Mitochondria determine the efficacy of anti-cancer agents that interact with DNA but not the cytoskeleton

Kenjirou Hara, Emiko Kasahara, Nozomi Takahashi, Masami Konishi, June Inoue, Mika Jikumaru, Shuji Kubo, Haruki Okamura, Eisuke Sato, Masayasu Inoue

K. H., E. K., N. T., M. K., J. I., M. J., E. S. and M. I.:
Department of Biochemistry and Molecular Pathology,
Osaka City University Medical School,
Asahimachi 1-4-3, Abeno, Osaka, Osaka, Japan

K. H.:
Iwata Chemical Co., Ltd, Iwata, Shizuoka, Japan

E. K., S. K. and H. O.:
Laboratory of Host Defenses, Institute for Advanced Medical Sciences, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

Running title: Role of mitochondria in cancer chemotherapy
Abbreviations: mtDNA, mitochondrial DNA; nuclear DNA, nDNA; LLC, Lewis lung carcinoma; DMEM, Dulbecco’s modified Eagle’s medium; RPMI-1640, Roswell Park Memorial Institute-1640; FBS, fetal bovine serum; FACS, Fluorescence-activated cell sorting; TBST, Tris-buffered saline Tween-20; Mn-TBAP, manganese (III) tetrakis (4-benzoic acid) porphyrin; JC-1, 5, 5’, 6, 6’-tetrachloro-1, 1’, 3, 3’-tetaethylbenzimidazolyld carbocyanine iodide; TBS, Tris-buffered saline

Correspondence: Masayasu Inoue, Department of Biochemistry and Molecular Pathology, Osaka City University Medical School, Asahimachi 1-4-3, Abeno, Osaka,
Osaka, Japan; Tel: +81-6-6645-3720; Fax: +81-6-6645-3721; E-mail: inoue@med.osaka-cu.ac.jp
Abstract

Although chemotherapy is an important method for the treatment of patients with cancer, its efficacy is limited due to different sensitivities of tumor cells to anti-cancer agents and/or side effects on normal tissues. The present work demonstrates that mitochondria play a crucial role in apoptosis of cancer cells induced by anti-cancer agents that interact with DNA but not with the cytoskeleton. Agents which interact with DNA selectively enhanced generation of reactive oxygen species (ROS) in mitochondria, released cytochrome c and activated caspase-9 and 3 to induce apoptosis of mesothelioma H2052 cells but not their $\rho^0$ cells which lack mitochondrial DNA (mtDNA). The sensitivity of a variety of cells to the agents showed positive correlation with the amounts of their mitochondria. In contrast, agents that selectively affect the cytoskeleton activated caspase-8 and 3, and equally induced apoptosis of both H2052 and their $\rho^0$ cells by a mitochondria-independent mechanism. The results suggest that mtDNA is a potential target for the anti-cancer agents that interact with DNA to induce ROS-dependent apoptosis of cancer cells, whereas agents that affect the cytoskeleton induce cell death by mitochondria- and ROS-independent mechanism. The present observation is important for the selection of medicine for chemotherapy of patients with cancer.
Introduction

Although chemotherapy is an important method for treating patients with cancer, its efficacy is limited partly due to different sensitivities of tumor cells to anti-cancer agents and side effects of the agents on normal tissues and cells. The mechanism of action of anti-cancer agents can be grouped depending on their affinity to cellular constituents, such as nuclear DNA (nDNA) (Momparler et al., 1976; Pacheco et al., 1989; Peters et al., 2002; Fuertes et al., 2003; Akiyama et al., 2008), the cytoskeleton (Chen and Horwitz, 2002), hormonal receptors, and specific epitopes on the surface of cancer cells (Mendelsohn and Baselga, 2000; Hudis, 2007).

Some anti-cancer agents that impair structure and function of DNA have been postulated to generate reactive oxygen species (ROS) and induce apoptosis of tumor cells (Gewirtz, 1999; Ravid et al., 1999; Caporossi et al., 2003; Wang et al., 2005). Since mitochondria are potential sites of ROS generation (Lenaz, 1998) and are highly enriched in mtDNA, oxidative injury occurs more rapidly and significantly with mtDNA than with nDNA (Saitou, 1991; Yakes and Van Houten, 1997). Therefore, agents that interact with DNA oxidatively impair mtDNA and/or the electron transport system thereby increasing ROS generation in mitochondria to form a vicious cycle leading to apoptosis. Based on such a concept, we tested the effect of cisplatin on a
variety of cancer cells and found that the amount of mitochondria in IEC-6 cells positively correlated with cellular sensitivity to the agent (Qian et al., 2005). Thus, we hypothesized that the amount of mitochondria may be a key factor determining the efficacy of anti-cancer agents that interact with DNA. To test this hypothesis, we analyzed the effects of several anti-cancer agents which impair either structure and function of DNA or the cytoskeleton on mesothelioma H2052 cells and their $\rho^0$ cells lacking mtDNA. We also compared the toxicity of the two types of anti-cancer agents in a variety of cancer cells with different amounts of mitochondria. The present work demonstrates that the amount of mitochondria and mtDNA in cancer cells are the important factors determining cell sensitivity to agents that preferentially impair structure and function of DNA but not the cytoskeleton.
Materials and Methods

Anti-cancer agents

Cisplatin was purchased from Sigma (St. Louis, MO, USA). Bleomycin, doxorubicin, methotrexate, busulfan, fluorouracil, paclitaxel, colchicine and vinblastine were purchased from Wako Pure Chemical Co. (Osaka, Japan).

Cells

Human mesothelioma cell lines, H2052 and H2452, were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa cells (human cervical cancer cell line), HepG2 cells (human hepatocyte cancer cell line), HL-60 cells (human leukemia cell line), LLC (murine lung cancer cell line) and HEK293 (human kidney embryonic cell line) were obtained from Riken Cell Bank (Tsukuba, Japan). B16 cells (murine melanoma cell line) were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were cultured at 37°C under 5% CO₂ in DMEM or RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (Nacalai Tesque, Kyoto, Japan).

mtDNA-depleted ρ₀ cells were established through long-term treatment of H2052 cells with ethidium bromide (25 ng/ml), as described previously (King and
Attardi, 1989). Depletion of mtDNA in the $p^0$ cells was confirmed by the PCR amplification method using a set of mtDNA-specific primers. Amounts of mitochondria in cells were analyzed by staining with MitoTracker Green FM (Invitrogen, Carlsbad, CA, USA).

**Evaluation of cell death**

Viability of cells was evaluated by the trypan blue exclusion test. After treatment of cells with various anti-cancer agents for 24 hr in the presence or absence of 100 $\mu$M manganese (III) tetrakis (4-benzoic acid) porphyrin (Mn-TBAP; Enzo Biochem Inc, Farmingdale, NY, USA), both floating cells and adherent cells collected by trypsinization were centrifuged at 1,500 rpm for 5 min. The cell pellets were resuspended in 50 $\mu$l of either DMEM or RPMI-1640, and 50 $\mu$L of 0.4% trypan blue was added. Dead cells were counted at four different areas in a hemocytometer.

**Analysis of ROS generation**

To analyze ROS generation, cells were loaded with dihydroethidium (DHE, Wako). After incubation of cells with various anticancer agents, both floating and adherent cells were collected and incubated in medium containing 5 $\mu$M of DHE at
37°C for 30 min in the dark. ROS generation was detected by using a FACSCalibur flow cytometer (Becton Dickinson Japan, Tokyo, Japan).

**Analysis of mitochondrial membrane potential**

Cells treated with various anticancer agents were incubated with 5 μM of 5, 5', 6, 6'-tetrachloro-1', 3', 3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen) at 37°C for 10 min and mitochondrial membrane potential (ΔΨm) was analyzed by flow cytometry. Cationic JC-1 accumulates in mitochondria and changes its fluorescence spectrum from orange to green depending on the decrease in membrane potential.

**Western blotting**

After incubation of cells with various anticancer agents, both floating and adherent cells were collected, lysed in a RIPA buffer containing protease inhibitors (Nacalai Tesque), and subjected to 10 - 15% SDS-PAGE. The electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) using a semidry blot system (2 mA/cm² for 0.5 hr in 0.2 M Tris-glycine buffer, pH 8.8). The activation of caspase-3, 8 and 9 was analyzed by using specific antibodies.
(Cell Signaling Technology, Beverly, MA, USA). After incubation of the sheets in TBS solution (140 mM NaCl, 50 mM Tris-HCl, pH 7.2) containing 0.1% Tween 20 (TBST) and 5% low-fat milk powder at 25°C for 1 hr, they were treated with anti-cleaved caspase-3, 8 and 9 antibodies, and anti-β-actin antibody (Sigma) at 25°C for 1 hr. The incubated membranes were washed five times with TBST to eliminate nonspecific binding of the antibodies. After incubation with horseradish peroxidase-conjugated anti-IgG antibody (1:1,000) (Dako, Glostrup, Denmark) at 25°C for 1 hr, immunoreactive spots were detected using ImmunoStar reagents (Wako) by LAS-3000 (FUJIFILM, Tokyo, Japan).

**Analysis of released cytochrome c**

To estimate the amount of cytochrome c released from mitochondria to cytosol, the harvested cells were incubated in an ice-cold cell lysis buffer (250 mM sucrose, 70 mM KCl, and 200 μg/ml digitonin in PBS) for 5 min as described previously (Waterhouse et al., 2001). The supernatant and pellet fractions containing cytosolic and mitochondrial proteins, respectively, were subjected to SDS-PAGE and analyzed by using anti-cytochrome c antibody (BD Pharmingen, San Diego, CA, USA).
Isolation of mitochondria

Mitochondria were isolated from male C57BL6/j mice liver by the method as described previously (Halestrap et al., 1997). Briefly, the liver was homogenized in an ice-cold 5 mM Tris-HCl buffer solution (pH 7.5) containing 250 mM sucrose and 0.1 mM EDTA. Subcellular fractions were separated using sucrose density gradient centrifugation. The mitochondrial fraction was suspended in a 5 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose. Mitochondria were used immediately after isolation with good conditions; respiratory control index of mitochondria were 4~6).

Analysis of mitochondrial ROS generation and ΔΨm

Isolated mitochondria were loaded with DHE to analyze ROS generation. Mitochondria were incubated with 5 μM of DHE at 37°C for 1 hr in the dark in the presence or absence of 50 μM cisplatin and 100 nM paclitaxel. ROS generation was analyzed by using Spectra Max Gemini (Molecular Devices, Sunnyvale, CA, USA). Isolated mitochondria were incubated with 50 μM cisplatin and 100 nM paclitaxel at 37°C. After incubation times, 50 nM JC-1 was added to the medium and ΔΨm was analyzed after 5 min using Spectra Max Gemini.
**Statistical analysis**

All experiments were repeated at least three times with similar results. Data were expressed as means ± SD and analyzed by Student's t-test (two-tailed) and $P<0.05$ was considered statistically significant.
Result

Effect of anti-cancer agents on mitochondrial generation of ROS and cell death

Since ROS is important in induction of apoptosis (Kane et al., 1993), we analyzed the effects of various anti-cancer agents on ROS generation and cell death using H2052 cells. A dye exclusion test revealed that incubation of cells with either cisplatin or paclitaxel for 24 hr dose-dependently induced cell death (Fig. 1). However, ROS generation was enhanced time-dependently by cisplatin but not by paclitaxel. Analysis using 5 μM MitoSOX also revealed similar results (data not shown). To elucidate the relationship between ROS generation and cell death, we determined the LD$_{50}$ of the two types of agents that affect structure and function of either DNA or the cytoskeleton (Table 1). Using LD$_{50}$ concentrations, we determined the activity of the agents to induce ROS and cell death after incubation for 24 hr (Fig. 2). Agents that interact with DNA, such as cisplatin, bleomycin, doxorubicin, methotrexate, busulfan and fluorouracil, strongly enhanced ROS generation. In contrast, agents that affect the cytoskeleton, such as paclitaxel, colchicine and vinblastine, failed to induce ROS.

To determine whether ROS is critical in inducing cell death, effect of Mn-TBAP, a membrane permeable superoxide scavenger (Faulkner et al., 1994), on cell death induced by the agents was investigated after incubation for 24 hr. Mn-TBAP
effectively suppressed the cell death induced by agents that interact with DNA. In contrast, Mn-TBAP failed to inhibit cell death induced by agents that affect the cytoskeleton. Thus, ROS generation seems to be responsible for the induction of cell death by agents that interact with DNA but not the cytoskeleton.

Effect of anti-cancer agents on mitochondrial membrane potential and cytochrome c localization

Overproduction of ROS often induces membrane permeability transition (MPT), an early event leading to apoptosis (Green and Kroemer, 2004). Thus, we analyzed mitochondrial MPT using a fluorescence probe JC-1 during the incubation of cells with either 50 μM cisplatin or 100 nM paclitaxel for 24 hr. Incubation with cisplatin but not paclitaxel increased the number of H2052 cells showing low ΔΨm (Fig. 3A). Kinetic analysis revealed that H2052 cells having low ΔΨm increased time-dependently during the incubation with cisplatin but not paclitaxel (Fig. 3B). Using LD₅₀ concentrations of anti-cancer agents, we also determined their activity to increase cells having low ΔΨm. Agents interacting with DNA increased the number of cells showing low ΔΨm, while agents affecting the cytoskeleton failed to increase depolarized cells (Fig. 3C). Under identical conditions, cytochrome c was released from
mitochondria to cytosol by agents interacting with DNA but not by agents affecting the cytoskeleton (Fig. 3D).

**Effect of anti-cancer agents on H2052 cells and their ρ^0 cells**

To elucidate possible involvement of mitochondria in apoptosis induced by anti-cancer agents, we analyzed the effect of the agents on the viability of H2052 cells and their ρ^0 cells using the trypan blue exclusion (Fig. 4). The cytocidal activity of agents interacting with DNA was significantly higher in H2052 cells than in ρ^0 cells. In contrast, agents affecting the cytoskeleton induced apoptosis equally in H2052 and ρ^0 cells. Analysis using annexin V also showed similar results (data not shown).

To determine signaling pathways leading to apoptosis, we analyzed the effect of agents on the activation of caspases. Western blotting analysis revealed that the agents that interact with DNA selectively activated caspase-9 but not caspase-8 in H2052 cells. Under identical conditions, the agents failed to activate caspase-9 in ρ^0 cells. Agents affecting the cytoskeleton selectively activated caspase-8 but not caspase-9 in H2052 and their ρ^0 cells. Although caspase-3 was activated by agents that interact with DNA in selectively H2052 cells, the enzyme was activated equally by agents that affect the cytoskeleton in H2052 and ρ^0 cells.
Effect of mtDNA depletion on ROS generation induced by anti-cancer agents

We compared the effect of the two types of anti-cancer agents on cellular generation of ROS using H2052 and their $\rho^0$ cells (Fig. 5). In the absence of anti-cancer agents, both types of cells generated small amounts of ROS though their generation was slightly higher with H2052 cells than with $\rho^0$ cells. Agents that interact with DNA increased ROS generation in H2052 cells but not in their $\rho^0$ cells. In contrast, agents that affect the cytoskeleton did not increase ROS generation in both types of cells (P=0.5389~0.9525).

Correlation between amounts of cellular mitochondria and drug sensitivity

To test the hypothesis that mitochondria are potential targets for agents that interact with DNA, we analyzed the relationship between the amounts of mitochondria and sensitivity of cells to the agents (Fig. 6). The result revealed that amounts of cellular mitochondria positively correlated with the sensitivity of cells to agents that interact with DNA; the higher the amount of mitochondria, the higher the sensitivity of cells to the agents. Cellular sensitivity to agents that affect the cytoskeleton did not correlate with the amounts of cellular mitochondria.
Discussion

The present work demonstrates that mitochondria play a crucial role in apoptosis induced by agents that interact with DNA but not with the cytoskeleton. The sensitivity of cells to the former type agents correlated positively with cellular contents of mitochondria (P=0.0060~0.0228).

Our hypothesis that mitochondria are potential targets for the agents interacting with DNA is consistent with the present findings that the agents specifically increased ROS, released cytochrome c into cytosol, and induced apoptosis by a mechanism inhibited by Mn-TBAP. The presence of membrane permeable N-acetylcysteine (10 mM) also inhibited apoptosis induced by agents interacting with DNA but not the cytoskeleton (data not shown). Furthermore, agents that interact with DNA failed to induce ROS generation and apoptosis of ρ₀ cells. Recent studies reported that superoxide generation by NADPH oxidase underlies the mechanism of in apoptosis (Gilleron et al., 2009; Kim et al., 2010). However, pretreatment of H2052 cells with 10 μM apocynin, an inhibitor of the oxidase, for 2 hr failed to suppress apoptosis induced by all agents tested (data not shown). Furthermore, the presence of membrane impermeable SOD1 (100 unit/ml) and catalase (100 unit/ml) showed no appreciable effect on apoptosis of H2052 cells induced by the agents interacting with DNA (data not
shown). Thus, intracellular ROS generated by mitochondria might be responsible for the induction of apoptosis by agents interacting with DNA.

The present work suggests that mitochondria are the primary target for the agents interacting with DNA. mtDNA is responsible for the synthesis of 13 subunits of mitochondrial electron transport chain (Anderson et al., 1981). Since turnover of mitochondrial proteins occurs independently (Ramachandran et al., 2002), imbalance and/or impairment of the electron transport system caused by mtDNA injury may facilitate leakage of electrons to generate the superoxide radical (Han et al., 2001). Although rotenone (50 μM) and antimycin (50 μM) instantaneously increase mitochondrial ROS (data not shown), its generation induced by the agents interacting with DNA increased fairly slowly (see Fig. 1B). We also tested effects of anti-cancer agents on isolated mitochondria. Antimycin rapidly but transiently increased ROS generation by mitochondria particularly during early periods of incubation (~3 hr) (see Fig. 7). In contrast, under identical conditions, both cisplatin and paclitaxel failed to induce ROS generation. Although mitochondrial membrane potential decreased rapidly after adding CCCP, it decreased slowly over a period of 6 hr irrespective of the presence of cisplatin and paclitaxel. It should be noted that cisplatin but not paclitaxel increased ROS generation (see Fig. 1B) and the number of depolarized cells (see Fig. 3B) after
incubation for longer than 6 hr. Thus, fairly long term incubation is required for the induction of ROS and decrease in membrane potential. This observation suggests that the lag for the production of mitochondrial ROS and decrease in membrane potential reflects the time required for impairment of mitochondrial electron transport proteins supplied by both mtDNA and nDNA. This possibility requires further study.

The agents that interact with DNA selectively activated caspase-9 in H2052 cells but not in \( \rho^0 \) cells, whereas those affecting the cytoskeleton preferentially activated caspase-8 in both types of cells. ROS generated in mitochondria has been shown to oxidize membranous cardiolipin to release cytochrome c (Petrosillo et al., 2003; Rostovtseva and Bezrukov, 2008). Pro-caspase-9 is activated by cytochrome c containing apoptosome (Li et al., 1997). Thus, agents that interact with DNA seem to activate this pathway resulting in apoptosis. Caspase-8 interacts with microtubules and induces apoptosis by a mitochondria-independent pathway (Wieder et al., 2001; Mielgo et al., 2009). These observations are consistent with our finding that the agents equally induced apoptosis of both H2052 and their \( \rho^0 \) cells by a ROS-independent mechanism. Recent studies demonstrated that paclitaxel activated caspase-8 to generate a cleaved form of Bid (Bhalla, 2003; von Haefen et al., 2003) that induced Bax-dependent apoptosis of HeLa cells and breast cancer cells (Luo et al., 1998; Yin et al., 1999; Kutuk...
and Letai, 2008). However, paclitaxel and other agents affecting the cytoskeleton failed to induce mitochondria-dependent apoptosis of H2052 cells under the present experimental conditions. The reason for the discrepancy of the involvement of mitochondria in the action mechanism of paclitaxel remains unknown. Possible involvement of mitochondria in the action mechanism of anticancer agents interacting with the cytoskeleton should be studied further.

Multi-drug resistance of cancer cells is a critical factor determining efficacy of chemotherapy of patients with cancers (Tomek et al., 2003). In this context, ATP-binding cassette transporters, including multi-drug resistance associated-proteins, participate in the mechanism for resistance to a wide variety of anti-cancer agents (Baldini, 1997). However, expression of ATP-binding cassette transporters is not sufficient to explain all mechanisms responsible for multi-drug resistance (Ogretmen et al., 1998; Soini et al., 2001). Preliminary experiments using specific antibody to multi-drug resistance protein 1 (MDR1) revealed that \( \rho^0 \) cells used in the present experiments did not express this protein (data not shown). We previously reported that cancer cells enriched in mitochondria showed higher sensitivity to cisplatin than did other cells having smaller amounts of mitochondria (Qian et al., 2005). The present work also demonstrates that malignant mesothelioma H2052 cells exhibited higher
sensitivity to all agents interacting with DNA than did their $\rho^0$ cells. This observation indicates that reduction in the amount of mitochondria in cancer cells underlies, at least in part, the mechanism for multi-drug resistance.

The present work demonstrates that anti-cancer agents used in the experiments can be classified into those affecting mtDNA which induce caspase-9 activation and mitochondria-dependent apoptosis, and those affecting the cytoskeleton which induce caspase-8 activation and mitochondria-independent cell death. Recent studies suggest that mitochondrial factors, including Bcl-2, SOD and other antioxidant enzymes may also be potential targets for anti-cancer agents (Fulda et al., 2010). Thus, analysis of mitochondrial status in cells is important for the selection of drugs for chemotherapy of patients with cancer. The present work also suggests that anti-cancer agents interacting with mtDNA may produce side-effects in normal tissues which are enriched with mitochondria. This hypothesis is consistent with the fact that mitochondria-enriched renal proximal tubules and intestinal mucosal cells are critical sites for the occurrence of side effect of cisplatin (Chang et al., 2002). Thus, such possibility should also be taken into account for the chemotherapy of patients with cancer.
Acknowledgement:

The authors thank Prof. Irwin M. Arias for his valuable suggestions during the preparation of this manuscript.
Authorship contributions:

Participated in research design: Hara, Kasahara, Takahashi, Konishi, J. Inoue, Jikumaru, Okamuro, Sato, M. Inoue.

Conducted experiments: Hara, Kasahara, Takahashi, Konishi, J. Inoue.

Performed data analysis: Hara, Kasahara, M. Inoue.

Wrote or contributed to the writing of the manuscript: Hara, Kasahara, Takahashi, Kubo, Sato, Inoue.
References


Halestrap AP, Woodfield KY and Connern CP (1997) Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by


Kutuk O and Letai A (2008) Alteration of the mitochondrial apoptotic pathway is key to acquired paclitaxel resistance and can be reversed by ABT-737. *Cancer Res*


Co-ordinated over-expression of the MRP and gamma-glutamylcysteine synthetase genes, but not MDR1, correlates with doxorubicin resistance in human malignant mesothelioma cell lines. *Int J Cancer* **75**:757-761.


von Haefen C, Wieder T, Essmann F, Schulze-Osthoff K, Dorken B and Daniel PT


(1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis.

Footnotes

This work was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (22590288 and 21700698), the 21st Century COE Program “Base to Overcome Fatigue” supported by MEXT, Japan, by the special funds from the Sakamoto Research Institute of Psychopathology and Nishiki Corporation, Osaka, Japan.

The authors declare no conflict of interest.
Figure Legends

**Fig. 1 Anti-cancer agents induced ROS generation and cell death.**

(A) H2052 cells were incubated with increasing concentrations of cisplatin and paclitaxel for 24 hr and analyzed for viability by trypan blue exclusion as described in the text. (B) After incubation of H2052 cells with 50 μM cisplatin or 100 nM paclitaxel, ROS generation was analyzed using DHE and flow cytometry. Data are expressed as means ± SD derived from three independent experiments. *, p < 0.05 versus paclitaxel.

**Fig. 2 Effect of various anticancer agents on ROS and cell death**

(A) H2052 cells were cultured for 24 hr in 50 μM cisplatin, 25 μM bleomycin, 1 μM doxorubicin, 100 μM methotrexate, 2000 μM busulfan, 4000 μM fluorouracil, 100 nM paclitaxel, 50 nM colchicine or 50 nM vinblastine. ROS generation was analyzed using DHE and flow cytometry as described in Fig. 1. (B) Cells were preincubated with 100 μM Mn-TBAP for 2 hr, subsequently cultured in the anti-cancer agents for 24 hr, and viability was determined. Data shows means ± SD derived from at least three independent experiments. *, p < 0.05 versus control.

**Fig. 3 Effect of the agents on membrane potential and cytochrome c localization**
(A) H2052 cells were incubated with 50 μM cisplatin or 100 nM paclitaxel for 24 hr and mitochondrial $\Delta \Psi_m$ was determined using JC-1 and flow cytometry. (B) During the incubation with the agents, $\Delta \Psi_m$ was determined. (C) Cells were incubated with various agents for 24 hr and $\Delta \Psi_m$. (D) The released cytochrome c into cytosol was analyzed by Western blotting. Data shows mean ± SD derived from at least three independent experiments. Other conditions were as in Fig. 2. *, p < 0.05 versus control.

**Fig. 4 Effect of agents on apoptosis and caspase activation**

(A) H2052 and $\rho^0$ cells were incubated with anti-cancer agents for 24 hr and viability was analyzed by dye exclusion. Under identical conditions, caspase activation was analyzed by SDS-PAGE and Western blotting using antibodies against caspase-3, 8 and 9 (B). Other conditions were as described in the text. Other conditions were as in Fig. 2. *, p < 0.05 versus control.

**Fig. 5 Effect of agents on cellular generation of ROS in H2052 and $\rho^0$ cells**

After incubation of H2052 and $\rho^0$ cells with the agents for 24 hr, ROS generation was analyzed using DHE and flow cytometry. The percentage of DHE-positive cells (high staining group) are expressed as means ± SD derived from at least three independent
experiments. Other conditions were as in Fig. 2. *, p < 0.05 versus control.

**Fig. 6** Correlation between the amounts of mitochondria and drug sensitivity of various cancer cells

LD$_{50}$ values to induce cell death were obtained for all agents as described in Table 1. The amounts of mitochondria in the cells were determined using MitoTracker Green FM and flow cytometry. Correlation coefficients between the two parameters were determined using linear regression and Pearson’s correlation coefficient analysis methods.

**Fig. 7** Effect of cisplatin and paclitaxel for ROS generation and \(\Delta\Psi_m\) on isolated mitochondria

After incubation of isolated mitochondria with 50 μM cisplatin or 100 nM paclitaxel ROS generation was detected DHE by using a spectrofluorophotometer (A). Under identical condition, \(\Delta\Psi_m\) was analyzed JC-1 by using a spectrofluorophotometer (B).
Table 1 Sensitivity of H2052 cells to anticancer agents

<table>
<thead>
<tr>
<th>Target</th>
<th>agents</th>
<th>LD$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>cisplatin</td>
<td>42.7</td>
</tr>
<tr>
<td></td>
<td>bleomycin</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td>doxorubicin</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>methotrexate</td>
<td>105.1</td>
</tr>
<tr>
<td></td>
<td>busulfan</td>
<td>1704.4</td>
</tr>
<tr>
<td></td>
<td>fluorouracil</td>
<td>5681.4</td>
</tr>
<tr>
<td>cytoskeleton</td>
<td>paclitaxel</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>colchicine</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>vinblastine</td>
<td>0.04</td>
</tr>
</tbody>
</table>

After incubation of H2052 cells with the agents for 24hr, LD$_{50}$ was determined by trypan blue exclusion test.
Fig. 1
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7