

Disulfiram Induces Apoptosis in Human Melanoma Cells: A Redox-related Process¹

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Abstract

Melanoma is highly resistant to conventional chemotherapy. We have demonstrated that redox regulation in melanoma cells is aberrant, and redox-modulating agents can induce cell apoptosis. We have currently explored the effect of disulfiram (DSF), a member of the dithiocarbamate family, on apoptosis of melanoma cells *in vitro*. Human metastatic melanoma cells c81-46A, c81-61, and c83-2C were treated with DSF and apoptosis measured. DSF, at a dose of 25–50 ng/ml, consistently caused a 4–6-fold increase in apoptosis. The same dose of DSF did not significantly affect apoptosis in melanocytes. Coincubation of *N*-acetyl-cysteine reversed the DSF-induced apoptosis. Buthionine sulfoximine (BSO), an inhibitor of γ -glutamyl-cysteine synthetase, as a single agent caused a ~2-fold increase in apoptosis when incubated with melanoma cells for 4 days. BSO slightly enhanced the level of apoptosis induced by DSF (4–10% higher than DSF alone). Intracellular glutathione was remarkably depleted with BSO treatment. DSF did not cause glutathione depletion; however, the ratio of reduced and oxidized glutathione was significantly decreased (14% of control), and *N*-acetyl-cysteine partially restored the ratio to 30% of control. There was a transient (2-fold) elevation of intracellular superoxide level after 24 h of DSF treatment (before the overt apoptosis). The intracellular H₂O₂ level progressively decreased with time. DSF decreased the mitochondrial membrane polarization in a time-dependent manner, and there was a significant inverse correlation between apoptosis and mitochondrial membrane polarization. We propose that DSF-induced apoptosis is redox related but involves a different mechanism from BSO-induced apoptosis in tumor cells. Our findings have provided new data for additional understanding of drug-induced apoptosis in melanoma cells and

suggests an alternative therapeutic approach to melanoma.

Introduction

Redox regulation has played an important role in cellular function, of which growth and proliferation, apoptosis, and response to oxidative stress are among the most extensively investigated (1–5). We have demonstrated previously that melanoma cells have a decreased ability to handle oxidative stress compared with normal melanocytes, resulting in a continuous build-up of ROS³ (6). The intracellular superoxide level was higher in melanoma cells than in melanocytes (7). The abnormal regulation of redox in melanoma cells should serve as a target for the therapeutic and/or pathogenic studies.

Glutathione homeostasis is of particular interest, because GSH protects the cell from the toxic effects of ROS formed during melanin synthesis. GSH depletion by BSO, an inhibitor of γ -glutamyl-cysteine synthetase, enhanced the cytotoxic effect of chemotherapeutic agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea, cisplatin, and melphalan (8–12) inhibited DNA synthesis or growth of melanoma cell lines *in vitro* (9–11, 13, 14) and prolonged survival of melanoma-bearing mice after *in vivo* administration (11, 13, 14). BSO alone caused significant apoptosis in tumor cells, especially neuroblastoma (15–17). The intracellular glutathione content is inversely proportional to the sensitivity of different tumor cell lines to arsenic trioxide, an apoptosis-inducing agent (18). Oxidizing a critical thiol residue in the mitochondrial membrane caused permeability transition pore opening and apoptosis (19). These findings suggest that reduced thiol groups are important for maintaining cellular function and/or mitochondrial integrity.

Dithiocarbamate induces apoptosis of thymocytes by oxidizing glutathione and creating an intracellular oxidative stress (20). Our previous experiments also showed that dithiocarbamates can induce melanoma apoptosis (7). DSF, an agent used for the treatment of alcoholism, is a member of the dithiocarbamate family. In this paper, we report the effect of DSF on apoptosis in melanoma cells and a study of the redox-related mechanism, including changes in mitochondrial membrane polarization. A comparative analysis of apoptosis and cellular glutathione content between DSF and BSO treatment was also conducted.

Received 8/7/01; revised 10/10/01; accepted 10/26/01.

¹ Supported in part by Grant P30CA62203, The Waltmar Foundation, and the Chao Family Comprehensive Cancer Center.

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³ The abbreviations used are: ROS, reactive oxygen species; DSF, disulfiram; NAC, *N*-acetyl-cysteine; BSO, buthionine sulfoximine; GSH, reduced glutathione; GSSG, glutathione disulfide; DDC, diethyldithiocarbamic acid; AV, Annexin V; PI, propidium iodide; DCF 2',7'-dichlorofluorescein; HE, dihydroethidium.

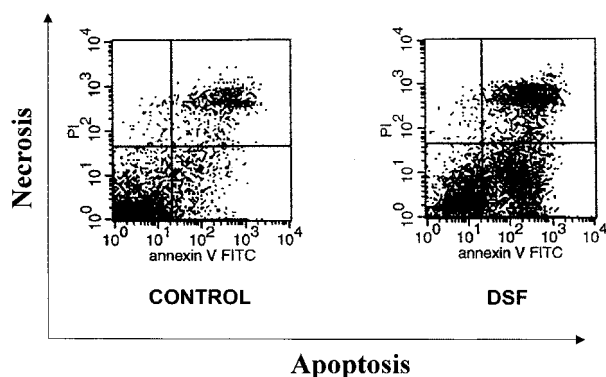


Fig. 1. Analysis of apoptosis and necrosis of melanoma cells treated with DSF. Cells (c81-46A, 3×10^5) were incubated with DSF (50 ng/ml) for 3 days and analyzed by flow cytometry with AV and PI as described in "Materials and Methods." Frequency of apoptosis is defined as AV+/PI-, necrosis as AV+/PI+, and viability as AV-/PI-.

Materials and Methods

Cell Culture. Metastatic melanoma cells (c81-46A, c81-61, and c83-2C) were cultured in F-10 medium (Fisher Scientific, Pittsburgh, PA) with 5% FCS, 5% newborn calf serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). These cell strains were derived from metastatic melanoma patients, and the number of passages used in our experiments was < 10 (21). Cells were split 1–2 days before drug treatment, with a density of $4\text{--}6 \times 10^4$ /ml (confluence level of ~75%). Melanocytes were processed from pooled neonatal foreskins and cultured in MCDB 153 (Sigma Chemical Co., St. Louis, MO) medium containing 2% FCS, 0.3% bovine pituitary extract (Clonetics, San Diego, CA), 10 ng/ml phorbol myristate-13-acetate, 2 mM calcium chloride, 5 μ g/ml insulin, and 0.1 mM 3-isobutyl-methylxanthine (Sigma Chemical Co.). Two commercial tumor cell lines, A375 (melanoma) and DU145 (prostate cancer) were cultured in DMEM with 10% FCS.

Drug Administration. DSF, NAC, BSO, and DDC were all from Sigma Chemical Co. Drugs were dissolved in the culture medium except that DSF was dissolved in DMSO (Sigma Chemical Co.) and added to the culture directly. Control samples were added with the same amount of medium/or DMSO (volume of DMSO was <0.2%).

Apoptosis Assay. Apoptosis assay was performed according to the manufacturer's protocol (PharMingen). Briefly, cells were trypsinized, washed twice in PBS, and resuspended in binding buffer [10 mM HEPES NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl_2] at a concentration of 1×10^6 cells/ml, of which 100 μ l was incubated with 5 μ l of AV conjugated to FITC (Molecular Probes, Eugene, OR) and 10 mM PI for 15 min at room temperature. Cells were then analyzed by flow cytometry using a Becton Dickinson FACScan. The proportion of apoptotic cells was estimated by the percentage of cells that stained positive for AV while remaining impermeable to PI (AV+/PI-); necrosis was defined as positive stain with both AV and PI (AV+/PI+); and viability was defined as AV-/PI- (Fig. 1). trypan blue stain was also used in parallel, and the number of trypan blue-positive cells was similar to the sum of AV+/PI- and AV+/PI+ cells. This

method was based on the literature published previously that in the early stages of apoptosis, phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane at the cell surface. AV has a high affinity for phosphatidylserine binding on the cell surface. In the late stage of cell death, membrane integrity is lost and taken up by PI (22).

Intracellular ROS Measurement. Hydrogen peroxide level was determined using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR). This fluorescence probe diffuses through cell membranes and is hydrolyzed by intracellular esterase to DCF, which remains trapped within cells, reacts with hydrogen peroxide, and generates the fluorescent DCF. Superoxide anion was measured using HE probe (Molecular Probes). HE is the sodium borohydride-reduced form of ethidium bromide that is permeable to viable cells, and it can be directly oxidized to ethidium bromide by superoxide anion. Cells were incubated with the probes (5 μ M of DCF and 10 μ M of HE) for 15 min, harvested, and analyzed by flow cytometry using a Becton Dickinson FACScan with Cell Quest software with an excitation of 488 nm (both DCF and HE) and filters of 530 nm/585 nm (DCF/HE). The fluorescence intensity is proportional to the intracellular ROS levels. The above methods were adapted from those described previously (23, 24).

Analysis of Mitochondrial Membrane Polarization. JC-1 (Molecular Probes) is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 nm) to red (590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in red fluorescence and an increase in green fluorescence. Cells were incubated with JC-1 (3 μ M) for 45 min and harvested as described above. The fluorescence is quantitated by flow cytometry and is proportional to the level of mitochondrial polarization in the cell. Data are expressed as a percentage of the control.

Quantitation of Intracellular Glutathione and GSSG. Cells grown in monolayer were trypsinized and washed twice with PBS. Viability and total viable cells were counted by trypan blue exclusion, and apoptosis assay (at 72 h of incubation) was performed as described above. Cell lysates were prepared by adding 200 μ l of 10 mM HCl to the pellet, followed by freeze and thaw three times in liquid nitrogen. Lysate (40 μ l) was used for protein assay, and the rest (160 μ l) was protein-precipitated by adding 40 μ l of 5-sulfosalicylate acid (6.5%, Sigma Chemical Co.) for 10 min on ice and centrifuged at $2000 \times g$, 4°C, for 15 min. Supernatants were stored at -80°C until tested. The DTNB-enzyme recycling assay protocol for total glutathione and GSSG) was performed according to methods published previously (25). Protein quantitation was performed in parallel according to the Bio-Rad D_c Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

Results

DSF-induced Apoptosis of Human Metastatic Melanoma Cells. Shown in Fig. 1 is the result of a typical experiment. Cells were treated with DSF and analyzed for apoptosis as described in "Methods and Materials." There was a good

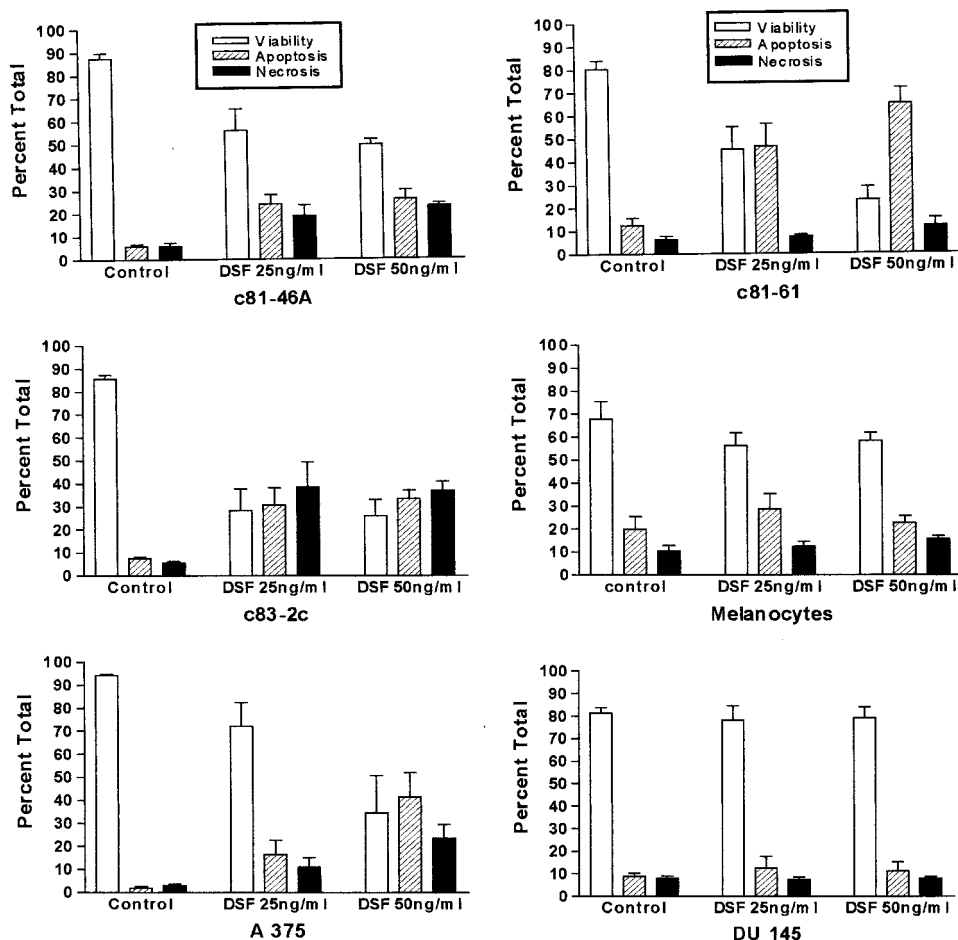


Fig. 2. DSF-induced apoptosis in human metastatic melanoma cells. Three human metastatic melanoma cell strains, ($2-3 \times 10^6$), melanocytes, and two tumor cell lines (A 375 and DU 145) were incubated with DSF (25–50 ng/ml) for 3 days and analyzed by flow cytometry with AV and PI as described in Fig. 1. Results are represented from 3–5 experiments; bars, \pm SD.

level of consistency in the control samples (Fig. 2). The viability was between 80–90%, and in most experiments, the sum of the control apoptotic and necrotic populations was <20%.

DSF increased the frequency of apoptosis in all three of the metastatic melanoma cells at a dose level of 25–50 ng/ml (Fig. 2). The maximum frequency of cell death (the sum of apoptosis and necrosis) ranged from 400% to 600% of the control level, and the viability from 28% to 55% of the control. A higher fraction of necrosis was observed in c83-2c, a more aggressive cell strain with the highest proliferation rate. Using trypan blue exclusion, total viable cells per culture in the DSF-treated c81-46a cells were 15.0% of the control (Table 2). DSF treatment of melanocytes caused maximum cell death and viability that were 137% and 83% of control, respectively (Fig. 2). The lowest DSF dose that caused maximum apoptosis in melanoma cell strains (in this culture system) was 50 ng/ml ($0.17 \mu\text{M}$).

To additionally test the selectivity of DSF-induced apoptosis, we have used two tumor cell lines. The melanoma cell line (A 375) was also sensitive to DSF treatment, with a maximum cell death of 1300% and viability of 36.7% of the control, respectively. A prostate cancer cell line (DU 145) was resistant to DSF treatment *in vitro* (cell death and viability

being 119% and 96% of control, respectively). This result indicates that DSF is not a universal killing agent *in vitro*. We do not know if DSF-induced apoptosis is melanoma-specific, because the culture conditions between melanoma cells and melanocytes (and other tumor cells) were different.

Melanoma cell strains and melanoma cell line (A375) were both sensitive to DSF, yet they were cultured in different medium. A375 and DU 145 were cultured in the same medium (DMEM), but only A375 was significantly sensitive to DSF treatment. However, cell culture using the same medium did not mean that the cysteine content was the same between two different cell lines. Even if the cysteine content in the culture medium was the same, different cell lines may require different amount of cysteine for optimal growth. It is our plan to measure the cysteine content before and after drug treatment on the same cell line.

One major metabolite of DSF, DDC, was also active in inducing apoptosis in melanoma cells (420% of control). The lowest dose that caused maximum apoptosis was ~ 200 ng/ml ($0.888 \mu\text{M}$; Fig. 3).

Redox Status in DSF-induced Apoptosis. In an attempt to study the role of thiol-redox in DSF-induced apoptosis, we have performed sequential experiments to address the central question: is glutathione depletion or oxidation an essen-

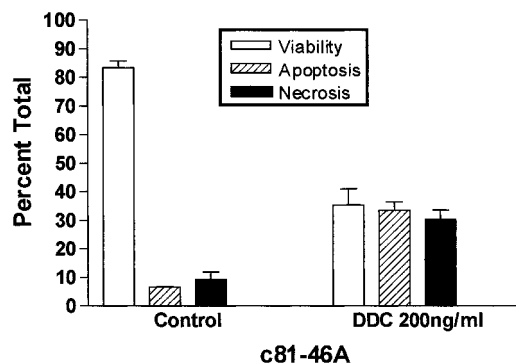


Fig. 3. DDC, a major metabolite *in vivo*, caused significant apoptosis in melanoma cells. Cells (3×10^5) were incubated with DDC (200 ng/ml) for 3 days and analyzed by flow cytometry with AV and PI as described in Fig. 1. Results are represented from four experiments; bars, \pm SD.

tial factor for inducing apoptosis? The addition of NAC, a glutathione precursor, significantly reversed the DSF-induced cell death (from 400% to 151% of control) and increased the viability (from 51.7% to 91.6% of control; Fig. 4).

The effect of DSF on apoptosis was compared with BSO, a GSH-depleting agent. BSO alone induced a level of apoptosis \sim 200% of the control, which required a 4-day incubation period (Table 1). BSO slightly enhanced the DSF-induced apoptosis (The level of apoptosis in the combination of DSF and BSO was about 4–10% higher than that treated with DSF alone; Table 1). On the basis of the finding that DSF caused a higher frequency of cell death than BSO-treated melanoma cells, we additionally analyzed the intracellular total glutathione and GSSG level, and compared the level with the viability/apoptosis. As shown in Table 2, total glutathione was depleted 24 h after BSO treatment, and only slight changes of cell viability and total viable cells were observed (95.5% and 97.7% of control, respectively). Treatment of melanoma cells with BSO for 72 h caused additional depletion of cellular glutathione, and viability was relatively maintained (93.8% of control); however, total viable cell number decreased to 57.4% of control. DSF did not deplete cellular glutathione after either 24 or 72 h of treatment, and the coinubation of NAC did not increase the glutathione level despite the fact that DSF caused significant cell death at 72-h incubation (viability, total viable cells, and apoptosis + necrosis were 53.4%, 15.0%, and 365.2% of control, respectively), and NAC reversed the DSF-induced effect. We additionally measured the cellular content of GSSG at 24 h of drug treatment. There was a significant increase in the GSSG level in the DSF-treated melanoma cells (727% of control), and the ratio of GSH:GSSG was decreased to 14% of control. NAC partially restored the ratio to 30% of control (Table 2). The GSSG level in BSO-treated cells was too low to be detected. It should be noted that the glutathione levels after 24 h of DSF incubation are the more reliable measurement of GSH:GSSG contents in viable cells because of higher frequency of cell death at 72-h incubation.

DSF-induced Apoptosis Was Associated with Decrease in Mitochondrial Membrane Potential. (Fig. 5). Melanoma cells (c83-2c) were incubated with DSF (50 ng/ml)

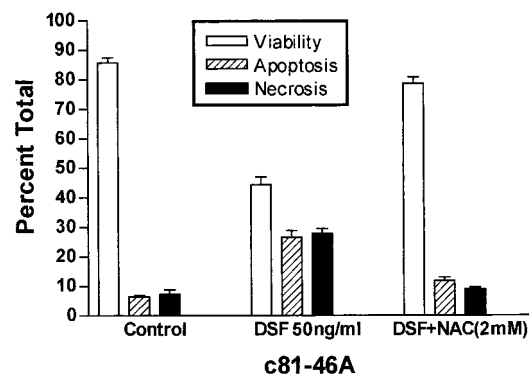


Fig. 4. Coinubation of NAC with DSF reversed the effect of apoptosis and restored the viability of melanoma cells. Cells (c81-46a, 3×10^5) were incubated with DSF alone (50 ng/ml) or the combination of DSF (50ng/ml) + NAC (2 mM) for 3 days and analyzed by flow cytometry with AV and PI as described in Fig. 1. Results are represented from six experiments; bars, \pm SD.

for a time course of 4–72 h, and apoptosis/necrosis and mitochondrial membrane potential were measured accordingly. There was a progressive decrease of membrane polarization with increasing apoptosis level during the course of DSF treatment with a significant inverse correlation ($r = -0.9838$; $P = 0.0025$). The inverse correlation between total cell death (apoptosis + necrosis) and mitochondrial membrane polarization was also significant ($r = -0.8930$; $P = 0.0413$).

DSF Caused a Transient Elevation of Cellular Superoxide Anion Level. Melanoma cells (c83-2c) were incubated with DSF (50 ng/ml) for a time course of 4–72 h, and intracellular ROS levels were measured. DSF caused a transient (2-fold) increase in the intracellular superoxide level at 24 h of incubation before overt apoptosis was observed. The intracellular H_2O_2 level decreased progressively over time to \sim 10% of the control values (Fig. 6).

Discussion

The abnormal redox regulation in melanoma cells may provide a candidate target for therapeutic purpose. Dithiocarbamate, as a redox-regulating agent, can induce apoptosis in melanoma cells (7). In this study, we demonstrated that DSF caused a consistent increase in the level of apoptosis (about 400–600% of control) in all three of the melanoma cell strains being tested at a very low dose (50 ng/ml; $0.17 \mu M$). This (*in vitro*) dose of DSF was \sim 12.5% of the serum concentration, when taking DSF p.o. at therapeutic dose (500 mg/day). The same dose of DSF did not have a significant apoptotic effect on melanocytes and the prostate cancer cell line DU 145. We are currently testing more tumor cell lines with DSF and studying the selectivity of DSF-induced apoptosis. Melanoma is largely resistant to conventional chemotherapy (26–31). New agents (or combinations) need to be developed for this refractory malignancy. The advantages of using DSF for this study, apart from being a redox-regulating agent, are several-fold. DSF is an oral agent that is convenient to administer; the pharmacokinetic status and toxicity profiles are well known; and it can cross the blood brain

Table 1 The effect of BSO and DSF on apoptosis

Melanoma cells (c81-46A) were incubated with BSO (100 μM), DSF (50 ng/ml), or DSF + BSO for 3 or 4 days, and analyzed by flow cytometry using AV and PI.

% Total	Day 3 (n = 3)				Day 4 (n = 3)			
	Cont	BSO	DSF	DSF + BSO	Cont	BSO	DSF	DSF + BSO
Viability	87.157 \pm 0.254	85.580 \pm 1.140	53.273 ^a \pm 1.156	43.747 ^a \pm 1.810	86.313 \pm 1.318	72.128 ^a \pm 2.773	33.525 ^a \pm 5.219	21.997 ^a \pm 3.026
P		0.268	0.0014	0.0021		0.034	0.0037	<0.0001
Apoptosis	6.250 \pm 0.28	7.097 \pm 0.35	20.647 ^a \pm 0.93	25.927 ^a \pm 2.463	7.380 \pm 0.63	15.163 ^a \pm 1.81	41.375 ^a \pm 3.51	51.317 ^a \pm 0.23
P		0.161	0.0024	0.0123		0.035	0.0033	<0.0001
Necrosis	5.807 \pm 0.44	6.097 \pm 0.76	24.703 ^a \pm 0.28	28.853 ^a \pm 0.378	5.887 \pm 0.69	11.823 ^a \pm 1.24	24.435 ^a \pm 2.17	26.047 ^a \pm 3.15
P		0.683	0.0012	0.0001		0.047	0.0074	0.0008

^a Statistically significant when compared with control, analyzed by *t* test.

Table 2 Intracellular glutathione content of melanoma cells

c81-46a cells were treated with DSF (50 ng/ml), DSF + NAC (50 ng/ml + 2 mM), or BSO (100 μM).

	24 h				72 h			
	Cont	DSF	DSF + NAC	BSO	Cont	DSF	DSF + NAC	BSO
n	5	3	3	5	5	3	3	4
Total Glutathione (nm/mg protein)	59.4 \pm 2.4	77.0 \pm 14.0	64.9 \pm 10.0	3.0 \pm 1.3	51.2 \pm 6.1	73.7 ^a	47.3 \pm 16.4	0.7 \pm 0.2
GSSG (nm/mg protein)	1.3 \pm 0.5	9.7 \pm 2.0	4.4 \pm 1.1					
GSH:GSSG ^b	42.5	5.9	12.8					
% Viability (Trypan blue)	91.700 \pm 1.445	91.5 \pm 1.99	94.0 \pm 3.35	87.6 \pm 1.61	88.840 \pm 2.322	47.4 \pm 6.15	88.9 \pm 2.28	83.3 \pm 1.53
Total viable cells (10 ⁵)	2.202 \pm 0.186	1.677 \pm 0.162	1.967 \pm 0.291	2.152 \pm 0.236	4.144 \pm 0.597	0.620 \pm 0.140	3.173 \pm 0.679	2.380 \pm 0.305
% Dead cells (AV+/PI- plus AV+/PI+)					16.104 \pm 1.664	58.8 \pm 2.08	17.3 \pm 2.06	21.7 \pm 0.87

^a Average of two experiments because of significant cell death and not enough protein for accurate quantitation of GSH in one sample. The exact values of cellular GSH of those two experiments were 68.39 and 78.96, respectively.

^b GSH content is calculated as total glutathione - (2 \times GSSG).

barrier with potential efficacy for central nervous system metastasis.

The effects of DSF on cellular redox changes have not been well studied. Dithiocarbamate induces apoptosis in rat thymocytes and astrocytes (20, 32) by chelating copper and creating an intracellular oxidative stress by oxidizing and/or depleting cellular glutathione. DSF binds to proteins via formation of mixed disulfides with free thiol groups of various proteins (33), and it may affect mitochondrial membrane proteins, which are redox sensitive (34). Disturbance in the glutathione redox may affect cellular ROS scavenging. We have performed sequential experiments to address the above questions. We coinubated NAC with DSF-treated melanoma cells. NAC significantly reversed the apoptotic effect and restored the viability (Fig. 4). These results suggested that the pool of glutathione or another thiol redox buffer is involved in the process of apoptosis induced by DSF. However, we do not know if cysteine in the medium interacts with DSF.

The redox-related apoptosis in melanoma cells induced by DSF was also compared with BSO treatment. Our experiments showed that BSO alone could induce apoptosis (2-fold of control level) in the melanoma cell strains, and this level of apoptosis required a 4-day treatment with BSO. Treatment of melanoma cells with BSO for 72 h decreased the total number of viable cells (57.4% of control), although the percentage of viability/apoptosis did not change significantly (Tables 1 and 2). The BSO-induced apoptosis was

associated with a significant (>90%) glutathione depletion (Table 2). The above result was consistent with the findings of other investigators that BSO is an active agent for inhibiting tumor cell growth and inducing apoptosis (9, 10).

Despite a higher level of apoptosis observed in DSF-treated melanoma cells, the total cellular glutathione level was not decreased. It is worth noting that NAC significantly reversed the DSF-induced apoptosis and increased the melanoma cell viability without increasing the cellular GSH level. Additional measurement of cellular GSSG, the oxidized form of glutathione, showed that DSF treatment significantly increased the GSSG content (727% of control). The ratio of GSH:GSSG was significantly decreased (14% of control), and NAC partially restored the ratio to 30% of control. These results suggested that glutathione redox balance contributed more to the DSF-induced apoptosis than glutathione depletion *per se*. The cellular GSSG level in BSO-treated melanoma cells was too low to be measured because of a significant depletion of total glutathione. Although the combination of DSF and BSO resulted in only slight increase in apoptosis than DSF alone, the long-term effect of this combination is not known. *In vivo* study (using this drug combination) to monitor the kinetics of tumor progression may provide additional information.

Glutathione depletion may not be the sole factor in the observed apoptosis. Sato *et al.* (35) had reported using Jurkat T cells and human peripheral blood lymphoblasts as the target for BSO and diamide treatment. Depletion of cellular

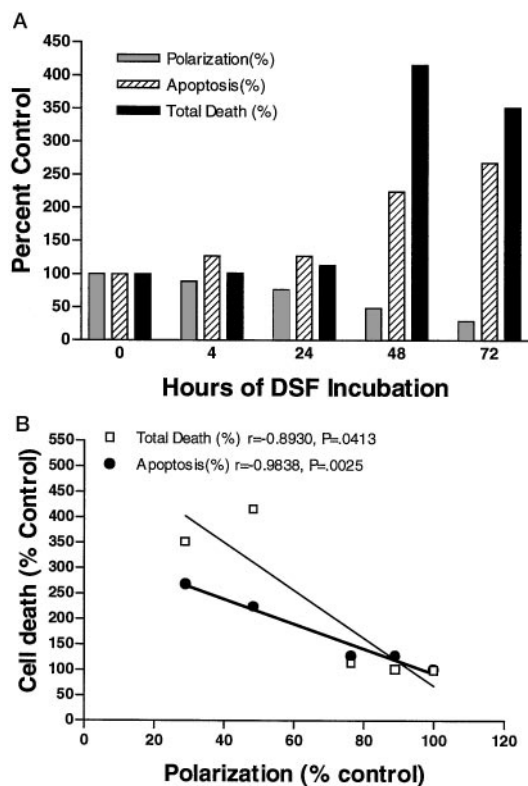


Fig. 5. DSF-induced apoptosis is associated with decrease in mitochondrial membrane potential. Cells (c83-2c) were incubated with DSF (50 ng/ml) for 4, 24, 48, and 72 h and analyzed for mitochondrial membrane potential (with JC-1 probe, as described in "Materials and Methods"), apoptosis, and total cell death (apoptosis + necrosis, as described in Fig. 1). Correlation between mitochondrial membrane potential and cell death (at corresponding time point) is illustrated in Fig. 5B. Results are expressed as the percentage of the control.

GSH with BSO failed to induce apoptosis despite a marked decrease of cellular GSH, which was greater than that observed in apoptosis induced by diamide. Thioredoxin was oxidized by incubation of these cells with diamide, suggesting that other cellular thiols may be involved in the diamide-induced T-cell apoptosis. Spyrou and Holmgren (36) also reported that BSO (0.1 mM) rapidly stopped GSH synthesis in exponentially growing 3T6 mouse fibroblasts, but the GSH depletion did not significantly affect the cell growth, DNA synthesis, and the pools of deoxyribonucleoside triphosphates, suggesting that GSH-glutaredoxin system is not the sole hydrogen donor system for ribonucleotide reductase. It is well known that thioredoxin and its reductase provides reducing equivalents for the cellular proteins and metabolites, including ribonucleotide reductase (37–40). The disturbance of glutathione balance in DSF-treated melanoma cells may reflect only one aspect of the thiol-redox alterations. Other components in the thiol-redox buffer system (such as thioredoxin and thioredoxin reductase, glutaredoxin and glutathione reductase) are also operating to maintain the cellular redox homeostasis. There are at least two major intracellular disulfide-reducing systems, the glutathione system and the thioredoxin system (38), and the latter one is

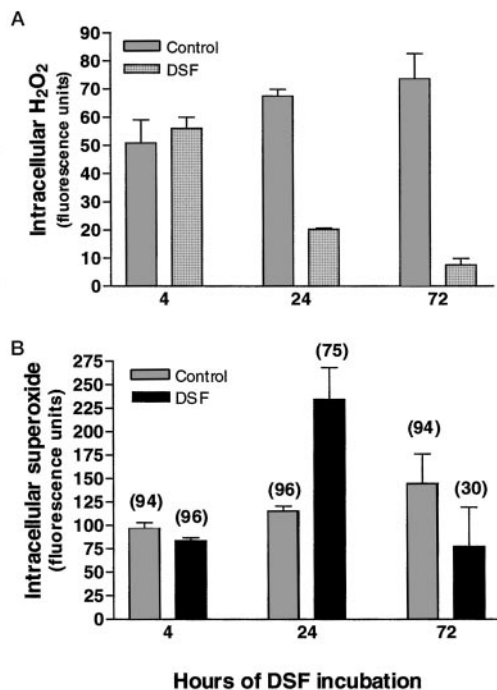


Fig. 6. DSF caused a transient increase in the cellular superoxide level. Cells (c83-2c) were incubated with DSF (50 ng/ml) for 4, 24, and 72 h, and analyzed for intracellular H₂O₂ (A) and superoxide (B) by 6-carboxy-2'-7'-dichlorodihydrofluorescein diacetate and HE, as described in "Materials and Methods." Shown in parentheses (B) are viabilities (performed by trypan blue exclusion). Results are represented from three experiments; bars, \pm SD.

regarded as an essential constituent of the cell-proliferating machinery in mammalian cells. It is our plan to additionally explore the role of those components on the DSF-treated melanoma cells.

The lack of significant DSF + BSO effect (as compared with DSF alone) can be attributable to maximal depletion of GSH by BSO. We have performed sequential incubation experiments (BSO followed by DSF) resulting in a more decreased apoptosis levels than coincubation treatments (data not shown). We have also used different doses of BSO (coincubation with DSF), and there were no significant changes in the apoptosis level.

We cannot exclude the possibility that DSF and NAC may affect the results of glutathione reductase-recycling assay. It has been reported that DSF may interfere with such an assay (41). Our next experiment is to incubate melanoma cells with DSF (or NAC) for 24 h and determine the intracellular uptake (content) of DSF (or NAC), then assay of GSH/GSSG by mixing untreated (control) cell lysate with known intracellular dose of DSF (or NAC) and compare the results with the untreated cells.

In this study, we also tested whether the redox-related apoptosis induced by DSF is associated with a decrease in the mitochondrial membrane potential in melanoma cells. Our finding showed that there was a significant inverse correlation of mitochondrial membrane polarization and the apoptosis/total cell death during the course of DSF incubation.

We hypothesize that changes in mitochondrial membrane potential might reflect apoptotic effects more accurately than late events of cell death (loss of membrane integrity). Experiments with other cell strains also showed a consistent decreased level of mitochondrial membrane polarization (approximately 40–60% of control) occurring at different time points (data not shown). The role of mitochondrion in the cell death control is being extensively investigated (42, 43). There are many different apoptosis-inducing conditions that can cause dissipation of mitochondrial $\Delta\Psi_m$ (33). Thiol cross-linking agents such as diamide, dithiodipyridine, or bis-maleimido-hexane can cause a covalent modification of a protein located in the inner mitochondrial membrane, adenine nucleotide translocator, leading to mitochondrial membrane permeabilization and cell death, and the process is Bcl-2 independent (19). It has been reported that DSF binds to proteins both *in vivo* and *in vitro* via formation of mixed disulfides with the free thiol groups of various proteins (33), and it is likely that this binding process may affect the function of the thiol-redox buffer (as mentioned above) and/or mitochondrial membrane integrity. Whether the DSF-induced apoptosis in melanoma cells is a direct action on mitochondria or an indirect effect through cellular redox changes remains to be answered. Study on purified mitochondria can additionally dissect the mechanisms of DSF on mitochondrial redox status and apoptosis.

There was a transient increase in the intracellular superoxide level after 24 h of incubation with DSF. Multiple experiments have shown that 24-h incubation is the earliest time point for detectable cell death (using AV and trypan blue stain, the viable fraction is approximately 75–95% of the control), and the findings were supported by the kinetic studies of apoptosis and the mitochondrial membrane potential (Fig. 5). Prolonged incubation of 48–96 h caused increasing frequency of apoptosis/necrosis, and the intracellular ROS content is the mixture from both viable and dead cells, and the leaking of ROS from dead cells may contribute to the decreased level observed. Dithiocarbamates, especially DDC, are known to inhibit copper/zinc superoxide dismutase (44), and oxidation of glutathione and/or other protein thiol groups may interfere with superoxide scavenging. Superoxide dismutase has been identified as a target for the selective killing of cancer cells, and the cellular level of superoxide is associated with oxidative-induced apoptosis of some tumor cells (45–47). Whether the transient increase in the superoxide anion in melanoma cells leads directly to apoptosis is not known. A cell-permeable superoxide scavenger, Trion, can be used to study the relationship of intracellular superoxide level and apoptosis (48).

DSF undergoes extensive metabolism after gastrointestinal absorption, and one major metabolite is DDC (33, 49). Our experiments showed that DDC also demonstrated a comparable capacity of inducing apoptosis in melanoma cells, although at a higher dose level (200 ng/ml or 0.888 μM). This finding may provide useful information for the possible *in vivo* use of DSF.

Our study showed that DSF induced apoptosis in metastatic melanoma cells, and NAC reversed the apoptotic effect. The apoptosis is associated with a decrease in the

GSH:GSSG ratio, decrease in mitochondrial membrane potential, and a transient increase in cellular superoxide level. In the future, we will additionally study the redox changes in the subpopulations (such as apoptotic, necrotic, and viable cells) when commercial fluorescence probe combinations are available. The differential mechanisms between DSF- and BSO-induced apoptosis may provide new information for selecting agents with complementary antimelanoma activity, and it will be interesting to study more drug combinations such as DSF with cisplatin, 1,3-bis(2-chloroethyl)-1-nitrosourea.

Acknowledgments

We thank Drs. John Fruehauf, Susan McNulty, and Pat Farmer for their helpful suggestions and discussions of this study and the manuscript.

References

- Davis, W., Jr., Ronai, Z., and Tew, K. D. Cellular thiols and reactive oxygen species in drug-induced apoptosis. *J. Pharmacol. Exp. Ther.*, 296: 1–6, 2001.
- Janssen-Heininger, Y. M., Poynter, M. E., and Baeuerle, P. A. Recent advances towards understanding redox mechanisms in the activation of nuclear factor κB . *Free Radic. Biol. Med.*, 28: 1317–1327, 2000.
- Mates, J. M., and Sanchez-Jimenez, F. M. Role of reactive oxygen species in apoptosis: implications for cancer therapy. *Int. J. Biochem. Cell Biol.*, 32: 157–170, 2000.
- Kamata, H., and Hirata, H. Redox regulation of cellular signalling. *Cell Signal.*, 11: 1–14, 1999.
- Nakamura, H., Nakamura, K., and Yodoi, J. Redox regulation of cellular activation. *Annu. Rev. Immunol.*, 15: 351–369, 1997.
- Meyskens, F. L., Jr., Chau, H. V., Tohidian, N., and Buckmeier, J. Luminol-enhanced chemiluminescent response of human melanocytes and melanoma cells to hydrogen peroxide stress. *Pigm. Cell Res.*, 10: 184–189, 1997.
- Meyskens, F. L., Jr., McNulty, S. E., Buckmeier, J. A., Tohidian, N. B., Spillane, T. J., Kahlon, R. S., and Gonzalez, R. I. Aberrant redox regulation in human metastatic melanoma cells compared to normal melanocytes. *Free Radic. Biol. Med.*, in press, 2001.
- Rodriguez-Vicente, J., Vicente-Ortega, V., and Canteras-Jordana, M. The effects of different antineoplastic agents and of pretreatment by modulators on three melanoma lines. *Cancer (Phila.)*, 82: 495–502, 1998.
- Fruehauf, J. P., Zonis, S., al-Bassam, M., Kyshtoobayeva, A., Dasgupta, C., Milovanovic, T., Parker, R. J., and Buzaid, A. C. Selective and synergistic activity of L-S, R-buthionine sulfoximine on malignant melanoma is accompanied by decreased expression of glutathione-S-transferase. *Pigm. Cell Res.*, 10: 236–249, 1997.
- Fruehauf, J. P., Zonis, S., al-Bassam, M., Kyshtoobayeva, A., Dasgupta, C., Milovanovic, T., Parker, R. J., and Buzaid, A. C. Melanin content and downregulation of glutathione S-transferase contribute to the action of L-buthionine-S-sulfoximine on human melanoma. *Chem.-Biol. Interact.* 111: 277–305, 1998.
- Palomares, T., Bilbao, P., del Olmo, M., Castro, B., Calle, Y., and Alonso-Varona, A. *In vitro* and *in vivo* comparison between the effects of treatment with adenosine triphosphate and treatment with buthionine sulfoximine on chemosensitization and tumor growth of B16 melanoma. *Melanoma Res.*, 9: 233–242, 1999.
- Thrall, B. D., Raha, G. A., Springer, D. L., and Meadows, G. G. Differential sensitivities of murine melanocytes and melanoma cells to buthionine sulfoximine and anticancer drugs. *Pigm. Cell Res.*, 4: 234–239, 1991.
- Prezioso, J. A., FitzGerald, G. B., and Wick, M. M. Melanoma cytotoxicity of buthionine sulfoximine (BSO) alone and in combination with 3,4-dihydroxybenzylamine and melphalan. *J. Investig. Dermatol.*, 99: 289–293, 1992.

14. Revesz, L., Edgren, M. R., and Wainson, A. A. Selective toxicity of buthionine sulfoximine (BSO) to melanoma cells *in vitro* and *in vivo*. *Int. J. Radiat. Oncol. Biol. Phys.*, **29**: 403–406, 1994.
15. Anderson, C. P., Tsai, J., Chan, W., Park, C. K., Tian, L., Lui, R. M., Forman, H. J., and Reynolds, C. P. Buthionine sulfoximine alone and in combination with melphalan (L-PAM) is highly cytotoxic for human neuroblastoma cell lines. *Eur. J. Cancer*, **33**: 2016–2019, 1997.
16. Anderson, C. P., Tsai, J. M., Meek, W. E., Liu, R. M., Tang, Y., Forman, H. J., and Reynolds, C. P. Depletion of glutathione by buthionine sulfoximine is cytotoxic for human neuroblastoma cell lines via apoptosis. *Exp. Cell Res.*, **246**: 183–192, 1999.
17. Anderson, C. P., Keshelava, N., Satake, N., Meek, W. H., and Reynolds, C. P. Synergism of buthionine sulfoximine and melphalan cell against neuroblastoma lines derived after disease progression. *Med. Pediatr. Oncol.*, **35**: 659–662, 2000.
18. Dai, J., Weinberg, R. S., Waxman, S., and Jing, Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. *Blood*, **93**: 268–277, 1999.
19. Costantini, P., Belzacq, A. S., Vieira, H. L., Larochette, N., de Pablo, M. A., Zamzami, N., Susin, S. A., Brenner, C., and Kroemer, G. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. *Oncogene*, **19**: 307–314, 2000.
20. Burkitt, M. J., Bishop, H. S., Milne, L., Tsang, S. Y., Provan, G. J., Nobel, C. S., Orrenius, S., and Slater, A. F. Dithiocarbamate toxicity toward thymocytes involves their copper-catalyzed conversion to thiuram disulfides, which oxidize glutathione in a redox cycle without the release of reactive oxygen species. *Arch. Biochem. Biophys.*, **353**: 73–84, 1998.
21. Meyskens, F. L., Jr., Buckmeier, J. A., McNulty, S. E., and Tohidian, N. B. Activation of nuclear factor- κ B in human metastatic melanoma cells and the effect of oxidative stress. *Clin. Cancer Res.*, **5**: 1197–202, 1999.
22. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. *J. Immunol. Methods*, **184**: 39–51, 1995.
23. Benov, L., Szejnberg, L., and Fridovich, I. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic. Biol. Med.*, **25**: 826–831, 1998.
24. Ubezio, P., and Civoli, F. Flow cytometric detection of hydrogen peroxide production induced by doxorubicin in cancer cells. *Free Radic. Biol. Med.*, **16**: 509–516, 1994.
25. Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., and Lorenzon, G. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biol. Toxicol.*, **10**: 415–421, 1994.
26. Balch, C. M., Houghton, A., and Peters, L. Cutaneous melanoma. *In: V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg (eds.), Cancer, Principles & Practice of Oncology*, Chapter 44, pp. 1499–1542. Philadelphia: J. P. Lippincott, 1989.
27. Balch, C. M., Reiretgen, D. S., Kirkwood, J. M., Houghton, A., Peters, C., and Ang, K. K. *In: V. T. DeVita, Jr., S. Hellman, and S. A. Rosenberg (eds.), Cancer, Principles & Practice of Oncology*, Chapter 44, pp. 1947–1994 Philadelphia: J. P. Lippincott, 1997.
28. Middleton, M. R., Grob, J. J., Aaronson, N., Fierlbeck, G., Tilgen, W., Seiter, S., Gore, M., Aamdal, S., Cebon, J., Coates, A., Dreno, B., Henz, M., Schadendorf, D., Kapp, A., Weiss, J., Fraass, U., Statkevich, P., Muller, M., and Thatcher, N. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. *J. Clin. Oncol.*, **18**: 158–166, 2000.
29. Middleton, M. R., Lorigan, P., Owen, J., Ashcroft, L., Lee, S. M., Harper, P., and Thatcher, N. A randomized phase III study comparing dacarbazine, BCNU, cisplatin and tamoxifen with dacarbazine and interferon in advanced melanoma. *Br. J. Cancer*, **82**: 1158–1162, 2000.
30. Jungnelius, U., Ringborg, U., Aamdal, S., Mattsson, J., Stierner, U., Ingvar, C., Malmstrom, P., Andersson, R., Karlsson, M., Willman, K., Wist, E., Bjelkengren, G., and Westberg, R. Dacarbazine-vindesine versus dacarbazine-vindesine-cisplatin in disseminated malignant melanoma. A randomised phase III trial. *Eur. J. Cancer*, **34**: 1368–1374, 1998.
31. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Seipp, C. A., Einhorn, J. H., White, D. E., and Steinberg, S. M. Prospective randomized trial of the treatment of patients with metastatic melanoma using chemotherapy with cisplatin, dacarbazine, and tamoxifen alone or in combination with interleukin-2 and interferon α -2b. *J. Clin. Oncol.*, **17**: 968–975, 1999.
32. Chen, S. H., Liu, S. H., Liang, Y. C., Lin, J. K., and Lin-Shiau, S. Y. Oxidative stress and c-Jun-amino-terminal kinase activation involved in apoptosis of primary astrocytes induced by disulfiram-Cu(2+) complex. *Eur. J. Pharmacol.*, **414**: 177–188, 2001.
33. Johansson, B. A review of the pharmacokinetics and pharmacodynamics of disulfiram and its metabolites. *Acta Psychiatr. Scand. Suppl.*, **369**: 15–26, 1992.
34. Loeffler, M., and Kroemer, G. The mitochondrion in cell death control: certainties and incognita. *Exp. Cell Res.*, **256**: 19–26, 2000.
35. Sato, N., Iwata, S., Nakamura, K., Hori, T., Mori, K., and Yodoi, J. Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. *J. Immunol.*, **154**: 3194–203, 1995.
36. Spyrrou, G., and Holmgren, A. Deoxyribonucleoside triphosphate pools and growth of glutathione-depleted 3T6 mouse fibroblasts. *Biochem. Biophys. Res. Commun.*, **220**: 42–46, 1996.
37. Mustacich, D., and Powis, G. Thioredoxin reductase. *Biochem. J.*, **346**: 1–8, 2000.
38. Becker, K., Gromer, S., Schirmer, R. H., and Muller, S. Thioredoxin reductase as a pathophysiological factor and drug target. *Eur. J. Biochem.*, **267**: 6118–6125, 2000.
39. Arner, E. S., and Holmgren, A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.*, **267**: 6102–6109, 2000.
40. Powis, G., Mustacich, D., and Coon, A. The role of the redox protein thioredoxin in cell growth and cancer. *Free Radic. Biol. Med.*, **29**: 312–322, 2000.
41. Nagendra, S. N., Shetty, K. T., Subhash, M. N., and Guru, S. C. Role of glutathione reductase system in disulfiram conversion to diethylthiocarbamate. *Life Sci.*, **49**: 23–28, 1991.
42. Fulda, S., Scaffidi, C., Susin, S. A., Krammer, P. H., Kroemer, G., Peter, M. E., and Debatin, K. M. Activation of mitochondria and release of mitochondrial apoptogenic factors by betulinic acid. *J. Biol. Chem.*, **273**: 33942–8, 1998.
43. Costantini, P., Jacotot, E., Decaudin, D., and Kroemer, G. Mitochondrion as a novel target of anticancer chemotherapy. *J. Natl. Cancer Inst.*, **92**: 1042–1053, 2000.
44. Kelner, M. J., Bagnell, R., Hale, B., and Alexander, N. M. Inactivation of intracellular copper-zinc superoxide dismutase by copper chelating agents without glutathione depletion and methemoglobin formation. *Free Radic. Biol. Med.*, **6**: 355–360, 1989.
45. Huang, P., Feng, L., Oldham, E. A., Keating, M. J., and Plunkett, W. Superoxide dismutase as a target for the selective killing of cancer cells. *Nature (Lond.)*, **407**: 390–395, 2000.
46. Shen, H. M., Yang, C. F., Ding, W. X., Liu, J., and Ong, C. N. Superoxide radical-initiated apoptotic signaling pathway in selenite-treated HepG(2) cells: mitochondria serve as the main target. *Free Radic. Biol. Med.*, **30**: 9–21, 2001.
47. Kuninaka, S., Ichinose, Y., Koja, K., and Toh, Y. Suppression of manganese superoxide dismutase augments sensitivity to radiation, hyperthermia and doxorubicin in colon cancer cell lines by inducing apoptosis. *Br. J. Cancer.*, **83**: 928–934, 2000.
48. Moreno-Manzano, V., Ishikawa, Y., Lucio-Cazana, J., and Kitamura, M. Selective involvement of superoxide anion, but not downstream compounds hydrogen peroxide and peroxyxynitrite, in tumor necrosis factor- α -induced apoptosis of rat mesangial cells. *J. Biol. Chem.*, **275**: 12684–91, 2000.
49. Kristenson, H. How to get the best out of antabuse. *Alcohol Alcohol*, **30**: 775–783, 1995.