Fig. 2a and c). CH-11 alone at 50 ng/ml induced only 6% cell loss after 24 h treatment, whereas there was about 25% cell death when applied with CPT (Fig. 2a). CH-11 also potentiated cell death induced by CPT in DAOY cells. At 10 μmol/l, CPT induced approximately 21% of cell death. Adding CH-11 (50 ng/ml) together with CPT at 10 μmol/l induced approximately 55% cell death (Fig. 2b). Clearly, the effect of the two drugs in combination was greater than the additive effect of either drug alone. Applying an isobologram analysis (as described in the Materials and methods) showed that $I_x = 0.91$, indicating that CH-11 and CPT act synergistically to induce cell death in DAOY cells.

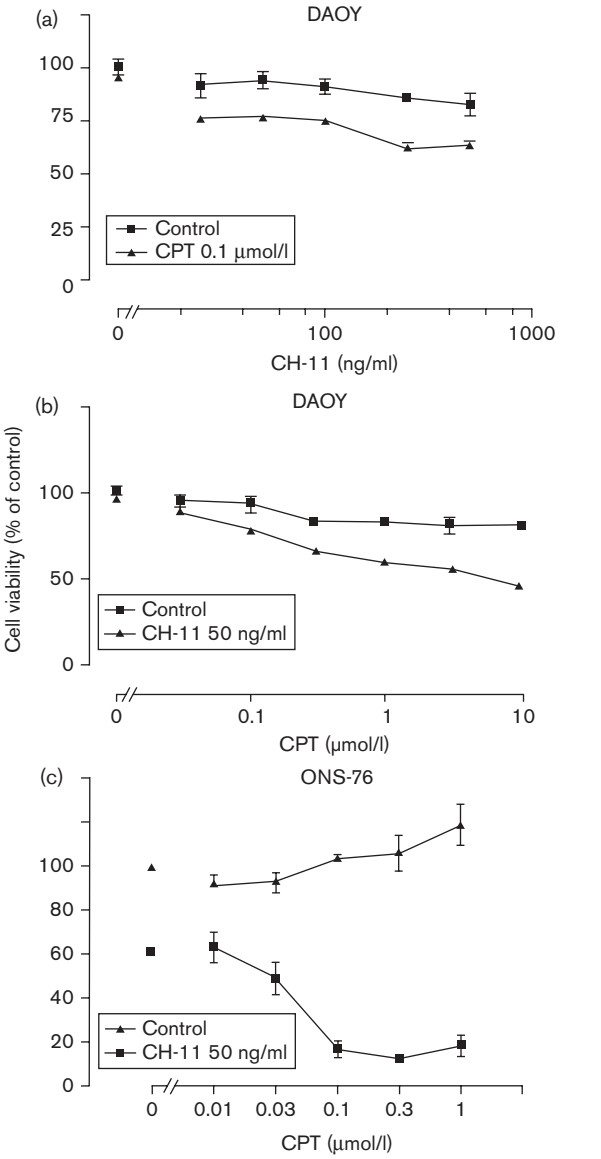
We observed an even greater cooperative/synergistic cell death response in ONS-76, another medulloblastoma cell line. ONS-76 cells respond to CH-11 + CPT similarly to DAOY. CPT at a concentration of less than 1 μmol/l did not induce visible cell death. CH-11 at 50 ng/ml induced

Fig. 1



Camptothecin (CPT) and CH-11 cooperate to induce cell death in DAOY medulloblastoma cells. (a–d) Phase contrast photomicrographs of DAOY cells treated for 24 h with CPT (0.1 μmol/l) alone (b), CH-11 (50 ng/ml) alone (c), and CH-11 + CPT (d). Compared with control cells (a), CPT or CH-11 alone caused little change in cell viability. Combining CPT and CH-11 induced dramatic cell death with typical features of apoptosis. Scale bar = 100 μm. (e) FACScan analysis of DAOY cell apoptosis using annexin V-FITC and propidium iodide staining. Annexin V-positive cells were found to be 9, 7, and 33% in the presence of CPT (0.1 μmol/l), CH-11 (50 ng/ml), and CH-11 + CPT, respectively. Experiments were repeated three times. Data represents mean ± SE ($N = 9$, $^*P < 0.001$ vs. control).

Fig. 2



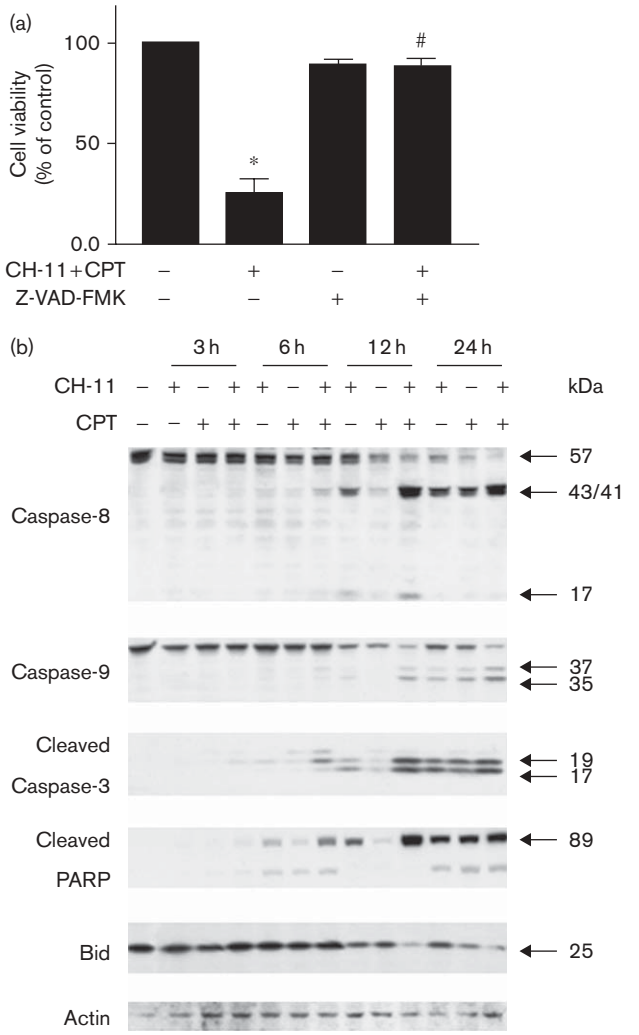
Concentration dependency of medulloblastoma cell death induced by CH-11, camptothecin (CPT), or their combination. (a) DAOY cells were treated for 24 h with different concentrations of CH-11 alone or in the presence of CPT (0.1 μmol/l). Cell viability was quantified by the MTT assay and presented as the percentage of absorbance measured in untreated control cultures. CPT alone induced 5% cell loss. CH-11 at 50 ng/ml caused only 6% cell death. The combination of two induced approximately 25% cell death. (b) Response of DAOY cells to different concentrations of CPT alone or in the presence of CH-11 (50 ng/ml). At 10 μmol/l, CPT induced approximately 21% cell death. Adding CH-11 together with CPT induced approximately 55% cell death. (c) CPT potentiates CH-11-induced ONS-76 medulloblastoma cell death. CPT at 0.1 μmol/l did not induce ONS-76 death. CH-11 at 50 ng/ml caused approximately 40% cells death. CH-11 + CPT induced approximately 80% cell death. Experiments were repeated three times. Data represents mean ± SE ($N = 9$, $^*P < 0.001$ vs. control).

approximately 40% cell death. CPT potentiated CH-11 toxicity in ONS-76 cells in a dose-dependent manner. Together with CPT (0.1 μmol/l), CH-11 induced approximately 80% cell death (Fig. 2c).

Cell death pathways involved in the synergistic cell death

We examined pathways involved in the synergistic cell death induced by CH-11 + CPT in DAOY cells. The pan-caspase inhibitor Z-VAD-FMK (50 μmol/l) completely reversed cell death induced by CH-11 + CPT, indicating that the synergistic death response was predominantly caspase-dependent apoptosis (Fig. 3a). Western blot analysis showed that caspase-3, caspase-8, caspase-9 and PARP were cleaved as early as 6 h after treatment with CH-11 + CPT (Fig. 3b). The substantial increase in

Fig. 3



Involvement of the intrinsic and extrinsic apoptosis pathways in the synergistic cell death induced by CPT + CH-11. (a) DAOY cells were preincubated with the pan-caspase inhibitor Z-VAD-FMK (50 μmol/l) for 30 min before treatment with CPT + CH-11. Apoptosis induced by CPT (1 μmol/l) + CH-11 (50 ng/ml) was completely rescued by Z-VAD-FMK. Cell viability is presented as the percentage of control cultures in MTT assay. (b) Western blot analysis shows the time course of caspase-8, caspase-9, caspase-3, PARP, and Bid cleavage in the presence of CH-11 (50 ng/ml) alone, CPT (1 μmol/l) alone, and CPT + CH-11. At 12 h, the combination of CPT + CH-11 induced cleavage of caspase-8, caspase-9, caspase-3, PARP, and Bid. Bid cleavage is evaluated by the reduction in total Bid protein. Experiments were repeated three times and representative data were shown ($N=9$, $^{*}P<0.001$ vs. control. $^{\#}P<0.001$ vs. CPT + CH-11). CPT, camptothecin.

PARP cleavage in response to CH-11 + CPT at 12 h correlated with the increase in caspase-3 cleavage. Cleavage of caspase-3 and caspase-8 indicates that the cell death induced by CH-11 + CPT involves both extrinsic and intrinsic cell death pathways. This was further confirmed by the cleavage of Bid in response to CH-11 + CPT. Total Bid levels decreased 12 h after cell exposure to CH-11 alone or to CH-11 + CPT (Fig. 3b).

Extrinsic and intrinsic pathway components affected by CPT

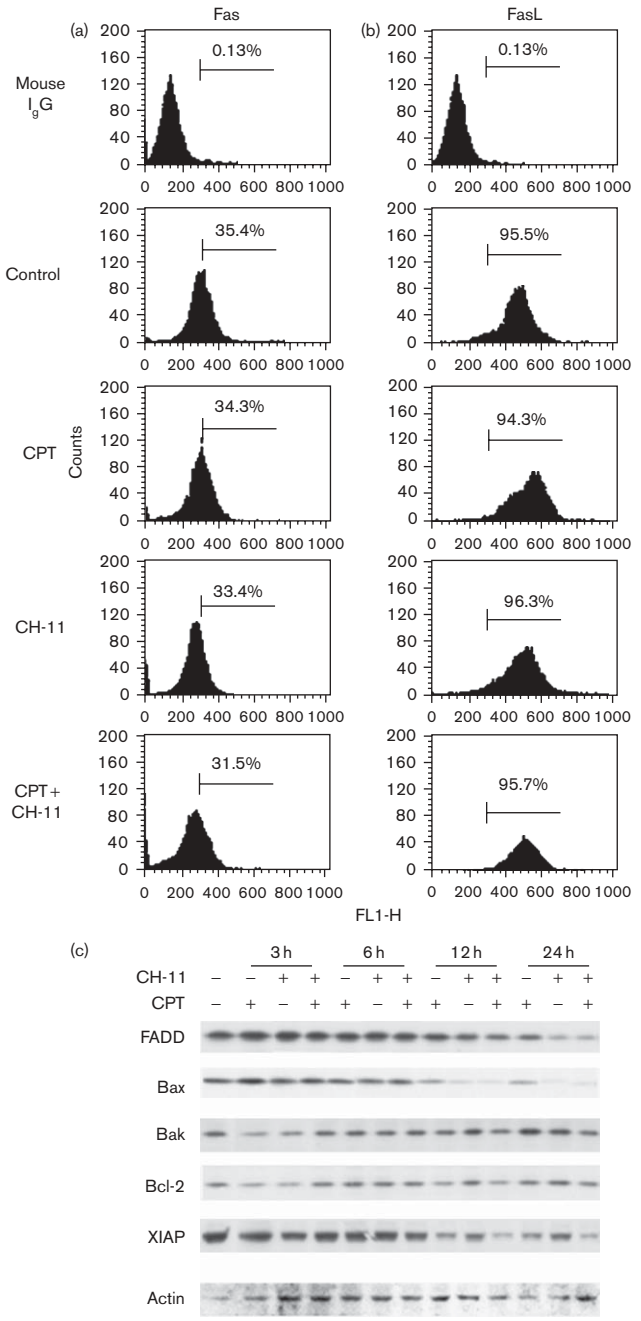
We further examined the molecular pathways involved in the synergistic cell death induced by CH-11 + CPT. Chemotherapeutic drugs can modulate cell death by upregulating or downregulating proteins involved in both extrinsic and intrinsic cell death pathways. Therefore, we used flow cytometry and Western blot analysis to determine whether CPT or the combination of CH-11 + CPT changed the expression of death receptor pathway proteins or mitochondrial pathway proteins. Expression of cell surface Fas or FasL in DAOY cells did not change after 24 h drug treatment (Fig. 4a and b). Western blot analysis showed that compared with the control, FADD decreased after 12 h incubation with CH-11 alone, or in combination with CPT. CH-11 + CPT did not induce a change in the FADD level compared with CH-11 alone (Fig. 4c). The expression of the proapoptotic protein Bax decreased in the presence of CPT or CH-11 after 12 h incubation, but the combination of CH-11 + CPT did not induce a more significant decrease in Bax expression. CH-11 + CPT did not change the expression level of Bak protein. The expression of the antiapoptotic protein Bcl-2 was not altered by CPT, CH-11, or their combination. However, both CPT and CH-11 alone decreased the XIAP protein level after 12 h incubation. The combination of CH-11 + CPT induced a significant decrease in XIAP level (2.5-fold) compared with either drug alone. Western blot showed no expression of Bad or survivin in DAOY cells (data not shown). Thus, CPT might increase the susceptibility of DAOY cells to CH-11 induced cell death by depleting the cells of XIAP.

Reactive oxygen species mediate synergism between CH-11 + CPT

We examined whether CPT + CH-11 affected the production of ROS in medulloblastoma cells and whether ROS generation was required for the synergistic death induced by CH-11 + CPT. The generation of O_2^- and H_2O_2 was quantified by analyzing the oxidation of DHE and DCFDA using FACScan. There was no change in DCFDA intensity in cells treated with CH-11 or CPT, either alone or in combination (data not shown). However, we found that DHE oxidation was increased 75% by CPT alone after 24 h of incubation (Fig. 5a–e). CH-11 alone did not change DHE oxidation. The combination of CH-11 + CPT induced a 3.5-fold increase in DHE intensity compared with untreated control cells. This indicated that O_2^- production was increased by CPT, and even more so by the CH-11 + CPT treatment. Generation of ROS in ONS-76 cells was also recorded by measuring DHE intensity change. In response to CH-11 + CPT, DHE oxidation increased six-fold after 24 h incubation (Fig. 5f).

DAOY cells were preincubated with the antioxidants GSH (10 mmol/l) or NAC (10 mmol/l) for 4 h before

Fig. 4



Effect of CPT + CH-11 on DAOY expression of apoptosis regulators. (a) Fas expression in DAOY cells treated with CH-11, CPT alone, or their combination as detected by FACsan analysis. Mouse IgG was used as negative control. In DAOY control cells, there were approximately 30% Fas-positive cells. CPT, CH-11 alone, or their combination did not change Fas expression. (b) Fas ligand (FasL) expression in DAOY cells treated with CH-11, CPT alone, or their combination. DAOY cells were permeabilized with Triton X-100 before incubation with anti-FasL antibody. Approximately 95% of DAOY cells express FasL. Compared to control, FasL levels in whole cells were not changed by CPT, CH-11, or their combination. (c) Western blot analysis showed the time course of expression of Fas-associated death domain (FADD), Bax, Bak, Bcl-2, XIAP after treatment with CH-11, CPT, and CPT + CH-11. There was a downregulation in FADD, Bax, and XIAP expression level with CPT + CH-11 at 24 h. Bcl-2 and Bak were not affected by treatment conditions. CPT, camptothecin.

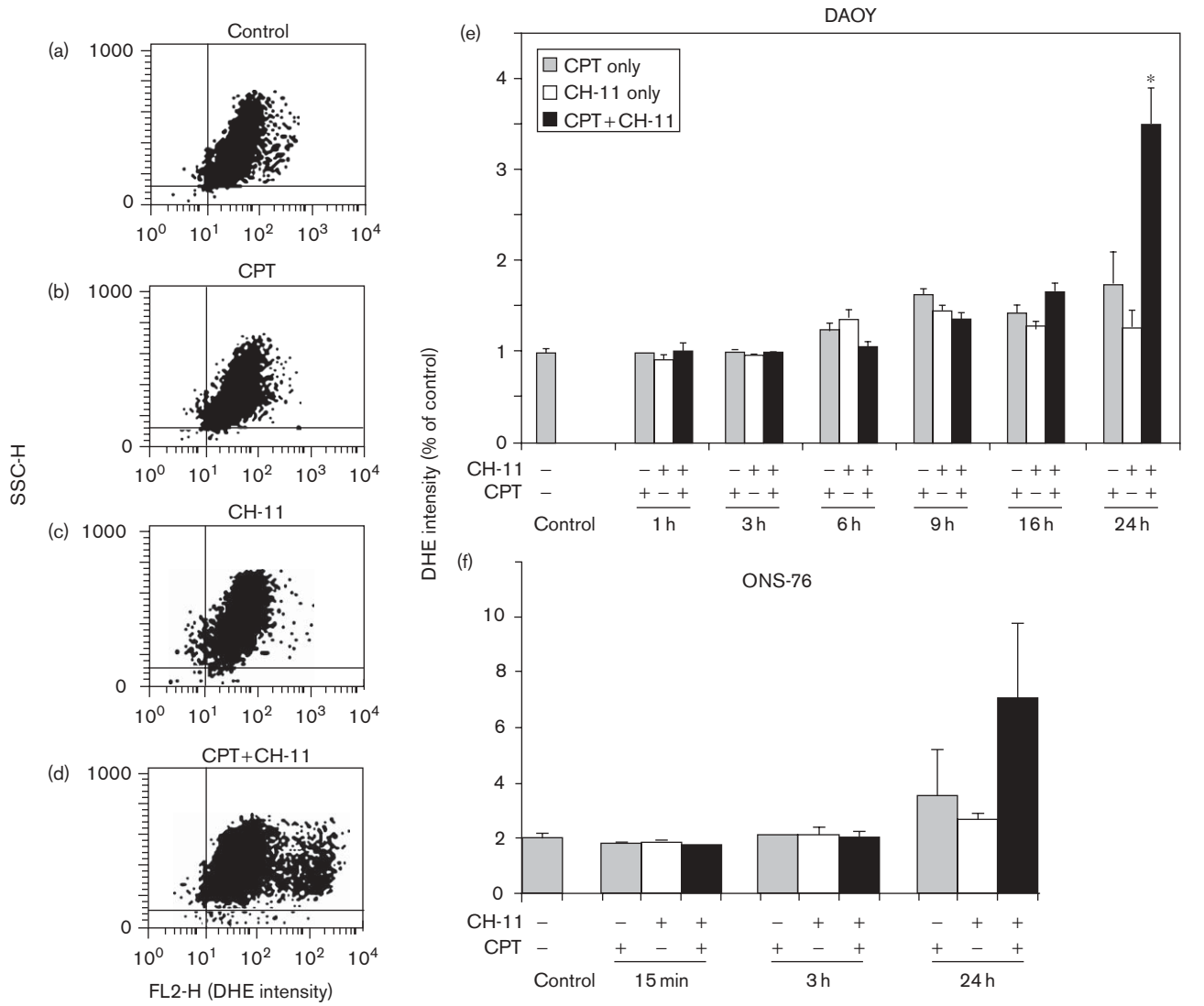
treatment with CH-11 + CPT. Both GSH and NAC prevented cell death induced by CH-11 + CPT as quantified by MTT assay (Fig. 6a). GSH or NAC at 10 mmol/l reversed cell death by approximately 54 and 55%, respectively ($P < 0.001$). This cytoprotection was confirmed with flow cytometry analysis, which showed that GSH or NAC decreased cell death by 35 and 45%, respectively (Fig. 6b, $P < 0.001$). The same results were obtained with ONS-76 cells. GSH and NAC were able to prevent ONS-76 cell death induced by CH-11 + CPT by 70 and 85%, respectively, as detected by MTT (Fig. 6c).

To further explore the role of ROS in potentiating apoptosis induced by CH-11, we examined the effect of exogenous ROS on CH-11-dependent apoptosis. We used the mitochondrial respiratory chain complex I inhibitor rotenone to generate ROS. In DAOY cells, coapplication of rotenone (0.3–1 μmol/l) with CH-11 was found to potentiate apoptosis in a dose-dependent manner (Fig. 6d). Taken together, these findings support the mechanism by which CPT potentiates Fas activation-mediated cell death in medulloblastoma cells by ROS generation and oxidative stress.

Discussion

Death receptor-based targeted therapy, especially TRAIL, has been combined with other sensitization methods to generate considerable antitumor effects in a variety of tumor models. For example, TRAIL has been combined with chemotherapeutic drug, radiation, or DNA methylation inhibitors to induce regression of breast, colon, glioblastoma, and prostate tumor growth *in vivo* [13–20,24]. CPT and its derivative irinotecan (CPT-11) are approved antitumor agents. The target of the CPT family is DNA topoisomerase I, a nuclear enzyme involved in the relaxation of the DNA double helix required for replication and transcription activities. These agents are particularly useful in advanced and metastatic colorectal carcinoma, lung cancer, and cervix cancer. Their main side effects are diarrhea and neutropenia [25]. To the best of our knowledge, so far there are no similar in-vivo studies in medulloblastoma. Although various cancer cells can be sensitized to death receptor ligand-mediated apoptosis with chemotherapeutic agents, the mechanisms responsible for this effect are poorly understood [13,14,18–20,26]. Here, we evaluated the mechanistic basis by which the chemotherapeutic agent CPT sensitizes medulloblastoma cells to death receptor activation-induced apoptosis. Our earlier findings in glioblastoma cells identified a role for ROS under similar experimental condition [13]. We applied similar strategies and techniques to study the synergistic cell death in medulloblastoma cell lines. We found that cell death induced by CH-11 + CPT was partially prevented by the antioxidant agent GSH and NAC, and that the combination of CH-11 and CPT causes significant ROS

Fig. 5

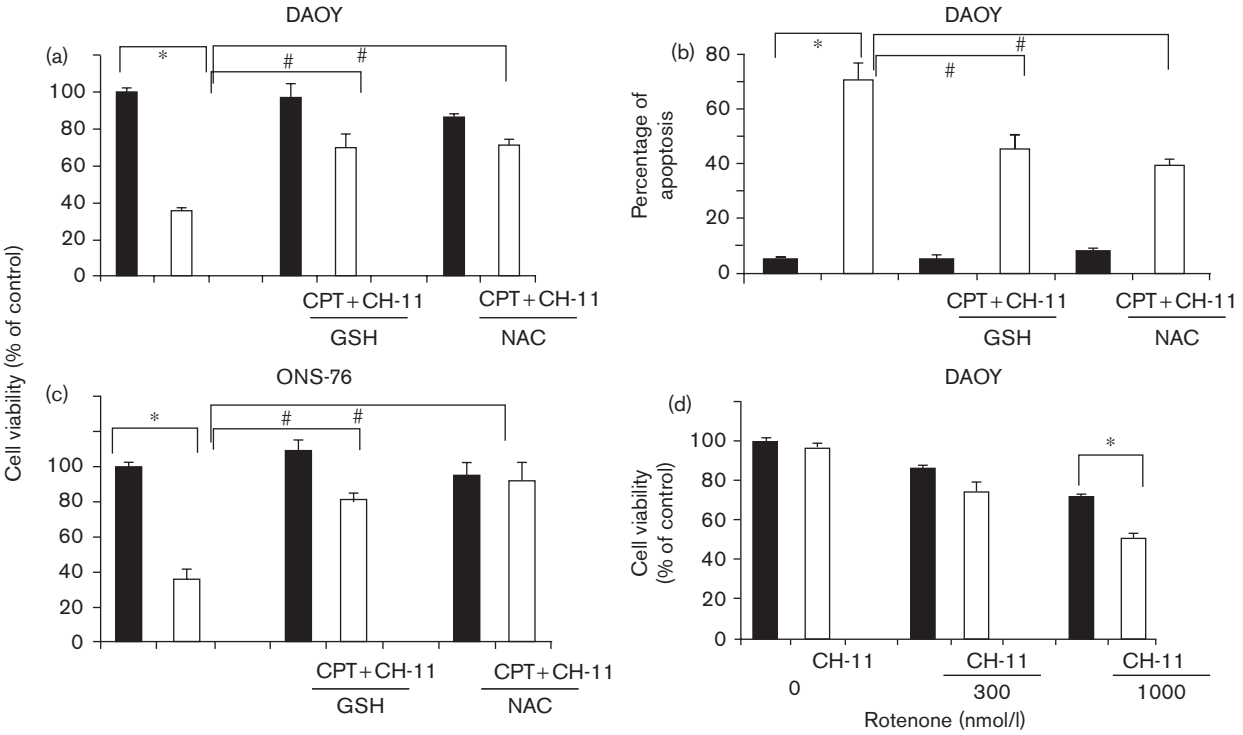


Camptothecin (CPT) and CH-11 cooperatively increase reactive oxygen species in medulloblastoma cells. (a–d) DAOY cells were treated with CH-11 (b), CPT (c), and their combination CPT + CH-11 (d) for 24 h and then incubated with the O_2^- oxidation fluorescence dye dihydroethidium (DHE, 10 μmol/l) for 30 min. DHE fluorescence intensity (FL2-H) was analyzed by FACScan and CellQuest software. There was a DHE intensity increase in cells treated with CPT + CH-11, consistent with increased O_2^- formation in the cells. (e) Quantification of DHE intensity change in DAOY cells during treatment by CPT, CH-11, or both. Intensity from control cells (after background subtraction) was set as 100%. CH-11 or CPT as single agents had little or no effect on DHE oxidation for up to 24 h of treatment. In response to CPT + CH-11, DHE oxidation increased 3.5-fold at 24 h. (f) Generation of ROS in ONS-76 cells as measured by DHE intensity change. In response to CPT + CH-11, DHE oxidation increased six-fold after 24 h incubation. Experiments were repeated three times and representative data were shown ($N=9$, $^*P<0.001$ vs. control).

production. Furthermore, the mitochondrial respiratory chain complex I inhibitor rotenone is known to generate ROS, especially superoxide and H_2O_2 in mitochondria. In DAOY cells, application of rotenone was found to sensitize cells to CH-11-induced apoptosis. Therefore, we suspect that CPT induces apoptosis in human medulloblastoma cells, through the induction of oxidative stress, followed by the activation of caspase-9 and caspase-8, which in turn, cleave caspase-3.

ROS (hydrogen peroxide, hydroxyl radicals, and superoxide anion) are highly reactive molecules with unpaired electrons that are generated in normal physiological processes or by external stress, such as UV light, ionizing irradiation, and DNA-damaging drugs [27,28]. There are two sources of ROS in most cell types: (i) the major source is the mitochondrial electron transport chain; and (ii) an alternate source of ROS is the small GTPase Rac–NADPH oxidase complex, which generates ROS in the plasma membrane. CPT and its derivatives have been found by others to induce oxidative stress in a variety of cell types and tumor models, including those derived from human breast cancer, small cell lung carcinoma, and T cell leukemia Jurkat [19,29]. Simizu *et al.* [29] showed

Fig. 6



Reactive oxygen species generation mediates the synergistic medulloblastoma cell death induced by CPT + CH-11. (a) Antioxidants protect DAOY cells from death induced by CPT + CH-11. DAOY cells were incubated with the antioxidants glutathione (GSH, 10 mmol/l) or *N*-acetyl-cysteine (NAC, 10 mmol/l) for 4 h before and during treatment (24 h) with CPT + CH-11. Cell death was quantified by the MTT assay. GSH and NAC protect cells from death caused by CPT + CH-11 by 54 and 55%, respectively. (b) Annexin V-FITC flow cytometry indicates that DAOY cell apoptosis induced by CPT + CH-11 was attenuated by GSH or NAC by approximately 35 and 45%, respectively. (c) The MTT assay shows that GSH and NAC protect ONS-76 cells from death induced by CH-11 + CPT by 70 and 85%, respectively ($N=6$, $^{*}P<0.001$ vs. control, $^{\#}P<0.001$ vs. CH-11 + CPT). (d). Rotenone enhanced cell death induced by Fas activation in a dose-dependent manner, as determined by the MTT assay. Rotenone alone at 1 μmol/l induced 28% cell death. Together with CH-11 (250 ng/ml), rotenone induced 50% cell death in DAOY cells. Data represent mean ± SE. ($N=6$, $^{*}P<0.001$ vs. control). CPT, camptothecin.

that generation of ROS by NADPH oxidase may be directly or indirectly related to CPT-induced apoptosis. Although it is proposed that CPT induces apoptosis by superoxide formation, the mechanism of ROS formation by CPT must be further elucidated.

ROS have the capacity to interact with death receptor pathways on at least two different nodes: DISC and mitochondria. It has been reported that ROS formation in the Jurkat cell line facilitates Fas receptor aggregation, which in turn, leads to the activation of caspase-8 and cell death [30,31]. In addition, oxidative stress-induced damage of proteins and lipids, particularly in mitochondria, can disrupt mitochondria and stimulate cytochrome c release [27,32]. Thus, ROS could sensitize cells to apoptosis through the mitochondrial pathway. We found that in DAOY cells, XIAP and Bax were downregulated by the combination of CH-11 + CPT, suggesting potential effect of ROS on mitochondria in the sensitization of medulloblastoma cells to CH-11.

ROS are capable of eliciting direct damage on macromolecules, such as DNA, proteins, and lipids. Higher levels of ROS result in slowed growth, cell cycle arrest, and apoptosis or even necrosis through signaling transduction pathways [32]. Among the signal transduction pathways influenced by ROS, c-Jun N-terminal kinase (JNK) and NF-kB appear to play important roles in the setting of death receptor activation by tumor necrosis factor-α [33,34]. ROS seem to play a critical role in the cross-talk between JNK and NF-kB. NF-kB acts as a suppressor of intracellular ROS formation. ROS are potent mediators of JNK activation, which further induces apoptosis through interaction with the mitochondrial apoptotic machinery. Therefore, it seems necessary to establish a more detailed understanding of the signaling pathways induced by ROS generation in medulloblastoma model systems.

In summary, we have shown that CPT sensitizes death receptor activation-induced apoptosis in human medulloblastoma cells by a cellular mechanism that involves the early production of ROS and concomitant activation of initiator caspase-8 and caspase-9, converging in the activation of the downstream effector caspase-3.

Although the precise molecular mechanism by which CPT exerts its oxidative action on cells is not yet clear, combining chemotherapeutic drugs with death receptor activation could be of considerable therapeutic interest. As the extent of synergized cytotoxicity is partially linked to the inherent cellular ROS level, and as tumor cells usually bear a higher ROS level owing to their hypermetabolism state when compared with their nontumor counterparts, tumor cells are more subject to apoptosis induced by elevated ROS level in response to the combination therapy. Thus far, antioxidants have been considered helpful in cancer treatment because they reduce the side effects of chemotherapy and radiotherapy. However, we propose that caution should be taken with antioxidants, as the production of ROS during chemotherapy is in fact beneficial to the cell death-inducing properties of chemotherapeutic drugs.

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