Necrotic pathway in human osteosarcoma Saos-2 cell death induced by chloroacetaldehyde

Kyohei Takahashi[a], Koichi Sakurai[a], Kiyoshi Takahashi[a], Hiroyuki Tanaka[a,b] and Yukio Fujimoto[a]

Chloroacetaldehyde, a metabolite of the anticancer drug ifosfamide, may be responsible for serious adverse effects like encephalopathy in ifosfamide chemotherapy. In this study, we demonstrate that chloroacetaldehyde, but not ifosfamide, induces cell death in human osteosarcoma Saos-2 cells and we investigated the mechanism by which this occurs. Chloroacetaldehyde above 30 μmol/l induced significant cell death in a time-dependent manner. Thiol compounds such as *N*-acetyl cysteine, glutathione and dithiothreitol protected the cells against chloroacetaldehyde-induced cell death, although other nonthiol compounds and the antioxidative enzymes superoxide dismutase and catalase did not, suggesting that reactive oxygen species might not mediate cell death. In cells exposed to chloroacetaldehyde, levels of both total thiols and glutathione were significantly reduced. Chloroacetaldehyde also collapsed the mitochondrial membrane potential of these cells, induced the release of cytochrome *c* from mitochondria to the cytosol and significantly reduced cellular ATP levels during the course of death. The mitochondrial potential collapse was also prevented by thiol compounds. Flow cytometric analyses by means of annexin-V and propidium iodide double staining and immunofluorescence staining of active caspase-3 revealed that cells subjected to a lethal dose of chloroacetaldehyde displayed features characteristic of necrosis and that caspase-3 was not activated in response to chloroacetaldehyde. Taken together, these findings suggest that Saos-2 cells exposed to chloroacetaldehyde die by necrosis resulting from a decrease in intracellular thiols, disruption of the mitochondrial membrane potential and the depletion of cellular ATP. *Anti-Cancer Drugs* 18:543–553 © 2007 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18:**543–553

Keywords: ATP, chloroacetaldehyde, cytochrome *c*, human osteosarcoma Saos-2 cells, mitochondrial membrane potential, necrosis, thiol

[a]Department of Biochemistry, Hokkaido Pharmaceutical University, Otaru and [b]National Hospital Organization Hokkaido Cancer Center, Sapporo, Hokkaido, Japan

Correspondence to Dr Koichi Sakurai, PhD, Department of Biochemistry, Hokkaido Pharmaceutical University, 7-1 Katsuraoka-cho, Otaru-City, Hokkaido 047-0264, Japan
Tel: +81 134 62 1891; fax: +81 134 62 5161;
e-mail: ks51@hokuyakudai.ac.jp

Sponsorship: This work was supported by Shionogi & Co., Ltd.

Received 18 July 2006 **Revised form accepted** 3 December 2006

Introduction

Ifosfamide (IFM) is an anticancer drug efficacious against a variety of cancers such as lung, breast and testicular carcinoma, sarcoma, and lymphoma [1]. 4-Hydroxy-IFM (4-OH-IFM) and 4-hydroperoxy-IFM (4-OOH-IFM), thought to be active metabolites of IFM, cause DNA alkylation and kill cancer cells by inhibiting DNA synthesis [2–4]. Chloroacetaldehyde (CAA), also a metabolite of IFM, is thought to cause the serious adverse effects of IFM chemotherapy because of its high toxicity [4–6]. On the other hand, in acute promyelocytic leukemia HL-60 cells, no significant differences were detected in cell injury between CAA and 4-OH-IFM [7]. Treatment of the breast carcinoma cell line MX1 with a combination of CAA and 4-OH-IFM decreased cell viability additively, compared with single administration of each drug [8]. In a study using mice that bore MX1 xenografts, CAA and 4-OH-IFM prevented tumor growth [9]. IFM was more effective than a structural isomer, cyclophosphamide (CPA), against testicular tumors, soft tissue sarcomas and some types of lung cancer [10]. As the rate of 4-hydroxylation of IFM is slower than that of CPA, the metabolic pathway to CAA is predominant and the amount of CAA produced from IFM was markedly greater than that from CPA [1,11]. From these results, we hypothesize that CAA could itself have anticancer activity.

Through the formation of complexes with thiol groups or induction of redox reactions that consume thiol groups, many aldehydes decrease cellular thiol levels, which play an important role in cell survival [12,13]. For example, CAA reacted with glutathione (GSH) to form *S*-formylmethyl-glutathione and thus decreased GSH levels in brain and liver of mice [14]. Glyoxal, a reactive α-oxoaldehyde, generated reactive oxygen species (ROS) and caused lipid peroxidation in isolated rat hepatocytes, resulting in oxidation of GSH [15]. Sood *et al.* [16] indicated that lipid peroxidation and depletion of GSH, proteinaceous thiols, and ATP were involved in the CAA-induced cell death of isolated rat hepatocytes. Moreover, this cell death was prevented by addition of the thiol-containing compound dithiothreitol (DTT),

0959-4973

suggesting that the decrease in intracellular thiol levels caused by CAA resulted in death [16].

CAA rapidly decreased glutamate-dependent mitochondrial respiration and induced release of Ca^{2+} from mitochondria to the cytosol in isolated rat liver [17]. CAA inhibited membrane transport of the long-chain fatty acid palmitic acid through the inhibition of the carnitine system in the mitochondrial inner membrane [18]. CAA inhibited β-oxidation of fatty acids in the presence of palmitoyl-L-carnitine, leading to suppression of mitochondrial respiration and decreased ATP production [19]. These findings strongly suggest that CAA causes mitochondrial dysfunction. Mitochondria play an important role in the induction of cell death. We have reported previously that the 1-hydroxyethyl radical (which resembles CAA in chemical structure except that it has a radicalized carbon atom, i.e. the chlorine atom of CAA is replaced with hydrogen) induced the mitochondrial permeability transition (MPT), which reportedly participates in apoptosis in isolated rat liver mitochondria [20]. Acrolein, a highly reactive α,β-unsaturated aldehyde, induced apoptosis through collapse of mitochondrial membrane potential, cytochrome *c* release and activation of caspase-9 [21]. Moreover, several aldehydes such as methylglyoxal and cinnamaldehyde induced apoptosis in cultured cells [22–24]. These results suggest that CAA could induce apoptosis through the disruption of mitochondrial function and subsequent activation of caspases. A recent study, however, reported necrotic cell death accompanied by mitochondrial dysfunction involving cytochrome *c* release in human epithelial A549 cells under hypoxic conditions [25]. When mice were fed drinking water containing CAA, hepatocellular necrosis was also observed [26]. Taking these findings into account, it is not clear which pathway, apoptosis or necrosis, is activated after induction of mitochondrial injury by CAA. As such, investigating CAA-induced cell death will contribute to clarification of the anticancer action and adverse effects of IFM as well as to an understanding of the biological action of CAA itself.

The goal of this study was to clarify the mode of cell death induced by CAA. As IFM is an effective treatment in patients with osteosarcoma, our experiments used Saos-2 cells – an established osteosarcoma-derived cell line that is highly sensitive to IFM. We demonstrated that CAA induced necrosis in Saos-2 cells, which was preceded by a decrease in thiol levels, collapse of mitochondrial membrane potential and ATP depletion. Our results suggest that CAA induces a mitochondrial injury-mediated necrotic pathway in Saos-2 cells.

Methods

Chemicals

IFM and 4-OOH-IFM were obtained from Shionogi (Osaka, Japan). Trolox, *N*-acetyl cysteine (NAC), superoxide dismutase, catalase, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), albumin, L-buthionine sulfoximine (BSO), fetal bovine serum (FBS) and McCoy's 5A medium were purchased from Sigma (St Louis, Missouri, USA). CAA, L-ascorbic acid, α-tocopherol, mannitol, thiourea, L-cysteine, 2-mercaptoethanol (ME), GSH, DTT, 5,5′-dithio-bis(2-nitrobenzoic acid) and 4% paraformaldehyde phosphate buffer solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). The polyclonal antibody against cleaved caspase-3 (Asp175) was from Cell Signaling Technology (Beverly, Massachusetts, USA). Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG was from Jackson ImmunoResearch Laboratories (West Baltimore Pike, Pennsylvania, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay kit was from Promega (Madison, Wisconsin, USA). BCA protein assay kit was from Pierce (Rockford, Illinois, USA). GSH assay kit was from OXIS (Portland, Oregon, USA). Cytochrome *c*, mitochondrial Membrane Sensor kit and Cell Fractionation kit were from BD Biosciences (Clontech, Palo Alto, California, USA). Annexin-FLUOS Staining kit was from Roche (Penzberg, Germany). The ATP assay kit was from Calbiochem (San Diego, California, USA). All other chemicals used in this study were of the highest grade available from commercial suppliers.

Cell culture

Human osteosarcoma Saos-2 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and were maintained in McCoy's 5A medium supplemented with 10% FBS in 75-cm^2 polystyrene flasks. The cells were cultured in an atmosphere of 5% CO_2 and 95% air at 37°C. The cells used were subconfluent cultures in exponential phase.

Cell viability

Cell viability was estimated by the ability of mitochondrial dehydrogenases to reduce MTT to formazan using an MTT assay kit. The cells were seeded onto 96-well plates at a density of 1×10^4 cells/100 µl culture medium. After incubating the cells in culture medium containing varying concentrations of CAA, IFM or 4-OOH-IFM for various times as described in the figure legends, the medium was removed and the cells were washed two times with phosphate-buffered saline (PBS). Culture medium consisting of 10% FBS (50 µl) and a dye solution (7.5 µl) was added to each of the 96 wells for a 4-h incubation at 37°C. A solubilization/stop solution (50 µl) was added to each well with overnight incubation at 37°C. The absorbance values at 540 nm (formation of formazan) and 690 nm (reference) were recorded with a microplate reader (Labsystems Bichromatic, Helsinki, Finland). Viability was determined as compared to control cells not treated with CAA.

Thiol content

Thiol content of Saos-2 cells was measured as described by Tanaka *et al.* [27]. Briefly, the cells were seeded onto six-well plates at a density of 5×10^6 cells/well. CAA at 30 μmol/l or DIDS at 50 μmol/l was added for various incubation times as described in the figure legends. Following the incubation, the cells were trypsinized and collected by centrifugation at 250*g* for 5 min. After washing with PBS, the cells were resuspended in 1.5 ml PBS and sonicated with an ultrasonic disrupter (model UR-200p; Tomy Seiko, Tokyo, Japan) three times for 10-s periods at 35 W in ice water. The lysate was centrifuged at 3000*g* for 10 min at 4°C. The supernatant was collected and protein content was determined using the BCA protein assay kit according to the manufacturer's instructions with bovine albumin as a standard.

After the addition of 5,5′-dithio-bis(2-nitrobenzoic acid) (2 mmol/l), the mixture was incubated for 10 min at 37°C. The thiol content of the mixture was measured by absorbance at 412 nm using a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The concentration of thiol groups in the mixture was calculated using the extinction coefficient of the resultant product, nitrothiobenzoate, $\varepsilon = 14\,100$ mol/l/cm. The GSH content of the cells was determined by a GSH assay kit according to the manufacturer's instructions. Briefly, to remove proteinaceous thiols, the cells (5×10^6) were washed and resuspended in 1 ml PBS containing 10% trichloroacetic acid. The suspension was centrifuged at 1750*g* at 4°C for 10 min. The supernatant was extracted three times with four volumes of diethyl ether. The remaining diethyl ether was removed by purging with N_2 gas at room temperature. After the sample was adjusted to 0.9 ml with *N*-2-hydroxyl piperazine-*N*′-2-ehane sulfonic acid buffer, pH 7.4, the GSH concentration was measured an authentic GSH as a standard.

Analysis of mitochondrial membrane potential

Mitochondrial membrane potential was estimated using a mitochondrial membrane sensor kit according to the manufacturer's instructions (Clontech, Palo Alto, California, USA). Briefly, Saos-2 cells were seeded onto a six-well plate at a density of 1×10^6 cells/well and incubated with CAA for various incubation times as described in the figure legends. Following the incubation, the cells were scraped and collected by centrifugation at 350*g* for 5 min. After washing with PBS, the cells were stained with 1 μl MitoSensor reagent (denoted JC-1) and incubated in a 5% CO_2 incubator at 37°C for 15 min. After removal of the unbound fluorescent dye by pelleting the cells (350*g* for 5 min), the cells were resuspended in 1 ml incubation buffer and analyzed using a flow cytometer (Beckman Coulter, Fullerton, CA, USA). The fluorescence of J-aggregates (see Results for explanation) was detected by the fluorescence channel 2 (FL-2) and the fluorescence of JC-1 monomers by the fluorescence channel 1 (FL-1).

Annexin-V and propidium iodide binding assay

The experimental conditions and cell sampling process were the same as described for the analysis of mitochondrial membrane potential. After washing with PBS, the cells (1×10^6) were double-stained with annexin-V fluorescein (20 μl) and propidium iodide (PI) (20 μl) at room temperature for 10 min. After centrifugation to remove the unbound dyes, the cells were resuspended in 0.5 ml incubation buffer and analyzed by flow cytometry. The fluorescence of FITC and PI was detected by FL-1 and FL-2, respectively.

Western blotting of cytochrome *c*

Cells were seeded onto 150-mm plates at a density of 5×10^7 cells/50 ml. CAA (30 μmol/l) was added for various incubation times as described in the figure legends. Following incubation, the cells were scraped and collected by centrifugation at 600*g* for 5 min and subsequently resuspended in 1 ml wash buffer containing 0.05% (w/v) Tween 20 in PBS. We then pelleted the samples as above and resuspended them in 600 μl fractionation buffer consisting of 10 mmol/l *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid, 0.15 mol/l NaCl, 1 mmol/l ethylene glycol tetraacetic acid, 0.5 mmol/l $MgCl_2$ and 1 mmol/l DTT at pH 7.0. After incubating the cells for 10 min on ice, we passed the cells through a 23-gauge needle five times to lyse the plasma membrane. We separated mitochondria from nuclei and broken membranes by centrifugation at 700*g* for 10 min at 4°C. We then centrifuged the supernatant at 10 000*g* for 25 min at 4°C. The supernatant was collected and used as the cytosolic fraction, and the pellet was resuspended in fractionation buffer and used as the mitochondrial fraction. Lactate dehydrogenase and succinate dehydrogenase were used as specific markers of the cytosolic and mitochondrial fractions, respectively. We carried out protein measurements using the BCA protein assay kit. The concentration of cytochrome *c* was determined by Western blot analysis as described in Tanel and Averill-Bates [21]. Briefly, 5 μg protein per lane was run on a 15% sodium dodecyl sulfate–polyacrylamide gel and electrotransferred to a polyvinylidine diflouride membrane for 40 min at 200 V. To block nonspecific binding sites, the membrane was immersed in Tris-buffered saline–Tween consisting of 20 mmol/l Tris, 0.14 mol/l NaCl, 0.1% Tween 20 and 5% nonfat dried milk for 1 h. After appropriate washings, the membrane was incubated with a polyclonal antibody against cytochrome *c* (1 : 500 dilution) and with a donkey anti-rabbit horseradish peroxidase-linked secondary antibody (1 : 2000 dilution), successively. The bands of cytochrome *c* were detected by enhanced chemiluminescence using the ECL Plus Western Blotting Detection System (Amersham, Little Chalfont, UK).

Detection of active caspase-3 by flow cytometry

The experimental conditions and sampling process of the cells were the same as described for the analysis of

mitochondrial membrane potential. After collecting the cells, they were resuspended with PBS, fixed in 1% formaldehyde for 10 min at 37°C, permeabilized with 90% methanol for 10 min at 4°C and washed two times with PBS. After blocking the cells with 0.5% bovine serum albumin in PBS for 10 min at room temperature, they were rinsed two times with PBS. Primary antibody against active caspase-3 (1 : 25 dilution) was added and incubated at room temperature for 30 min followed by incubation with FITC-conjugated donkey anti-rabbit IgG (1 : 200 dilution) at room temperature for 30 min. After removal of antibodies, the cells were rinsed two times with PBS, resuspended in 0.5 ml PBS and analyzed by flow cytometry with FL-1.

ATP content

The ATP content of Saos-2 cells was determined using an ATP assay kit based on the luciferin–firefly luciferase method [28]. The principle of the assay is the firefly luciferase-catalyzed oxidation of D-luciferin in the presence of an ATP-magnesium salt and oxygen, whereby the amount of ATP can be readily quantitated by the amount of luminescence produced. The experimental conditions and the sampling process of the cells were the same as described for the analysis of mitochondrial membrane potential. After collecting the cells, they were resuspended in 1 ml PBS, and an aliquot (200 µl) of the suspension was subjected to the measurement of ATP. The luminescence was recorded by ATP Photometer model 2000 (SAI Technology, La Jolla, California, USA).

Statistical analysis

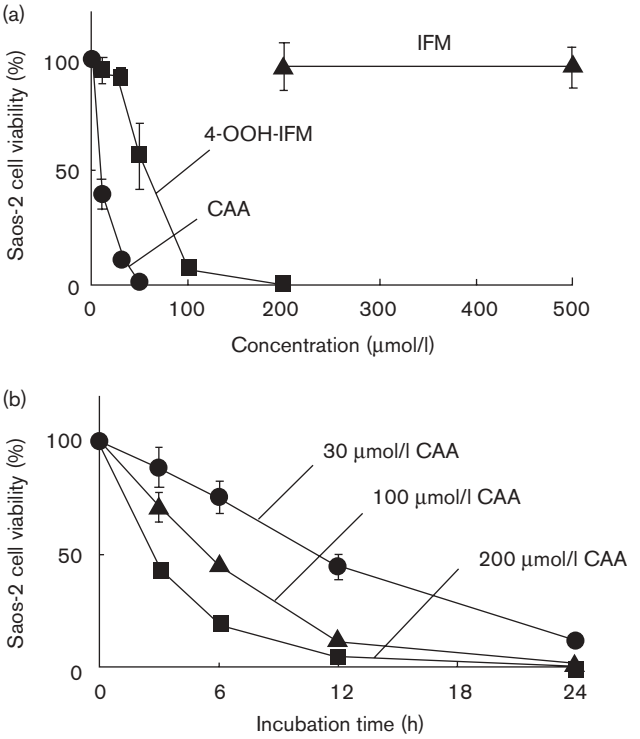
Data are expressed as the mean ± SD and were analyzed using the Student's *t*-test for paired data. $P < 0.05$ was considered statistically significant.

Results

Viability of Saos-2 cells treated with chloroacetaldehyde

First we investigated the effect of IFM, 4-OOH-IFM and CAA on viability of human osteosarcoma Saos-2 cells (Fig. 1a) exposed to 10–500 µmol/l of these compounds for 24 h. At CAA concentrations at or above 10 µmol/l, cell viability decreased substantially. At 4-OOH-IFM concentrations of 50 µmol/l, the viability was 57.2%. On the other hand, IFM at concentrations up to 500 µmol/l had no effect on viability. These results demonstrate that CAA is the most powerful inducer of cell death among the three compounds tested. Therefore, we conducted the following experiments to clarify the mechanism by which CAA induces Saos-2 cell death. Figure 1b shows that CAA induces cell death in a dose- and time-dependent manner. We detected a slight decrease in cell viability at 3 h following 30 µmol/l CAA treatment, and a significant reduction after 6 h of incubation. Therefore, in subsequent experiments we selected 30 µmol/l CAA as the dose and 24 h as the incubation period.

Fig. 1

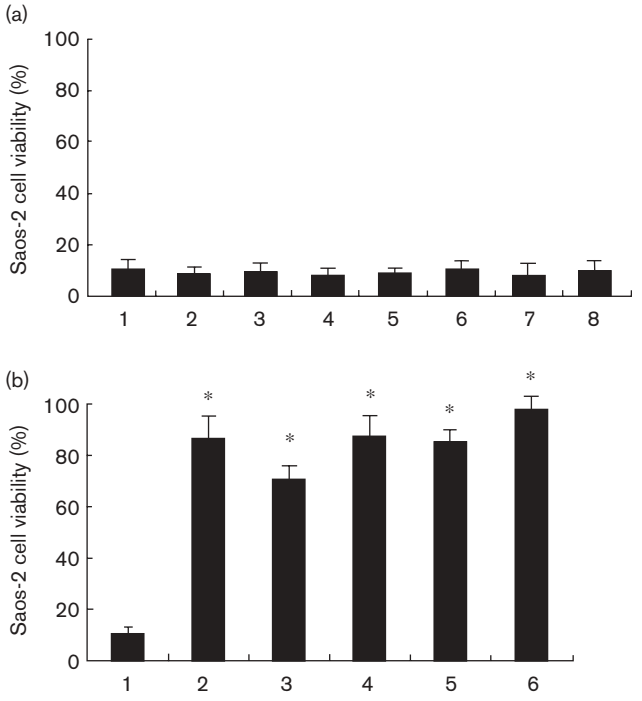


Viability of Saos-2 cells treated with ifosfamide (IFM), 4-hydroperoxy-IFM (4-OOH-IFM) and chloroacetaldehyde (CAA). (a) Human osteosarcoma Saos-2 cells were incubated with various concentrations of IFM, 4-OOH-IFM and CAA for 24 h. (b) Cells were incubated with 30, 100 or 200 µmol/l of CAA for the indicated times. The viability of the cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay as described in Methods. Data are mean ± SD of six independent experiments.

Effect of antioxidative enzymes and compounds on chloroacetaldehyde-induced Saos-2 cell death

Several aldehydes induce cell death via generation of ROS [15,21,29,30]. We thus investigated the effect of antioxidative enzymes or compounds on CAA-induced cell death to clarify whether ROS might mediate the cytotoxicity of CAA. Antioxidative compounds were added both 1 h before the addition of CAA and at the same time as addition of CAA. In this treatment paradigm, these enzymes and compounds had no protective effect against CAA-induced cell death (Fig. 2a). These compounds had no effect on the viability of control cells in the absence of CAA. Flow cytometric detection of peroxides using a specific fluorescent probe 2′,7′-dichlorofluorescein diacetate also revealed that the 2′,7′-dichlorofluorescein fluorescence did not increase after CAA treatment (data not shown). These results suggest that ROS might not participate in CAA-induced cell death.

Fig. 2



Effect of thiol compounds and antioxidants on chloroacetaldehyde (CAA)-induced Saos-2 cell death. (a) Antioxidative enzymes were added at the same time as CAA and antioxidative compounds were added 1 h before the addition of CAA. Cells were incubated with 30 μmol/l CAA for 24 h. Column 1, cells treated with CAA; 2, + 1000 U/ml superoxide dismutase; 3, + 1000 U/ml catalase; 4, + 0.1 mmol/l ascorbic acid; 5, + 0.025 mmol/l α-tocopherol; 6, + 0.05 mmol/l trolox; 7, + 1 mmol/l mannitol; 8, + 1 mmol/l thiourea. (b) Cells were preincubated with various thiol compounds for 1 h and then incubated with 30 μmol/l CAA for 24 h. Column 1, cells treated with CAA; 2, + 1 mmol/l cysteine; 3, + 1 mmol/l 2-mercaptoethanol; 4, + 1 mmol/l glutathione; 5, + 0.1 mmol/l dithiothreitol; 6, + 0.5 mmol/l *N*-acetyl cysteine. The viability of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Data are mean ± SD of six independent experiments. *$P < 0.05$ compared with CAA alone.

Effect of thiol compounds on chloroacetaldehyde-induced Saos-2 cell death

We next examined the effect of the thiol compounds cysteine, ME reduced GSH (each 1 mmol/l), DTT (0.1 mmol/l), and NAC (0.5) mmol/l on cell death (Fig. 2b). All thiol compounds significantly prevented CAA-induced cell death. Although 30 μmol/l CAA alone lowered cell viability to 10.2%, preincubation with cysteine, ME, GSH or DTT reversed the viability to 86.6, 70.7, 88.0 and 85.2%, respectively (Fig. 2b). These results suggest that CAA might induce cell death by affecting cellular thiol groups, which are indispensable for cell function.

Effect of chloroacetaldehyde on thiol groups in Saos-2 cells

We next investigated the interaction of CAA with cellular thiol groups. Figure 3a shows changes in thiol content over time in Saos-2 cells exposed to 30 μmol/l CAA or 50 μmol/l DIDS, which have almost the same oxidization capacity. Thiol content of the cells exposed to CAA decreased with time; thiol content was 0.41 μmol/mg protein at the beginning of the incubation, and gradually decreased to 0.35, 0.30, 0.16 and 0.09 μmol/mg protein at 3, 6, 12 and 24 h, respectively. When the cells were exposed to 50 μmol/l DIDS, a plasma membrane-impermeable thiol oxidant, thiol content decreased by only 17% during the 24-h incubation. These results suggest that CAA can oxidize thiols in Saos-2 cells.

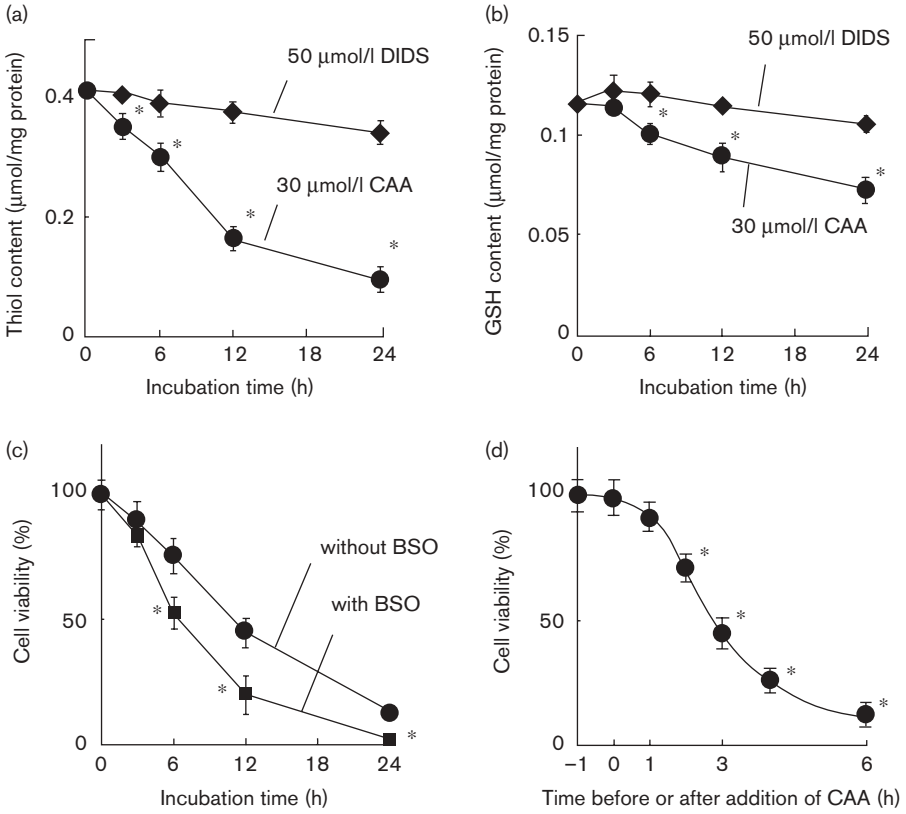
As GSH is at high concentration (i.e. millimolar) in cells, we investigated GSH content in Saos-2 cells exposed to 30 μmol/l CAA or 50 μmol/l DIDS. CAA decreased GSH content in a time-dependent manner; GSH content was 0.12 μmol/mg protein at the beginning of incubation, and gradually decreased to 0.11, 0.10, 0.09 and 0.07 μmol/mg protein at 3, 6, 12 and 24 h, respectively (Fig. 3b). CAA decreased GSH in Saos-2 cells about 37% during the 24-h incubation, but DIDS decreased it only 9%. Figure 3c shows viability of CAA-treated Saos-2 cells pretreated overnight with or without BSO, an inhibitor of GSH synthesis. The concentration of GSH was reduced in cells pretreated with BSO compared with untreated cells ($P < 0.05$, data not shown). Time-dependent cell death was observed in both cell groups, whereas viability of BSO-pretreated cells was slightly lower than that of untreated cells, indicating that depletion of cellular GSH did not influence CAA-induced cytotoxicity.

The cell-permeable reductant NAC was added to Saos-2 cells at different times before or after the addition of CAA. NAC prevented and partially reversed the CAA-induced cytotoxicity when it was added up to 3 h after the addition of CAA (Fig. 3d).

Effect of chloroacetaldehyde on mitochondrial function in Saos-2 cells

Mitochondria play a central role in maintenance of cellular life. We evaluated the effect of CAA on mitochondrial membrane potential ($\Delta\Psi_m$) in Saos-2 cells by flow cytometry. Cells were stained with a mitochondrial membrane sensor, the specific mitochondrial cationic dye JC-1, which accumulates in the transmembrane space of polarized mitochondria and forms so-called 'J-aggregates' that emit orange fluorescence recorded by the FL-2 channel. A decrease in $\Delta\Psi_m$ results in disappearance of J-aggregates and formation of JC-1 monomers, which emit a greenish-yellow fluorescence recorded by the FL-1 channel. The distribution of cells was divided into region A containing normal cells with intact $\Delta\Psi_m$ and region B containing cells with disrupted $\Delta\Psi_m$ (Fig. 4a). As incubation time proceeded, FL-1 fluorescence increased and FL-2 fluorescence decreased (Fig. 4a), indicating that CAA disrupted $\Delta\Psi_m$ in a time-dependent manner. Clear loss of $\Delta\Psi_m$ was detected 3 h after the

Fig. 3



Cellular thiol levels and effect of L-buthionine sulfoximine (BSO) on chloroaceteldehyde (CAA)-induced cell death. Time-course changes of cellular thiol (a) and cellular glutathione (GSH) (b) levels in Saos-2 cells treated with 30 μmol/l CAA or 50 μmol/l 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) for the indicated times. Cellular thiol was measured using the 5,5′-dithio-bis(2-nitrobenzoic acid) method. Cellular GSH was measured using a GSH assay kit. Data are mean ± SD of three independent experiments. *$P<0.05$ compared with the 50 μmol/l DIDS–treated group. (c) Effect of BSO on CAA-induced Saos-2 cell death. Cells were preincubated with or without 0.1 mmol/l BSO overnight and then incubated with 30 μmol/l CAA. Data are mean ± SD of six independent experiments. *$P<0.05$ compared with untreated cells or cells treated without BSO group. (d) *N*-acetyl cysteine was added to cells at different times before or after the addition of 30 μmol/l CAA. Cells in all groups were treated with CAA for 24 h. Data are mean ± SD of six independent experiments. *$P<0.05$ was compared 1 h before the addition of CAA.

addition of CAA, but significant decreases in viability were detected only after 6 h (Fig. 1b), suggesting that disruption of $\Delta\Psi_m$ precedes CAA-induced Saos-2 cell death and that CAA injures mitochondria.

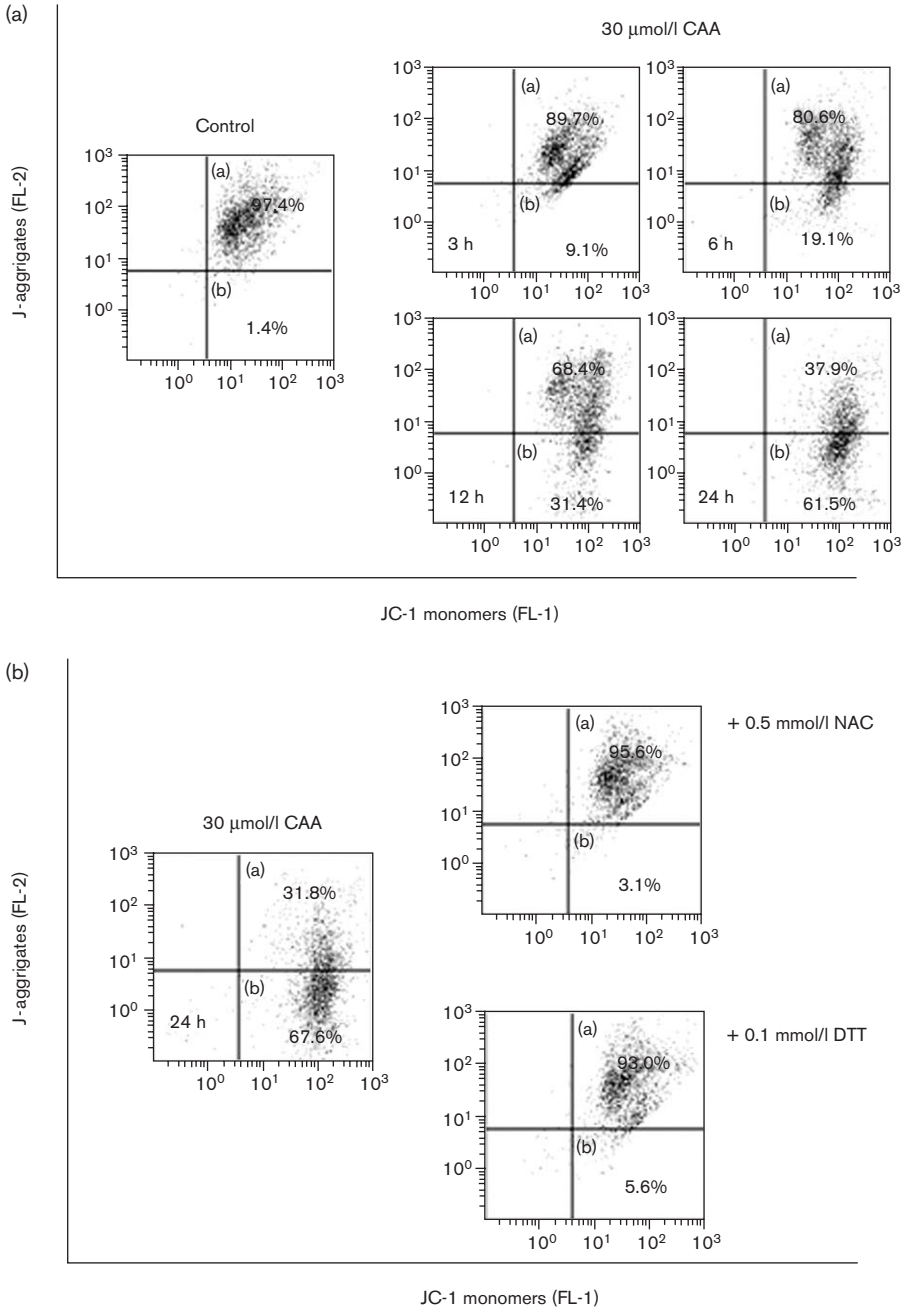
We studied the effect of thiol compounds on the CAA-induced disruption of $\Delta\Psi_m$. Few cells treated with 30 μmol/l CAA in the presence of 0.5 mmol/l NAC or 0.1 mmol/l DTT moved from region A to region B, indicating that these thiol compounds almost completely prevent CAA-induced disruption of $\Delta\Psi_m$ (Fig. 4b).

We next conducted Western blotting to detect the release of cytochrome *c*, which binds to the mitochondrial inner membrane. Before CAA treatment, cytochrome *c* was found predominantly in the mitochondrial fraction. After 6 h of CAA treatment, however, cytochrome *c* in the mitochondrial fraction gradually disappeared and in turn cytochrome *c* in the cytosolic fraction gradually increased (Fig. 5). These data indicate that CAA causes mitochondrial injury.

Chloroacetaldehyde induces necrosis in Saos-2 cells

Although mitochondrial cytochrome *c* release plays an important role in promoting apoptosis, mitochondria are involved in the induction of necrosis as well [31]. We performed two flow cytometric analyses to determine the mode of cell death that CAA induces in Saos-2 cells. First, we used an Annexin-V and PI double-staining method to distinguish between necrosis and apoptosis in the early stages of cell death. Necrotic cells are Annexin-positive and PI-positive, whereas apoptotic cells are Annexin-positive and PI-negative. The distribution of cell populations exposed to CAA changed from the Annexin and PI-negative region (a) to Annexin and PI-positive region (c), depending on incubation time (Fig. 6a). Additionally, the presence of 0.5 mmol/l NAC or 0.1 mmol/l DTT prevented the CAA-induced changes in the distribution of cell populations (Fig. 6b).

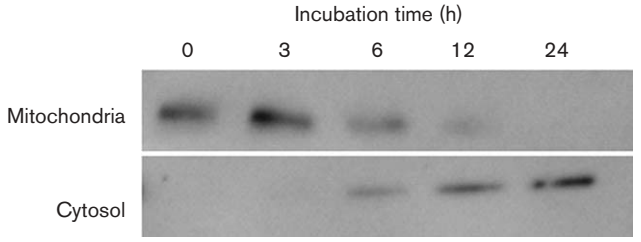
Fig. 4



Effect of thiol compounds on disruption of mitochondrial membrane potential ($\Delta\Psi_m$) in Saos-2 cells treated with chloroacetaldehyde (CAA). (a) Disruption of $\Delta\Psi_m$ of Saos-2 cells treated with 30 μmol/l CAA for the indicated times. (b) Effect of thiol compounds on CAA-induced disruption of $\Delta\Psi_m$. Cells were preincubated with 0.5 mmol/l *N*-acetyl cysteine or 0.1 mmol/l dithiothreitol for 1 h and then incubated with 30 μmol/l CAA for 24 h. The $\Delta\Psi_m$ was detected by flow cytometry using the JC-1 dye. Regions (a) and (b) contain cells with intact $\Delta\Psi_m$ and disrupted $\Delta\Psi_m$, respectively. Similar results were obtained in two other independent experiments using different cell preparations.

Second, we attempted detection of active caspase-3, which is involved in apoptotic cell death, using an antibody against active caspase-3 in combination with a FITC-conjugated secondary antibody. CAA did not activate caspase-3 even after a 24-h incubation (Fig. 7), at which time the majority of cells were dead (Fig. 1b). Moreover, Hoechst 33258 staining of nuclei revealed no condensation or fragmentation of nuclei, and formation of a DNA smear in Saos-2 cells exposed to CAA for 3 or 6 h was detected by gel electrophoresis (data not shown).

Fig. 5



Release of cytochrome *c* from mitochondria in cells treated with chloroacetaldehyde (CAA). Cells were incubated with 30 μmol/l CAA for the times indicated. Cytochrome *c* was examined by Western blotting after separation of the mitochondrial and cytosolic fractions of the cell lysates. Similar results were obtained in two other independent experiments using different cell preparations.

These results suggest that CAA-induced cell death in Saos-2 cells occurs by necrosis and that thiol compounds prevent necrotic cell death.

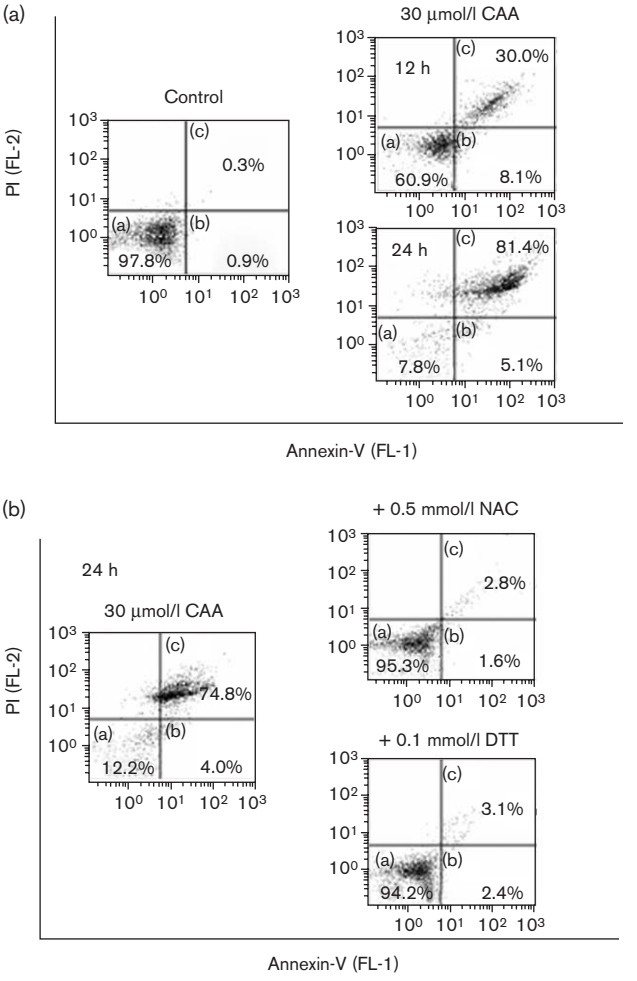
ATP levels in Saos-2 cells after chloroacetaldehyde treatment

Necrotic cell death is accompanied by a decrease in ATP level. We therefore determined ATP levels in the cells exposed to CAA. CAA decreased ATP levels in a time-dependent manner; ATP levels were 2.4 nmol per 2×10^5 cells at the beginning of incubation, and 2.1, 1.5, 0.8 and 0.3 nmol per 2×10^5 cells at 3, 6, 12 and 24 h, respectively (Fig. 8). A significant decrease in ATP level was observed after 6 h, demonstrating that CAA-induced Saos-2 cell death is accompanied by a decrease in cellular ATP levels. These results are also consistent with CAA-induced necrosis as the cause of death in Saos-2 cells.

Discussion

In this study, we demonstrated that CAA, a metabolite of IFM, induces cell death in Saos-2 cells, an established human osteosarcoma cell line, at lower concentrations compared with 4-OOH-IFM, an active metabolite of IFM (Fig. 1). CAA reduces ATP levels, resulting in inhibition of glycolysis and DNA strand breaks in the breast carcinoma cell line MX-1 [32]. The cell toxicity of CAA to MX1 and the thyroid gland sarcoma cell line S117 was about 1.2 and 1.6 times higher, respectively, compared with that of 4-OH-IFM [8]. On the contrary, the cell toxicity of CAA to the acute promyelocytic leukemia cell line HL-60 and the Burkitt B cell lymphoma line HS-Sultan was about 1.3 and 2.3 times lower, respectively, compared with that of 4-OH-IFM [7]. These results suggest that the sensitivity of cells to CAA differed according to tumor cell line. On the other hand, the concentration of CAA in the blood of a patient administered IFM (1.6 g/m^2/day) increased to 109 μmol/l [33]. It is therefore possible that the concentrations of CAA we used in this study would be observed in tumor tissues of IFM-administered patients, suggesting that CAA itself might exert anticancer action when IFM is administered to patients.

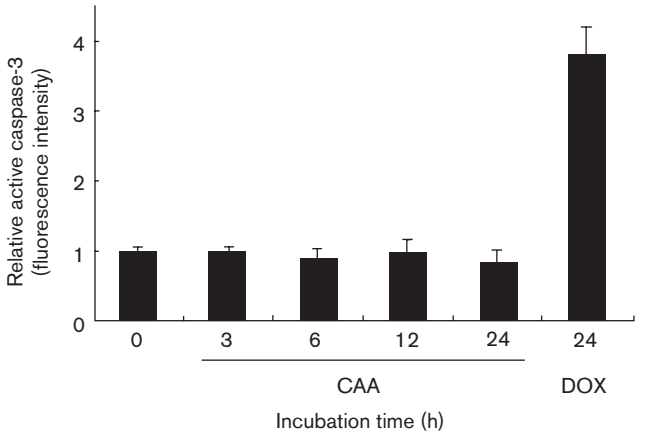
Fig. 6



Chloroacetaldehyde (CAA) induces necrosis in Saos-2 cells. (a) Cells were incubated in 30 μmol/l CAA for 12 or 24 h and then analyzed by flow cytometry using an Annexin-V/propidium iodide (PI) double-staining method. Regions (a), (b) and (c) contain viable, apoptotic and necrotic cells, respectively. Similar results were obtained in two other independent experiments using different preparations of cells. (b) Effect of thiol compounds on CAA-induced necrosis in Saos-2 cells. Cells were preincubated with 0.5 mmol/l *N*-acetyl cysteine (NAC) or 0.1 mmol/l dithiothreitol (DTT) for 1 h and then incubated with 30 mmol/l CAA for 24 h. Similar results were obtained in two other independent experiments using different cell preparations.

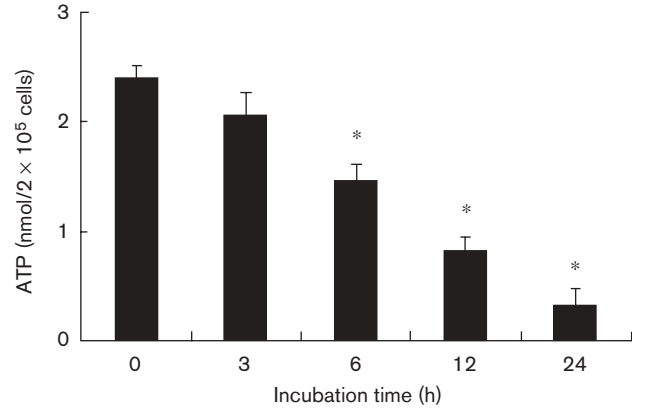
In this study, CAA rapidly decreased cellular thiol levels (Fig. 3), and thiol compounds such as GSH and NAC abolished CAA-induced cell death in Saos-2 cells (Fig. 2b). DIDS, a membrane-impermeable thiol oxidant, however, only slightly decreased cellular thiol levels (Fig. 3a), but did not induce cell death (data not shown). These results suggest that CAA decreases intracellular thiol levels, leading to cell death. When cells were

Fig. 7



Activation of caspase-3 in Saos-2 cells treated with CAA. Measurement of active caspase-3 in Saos-2 cells treated with 30 μmol/l CAA for the times indicated. Active caspase-3 was detected by flow cytometry using a specific antibody. Doxorubicin (DOX) (5 μmol/l) was used as the positive control. Data are mean ± SD of three independent experiments. *$P<0.05$ compared with control.

Fig. 8



ATP levels in Saos-2 cells after chloroacetaldehyde (CAA) treatment. Cells were incubated with 30 μmol/l CAA for the times indicated. The assay for cellular ATP levels is described in Methods. *$P<0.05$ compared with control.

incubated with CAA for 24 h, the content of GSH, a main intracellular thiol compound, was decreased by 37% (Fig. 3b). The viability of BSO (an inhibitor of GSH synthesis)-pretreated cells that contained a low level of GSH did not substantially differ from that of normal cells upon CAA treatment (Fig. 3c). These results suggest that CAA might induce cell death by causing the reduction of level of thiol compounds other than GSH.

Several aldehyde-containing compounds that have a similar structure to CAA induce cell death by generating ROS [15,29,30,32]. In our experiments, superoxide dismutase, catalase and various antioxidants did not prevent CAA-induced cell death (Fig. 2a), and we detected no apparent generation of intracellular peroxides upon CAA treatment (data not shown). In in-vitro experiments, CAA rapidly reacts with a thiol group and forms a complex with thiols under anaerobic and aerobic conditions (data not shown). These data suggest that CAA decreases the intracellular thiol level independently of the generation of ROS. Whereas NAC abolished CAA-induced cell death when added to the cells at the same time as CAA or 1 h before the addition of CAA, it did not completely prevent cell death when it was added 1 h or longer after the addition of CAA (Fig. 3d), suggesting that the rapid decrease in intracellular thiol level that occurs within 1 h after CAA treatment might be one of the key events in cell death. The redox state of thiol groups, which exist in transcription factors and signaling molecules, plays an important role in cell function [34]. We confirmed that the mitogen-activated protein kinase, JNK, which is often involved in cell death as a critical regulator and contains key thiol groups in its active moiety, was not activated upon CAA treatment (data not shown), suggesting that CAA induces cell death through a JNK-independent signal transduction pathway. Further studies are necessary to identify the intracellular thiol compounds that are involved in cell death.

CAA collapsed mitochondrial membrane potential at 3 h of incubation (Fig. 4a), but thiol compounds abolished the collapse (Fig. 4b). Mitochondria are important organelles in determining cell survival or death, and the collapse of $\Delta\Psi_m$ and the induction of MPT are accepted as early events in mitochondria-mediated cell death [34,35]. Oxidation of cysteine residues in the adenine nucleotide translocator existing in the mitochondrial inner membrane and a decrease in the amount of reduced thioredoxin might lead to the induction of MPT and cytochrome *c* release [36,37]. Although the 1-hydroxyethyl radical, a compound similar to CAA in chemical structure, induced MPT through oxidation of the thiol group, thiol-containing compounds prevented radical-induced MPT [20]. These results suggest that CAA induces mitochondrial injury by lowering intracellular thiol levels. Cytochrome *c* release was observed in CAA-treated Saos-2 cells (Fig. 5). The strength of fluorescence of both Annexin-V and PI, however, was increased in CAA-treated cells, indicating that necrosis rather than apoptosis was in progress (Fig. 6). Moreover, we did not detect apoptotic features such as caspase-3 activation (Fig. 7), caspase-8 and caspase-9 activation (at 3, 6 and 12 h of incubation), chromatin condensation, the formation of a DNA ladder and nuclear fragmentation (at 3 and 6 h of incubation) (data not shown). Although it is generally supposed that mitochondrial injury is involved in the induction of apoptosis, the possibility has also been raised that it leads to necrosis [38–41]. Wang *et al.* [25] demonstrated that necrotic cell death was executed in human epithelial A549 cells under hyperoxia conditions,

though activation of the caspase-8/Bid pathway led to mitochondrial injury followed by cytochrome *c* release and caspase-9 activation.

In this study, a decrease in cellular ATP level was rapidly observed following damage to mitochondria, such as collapse of $\Delta\Psi_m$, when cells were treated with CAA (Figs 4 and 8), suggesting that mitochondrial dysfunction was responsible for the decrease in ATP levels. Apoptosis is an energy-dependent mode of cell death, and ATP plays an important role in the mitochondrial-mediated apoptotic cell death pathway via formation of the apoptosome and activation of caspase-9 [42,43]. Leist *et al.* [44] described how the mode of cell death induced by an apoptosis-inducing agent was shifted from apoptosis to necrosis when the cellular ATP level was decreased by oligomycin, an inhibitor of mitochondrial ATP synthesis. Han *et al.* [45] showed that usnic acid-induced necrosis in hepatocytes was preceded by a rapid decrease in the intracellular ATP level. Kim *et al.* [46] demonstrated that the supply of ATP via glycolytic breakdown of fructose prevented ischemia–reperfusion-induced necrosis in hepatocytes. Considering the possibility that the intracellular ATP level decides the mode of cell death, we speculate that CAA induces necrotic cell death in Saos-2 cells owing to ATP depletion arising from mitochondrial injury.

Our present study clarifies that CAA decreases the intracellular thiol level and induces mitochondrial injury, leading to necrotic cell death in Saos-2 cells owing to ATP depletion. Considering that CAA, even at low concentrations, decreased the viability of these cells, it is possible that CAA might have anticancer activity in patients who receive IFM chemotherapy. When necrosis occurs, integrity of the plasma membrane declines and leakage of dying cell contents into surrounding tissues may cause inflammation. Therefore, CAA can contribute to the adverse effects of IFM therapy. It is desirable that anticancer drugs induce apoptosis, a mode of cell death not accompanied by inflammation, to avoid serious adverse effects resulting from inflammation and to increase specificity of the drug to tumor cells. As such, prevention of CAA-induced depletion of intracellular ATP might switch the mode of tumor cell death from necrosis to apoptosis.

References

1 Dechant KL, Brogden RN, Pilkington T, Faulds D. Ifosfamide/mesna. *Drugs* 1991; **42**:428–467.
2 Furlanut M, Franceschi L. Pharmacology of ifosfamide. *Oncology* 2003; **65**:2–6.
3 Yazawa Y, Takagi T, Asakura S, Suzuki K, Kano Y. Effect of 4-hydroperoxy ifosfamide in combination with other anticancer agents on human cancer cell lines. *J Orthop Sci* 1999; **4**:231–237.
4 Huitema AD, Smits KD, Mathot RA, Schellens JH, Rodenhuis S, Beijnen JH. The clinical pharmacology of alkylating agents in high-dose chemotherapy. *Anticancer Drugs* 2000; **11**:515–533.
5 Dubourg L, Michoudet C, Cochat P, Baverel G. Human kidney tubules detoxify chloroacetaldehyde, a presumed nephrotoxic metabolite of ifosfamide. *J Am Soc Nephrol* 2001; **12**:1615–1623.
6 Wagner T. Ifosfamide clinical pharmacokinetics. *Clin Pharmacokinet* 1994; **26**:439–456.
7 Brueggemann SK, Schlenke P, Klich S, Deeken M, Peters SO, Wagner T. Stem cell toxicity of oxazaphosphorine metabolites in comparison to their antileukemic activity. *Biochem Pharmacol* 2002; **63**:1337–1341.
8 Bruggemann SK, Kisro J, Wagner T. Ifosfamide cytotoxicity on human tumor and renal cells: role of chloroacetaldehyde in comparison to 4-hydroxyifosfamide. *Cancer Res* 1997; **57**:2676–2680.
9 Borner K, Kisro J, Bruggemann SK, Hagenah W, Peters SO, Wagner T. Metabolism of ifosfamide to chloroacetaldehyde contributes to antitumor activity *in vivo*. *Drug Metab Dispos* 2000; **28**:573–576.
10 Moore MJ. Clinical pharmacokinetics of cyclophosphamide. *Clin Pharmacokinet* 1991; **20**:194–208.
11 de Jonge ME, Huitema AD, van Dam SM, Rodenhuis S, Beijnen JH. Effects of co-medicated drugs on cyclophosphamide bioactivation in human liver microsomes. *Anticancer Drugs* 2005; **16**:331–336.
12 Yang X, Wu X, Choi YE, Kern JC, Kehrer JP. Effect of acrolein and glutathione depleting agents on thioredoxin. *Toxicology* 2004; **204**: 209–218.
13 Kondo M, Oya-Ito T, Kumagai T, Osawa T, Uchida K. Cyclopentenone prostaglandins as potential inducers of intracellular oxidative stress. *J Biol Chem* 2001; **276**:12076–12083.
14 Sood C, O'Brien PJ. 2-Chloroacetaldehyde-induced cerebral glutathione depletion and neurotoxicity. *Br J Cancer* 1996; **74**:287–293.
15 Shangari N, O'brien PJ. The cytotoxic mechanism of glyoxal involves oxidative stress. *Biochem Pharmacol* 2004; **68**:1433–1442.
16 Sood C, O'Brien PJ. Chloroacetaldehyde-induced hepatocytes cytotoxicity. *Biochem Pharmacol* 1994; **48**:1025–1032.
17 Sood C, O'Brien PJ. Molecular mechanisms of chloroacetaldehyde-induced cytotoxicity in isolated rat hepatocytes. *Biochem Pharmacol* 1993; **46**:1621–1626.
18 Visarius TM, Stucki JW, Lauterburg BH. Inhibition and stimulation of long-chain fatty acid oxidation by chloroacetaldehyde and methylene blue in rats. *J Pharmacol Exp Ther* 1999; **289**:820–824.
19 Visarious TM, Stucki JW, Lauterburg BH. Stimulation of respiration by methylene blue in rat liver mitochondria. *FEBS Lett* 1997; **412**: 157–160.
20 Sakurai K, Stoyanovsky DA, Fujimoto Y, Cederbaum AI. Mitochondrial permeability transition induced by 1-hydroxyethyl radical. *Free Radic Biol Med* 2000; **28**:273–280.
21 Tanel A, Averill-Bates DA. The aldehyde acrolein induces apoptosis via activation of the mitochondrial pathway. *Biochim Biophys Acta* 2005; **1743**:255–267.
22 Al-Maghrebi MA, Al-Mulla F, Benov LT. Glycolaldehyde induces apoptosis in a human breast cancer cell line. *Arch Biochem Biophys* 2003; **417**: 123–127.
23 Fukunaga M, Miyata S, Liu BF, Miyazaki H, Hirota Y, Higo S, *et al.* Methylglyoxal induces apoptosis through activation of p38 MAPK in rat Schwann cells. *Biochem Biophys Res Commun* 2004; **320**: 689–695.
24 Wu SJ, Ng LT, Lin CC. Cinnamaldehyde-induced apoptosis in human PLC/PRF/5 cells through activation of the proapoptotic Bcl-2 family proteins and MAPK pathway. *Life Sci* 2005; **77**:938–951.
25 Wang X, Ryter SW, Dai C, Tang ZL, Watkins SC, Yin XM, *et al.* Necrotic cell death in response to oxidative stress involves the activation of the apoptogenic caspase-8/Bid pathway. *J Biol Chem* 2003; **278**: 29184–29191.
26 Daniel FB, DeAngelo AB, Stober JA, Olson GR, Page NP. Hepatocarcinogenicity of chloral hydrate, 2-chloroacetaldehyde, and dichloroacetic acid in the male B6C3F1 mouse. *Fundam Appl Toxiccol* 1992; **19**:159–168.
27 Tanaka H, Sakurai K, Takahashi K, Fujimoto Y. Requirement of intracellular free thiols for hydrogen peroxide-induced hypertrophy in cardiomyocytes. *J Cell Biochem* 2003; **89**:944–955.
28 Farber JL. Biology of disease: membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. *Lab Invest* 1982; **47**:114–123.
29 Du J, Suzuki H, Nagase F, Akhand AA, Ma XY, Yokoyama T, *et al.* Superoxide-mediated early oxidation and activation of ASK1 are important for initiating methylglyoxal-induced apoptosis process. *Free Radic Biol Med* 2001; **31**:469–478.
30 Saito Y, Nishio K, Yoshida Y, Niki E. Cytotoxic effect of formaldehyde with free radicals via increment of cellular reactive oxygen species. *Toxicology* 2005; **210**:235–245.

31 Jemmerson R, LaPlante B, Treeful A. Release of intact, monometric cytochrome *c* from apoptotic and necrotic cells. *Cell Death Differ* 2002; **9**:538–548.
32 Bruggemann SK, Radike K, Braasch K, Hinrichs J, Kisro J, Hagenah W, *et al.* Chloroacetaldehyde: mode of antitumor action of the ifosfamide metabolite. *Cancer Chemother Pharmacol* 2006; **57**:349–356.
33 Goren MP, Wright RK, Pratt CB, Pell FE. Dechloroethylation of ifosfamide and neurotoxicity. *Lancet* 1986; **2**:1219–1220.
34 Sen CK. Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochem Pharmacol* 1998; **55**:1747–1758.
35 Kim JS, He L, Lemasters JJ. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. *Biochem Biophys Res Commun* 2003; **304**:463–470.
36 Constantini P, Belzacq AS, Vieira HLA, Larochette N, dePablo MA, Zamzami N, *et al.* Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. *Oncogene* 2000; **19**:307–314.
37 Rigobello MP, Scutari G, Folda A, Bindoli A. Mitochondrial thioredoxin reductase inhibition by gold(I) compounds and concurrent stimulation of permeability transition and release of cytochrome *c*. *Biochem Pharmacol* 2004; **67**:689–696.
38 Matsumura H, Shimizu Y, Ohsawa Y, Kawahara A, Uchiyama Y, Nagata S. Necrotic death pathway in Fas receptor signaling. *J Cell Biol* 2000; **151**:1247–1255.
39 Kumar S, Bharti A, Mishra NC, Raina D, Kharbanda S, Saxena S, *et al.* Targeting of the c-Abl tyrosine kinase to mitochondria in the necrotic cell death response to oxidative stress. *J Biol Chem* 2001; **276**:17281–17285.
40 Ogbourne SM, Suhrbier A, Jones B, Cozzi SJ, Boyle GM, Morris M, *et al.* Antitumor activity of 3-ingenyl angelate: plasma membrane and mitochondrial disruption and necrotic cell death. *Cancer Res* 2004; **64**:2833–2839.
41 Troyano A, Fernandez C, Sancho P, deBlas E, Aller P. Effect of glutathione depletion on anticancer drug toxicity (apoptosis and necrosis) in U-937 human Promonocytic cells. *J Biol Chem* 2001; **276**:47107–47115.
42 Hu Y, Benedict MA, Ding L, Nunez G. Role of cytochrome *c* and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis. *EMBO J* 1999; **18**:3586–3595.
43 Jiang X, Wang X. Cytochrome *c* promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J Biol Chem* 2000; **275**:31199–31203.
44 Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* 1997; **185**:1481–1486.
45 Han D, Matsumaru K, Rettori D, Kaplowitz N. Usnic acid-induced necrosis of cultured mouse hepatocytes: inhibition of mitochondrial function and oxidative stress. *Biochem Pharmacol* 2004; **67**:439–451.
46 Kim JS, Qian T, Lemasters JJ. Mitochondrial permeability transition in the switch from necrotic to apoptotic cell death in ischemic rat hepatocytes. *Gastroenterology* 2003; **124**:494–503.