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Disulfiram, an old drug with new potential therapeutic uses for human hematological malignancies

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Disulfiram (DSF) is an aldehyde dehydrogenase inhibitor currently used for the treatment of alcoholism. Here, we show that multiple myeloma (MM) cell lines and primary cells from newly diagnosed and relapsed/resistant patients affected by MM, acute myeloid and lymphoblastic leukemia are significantly sensitive to DSF alone and in combination with copper. These effects are present at doses lower than those achievable *in vivo* after DSF standard administration. The cytotoxic effect achieved by this treatment is comparable to that obtained by conventional chemotherapy and is absent in normal hematopoietic cells. In addition, we found that DSF plus copper induces loss of mitochondrial membrane potential, triggers reactive oxygen species (ROS) production and activates executioner caspases. DSF-copper-induced apoptosis and caspases activation are strongly reversed by antioxidant *N*-acetylcysteine, thus indicating a critical role of ROS. These results might suggest the use of the old drug DSF, alone or in combination with copper, in the treatment of hematological malignancies.

The commonly used alcohol-abuse deterrent disulfiram (DSF) is a member of the dithiocarbamate family, a broad class of molecules with the ability to complex metals, such as copper, and react with sulfhydryl groups and glutathione (GSH). DSF has been used for decades in the treatment of alcoholism for its ability to inhibit irreversibly the aldehyde dehydrogenase.[1,2] Several studies from 1970s (*e.g.*, Lewison 1977)[3] have shown that DSF and its metabolites can enhance the effect of some chemotherapeutics, and it is considered a convincing anticancer drug both *in vivo* and in human patients. The case report described by Lewison was the first on DSF activity against breast cancer.[3] Then the compound or its main metabolite was successfully used in a phase II clinical trial with 64 high-risk breast cancer patients and in hepatic metastases and in a patient with stage IV metastatic ocular melanoma.[4] More recently, there are ongoing clinical trials for DSF (Antabuse) as an adjuvant therapy (ClinicalTrials.gov Identifier NCT00312819) or combined with copper gluconate (ClinicalTrials.gov Identifier NCT00742911) against advanced solid cancers. However, a clear explanation for the antitumor effect of DSF is still missing.[2,4]

It has been shown that association of DSF with copper forms a complex with potent proteasome inhibiting and apoptosis-inducing activity on several tumors, both *in vitro* and *in vivo*.[1,2,4–6]

Moreover, DSF has also been found to reverse the resistance of human tumors to chemotherapeutic drugs by blocking maturation of the *P*-glycoprotein membrane pump; inhibit activation of nuclear factor-kB; decrease angiogenesis, tumor growth *in vivo*, matrix metalloproteinases activity and cancer cell invasiveness.[4,5]

The proapoptotic activity of DSF has been attributed to redox-related mitochondrial membrane permeabilization, followed by complexation with zinc/copper and inhibition of Zn-dependent matrix metalloproteinases or Cu/Zn superoxide dismutase. The consequent decrease in the production of H_2O_2 from dismutation of superoxide anion causes apoptosis.[2,4,5,7]

In this study, we have examined the effects and mechanism of action of DSF on myeloma cell lines and primary cells from multiple myeloma (MM) patients as well as primary cells obtained from acute myeloid and lymphoblastic leukemia (AML and ALL) patients.

Key words: acute leukemia, apoptosis, disulfiram, mitochondria, multiple myeloma
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Material and Methods

Primary cells and cell lines

Twenty-two MM, 19 AML and 11 ALL (Supporting Information Tables I and II) marrow aspirates were obtained from patients who gave informed consent and were managed at

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the Division of Hematology, Ospedale Ferrarotto, University of Catania. Cells were isolated by Ficoll Hypaque (Cedarlane Labs, Ontario, Canada) density gradient centrifugation.

Primary cells and the human MM cell lines KMS-18, KMS-27 and ARH-77 (ATCC, Rockville, MD) were grown in RPMI 1640 medium (Gibco, Invitrogen) supplemented with L-glutamine and penicillin–streptomycin and 10% heat-inactivated fetal bovine serum (Invitrogen, Cartsbad, CA).

Adult peripheral blood and bone marrow were obtained from healthy male donors (according to the Hospital ethical guidelines) after their informed consent. Human CD34+ precursor cells were purified from peripheral blood by using the midi-MACS immunomagnetic separation system (Miltenyi Biotec). Peripheral blood mononuclear cells (PBMCs) and bone marrow cells (BMs) were isolated by Ficoll Hypaque (Cedarlane Labs) density gradient centrifugation and maintained as cell lines.

Reagents

Antineoplastic agents and *N*-acetyl cysteine were from Sigma-Aldrich (St. Louis, MO). Bortezomib was from Millennium Pharmaceuticals (Cambridge, MA). Primary antibodies anti-Bcl-xl, anti-IAP1, anti-Mcl-1, anti-ERK-1, anti-phospho-ERK 1-2, anti-p21, anti-Akt and anti-Smac were from Santa Cruz Biotechnology, antiactin from Sigma (St Lous, MO), anti-Bcl2 from Pharmingen (S. Diego, CA) anti-FLIP from Alexis (San Diego, CA) and anti-phospo-Akt (Ser^{473}), anti-p38 and anti-phospho-p38 from Cell Signaling Technology.

Cell proliferation, apoptosis, mitochondrial membrane potential, reactive oxygen species and caspase assays

Cell proliferation was determined using the CellTiter 96 Aqueous One solution cell proliferation assay (Promega, Madison, WI). Apoptosis was determined by Annexin-V Alexa Fluor 488 conjugated (Molecular Probes, Leiden, The Netherlands) and propidium iodide (Sigma Chemical Co), mitochondrial membrane potential (MMP) was determined by JC-1 staining (Molecular Probes), and data were collected and analyzed with a FACSAria II cell sorter (Becton Dickinson).

Intracellular reactive oxygen species (ROS) and GSH levels were detected using the dye 2′-7′DCFH-DA and [1-(4-chloromercuryphenyl-azo)-2-naphthol] (Sigma). Data were then analyzed as previously described.[8]

Caspase activity was determined with the Apo-ONE homogeneous caspase-3/7 kit (Promega) and analyzed on a plate fluorometer.

Statistical analysis

Two-tailed paired *t* test was used. $p < 0.005$ was considered statistically significant. One asterisk indicates $p < 0.005$ and two asterisks $p < 0.0005$.

Results and Discussion

To evaluate the potential cell growth inhibition of DSF or a mixture of DSF with Cu, three MM cell lines and MM primary cells from 22 patients (19 newly diagnosed, 3 resistant/relapsed) were treated with Cu, DSF and the combination of both at concentrations ranging from 0.1 to 5 μM for DSF and from 0.2 to 1.2 for Cu. The number of viable cells was greatly reduced after treatment with the DSF-Cu mixture in a time and concentration-dependent manner, without any correlation with the features of the patients from whom cells were derived. Moreover, the combination of DSF and Cu slightly affects cell cycle (data not shown) as previously described by Brar *et al.*[5] The antiproliferative IC_{50} for DSF–copper combination was 0.5 μM for both agents (Fig. 1*a*, upper panels, Supporting Information Fig. S1), significantly lower than the concentration achieved with a normal adult dose for the treatment of alcoholism (500 mg as maximum daily dose[4]) and for Cu recommended dietary intake.[9] In addition, we found that the cytotoxic effect exerted by DSF-Cu mixture is similar to that of chemotherapeutic drugs currently used for the treatment of MM, at dosage that are consistent with their achievable serum concentration (FDA 2003)[10] (Fig. 1*a*, lower panel). Human samples, differently from cell lines, can present different behaviors, as exemplified by the three samples that are resistant to DSF (Supporting Information Fig. S2*a*). Similar results were obtained with samples from 19 AML (14 newly diagnosed, 5 resistant/relapsed) and 11 ALL patients (4 newly diagnosed, 7 resistant/relapsed) treated with DSF, Cu or DSF-Cu combination (Figs. 1*b*–1*c*, upper panels) or with other cytotoxic drugs (Figs. 1*b*–1*c*, lower panel, Supporting Information Figs. S2*b* and S2*c*). Conversely, the combination of DSF and Cu had only a weak effect on normal CD34, PBMCs and BMs (Supporting Information Figs. S3*a*–S3*c*). Importantly, DSF-Cu-treated cells undergo apoptosis (Fig. 1*d*, Supporting Information Fig. S4*a*) with typical signs of apoptotic cells, such as, nuclear condensation, cell shrinkage and membrane blebbing (Supporting Information Fig. S4*b*), in addition to loss of MMP (Fig. 1*e*) and caspases activation, without any modification in the expression level of apoptotic-related proteins except antiapoptotic protein Bcl-xL (data not shown, Supporting Information Fig. S5).

DSF has been described as a promising anticancer drug.

The apoptotic effect against tumor cells of a combination of DSF and heavy metal ions has already been described by different authors.[1,2,4,5,7,11]

It has been widely reported that DSF possesses a strong proteasomal inhibiting activity on several tumors.[1,2,4–6,11–13] Moreover, DSF is in the hit of proteasome inhibitors against MM,[6] where proteasome inhibitor bortezomib is a clinically approved drug. Proteasome inhibitors are generally held to block NF-kB activation by inhibiting IkB degradation and consequently preventing the translocation of NF-kB to the nucleus. One proposed mechanism to explain the antiproliferative activity of DSF could therefore be a bortezomib-like effect through inhibition of translocation of transcription factor NF-kB to the nucleus.

We have therefore explored both the 20S proteasome activity and the NF-kB status in MM and acute leukemia (AL)

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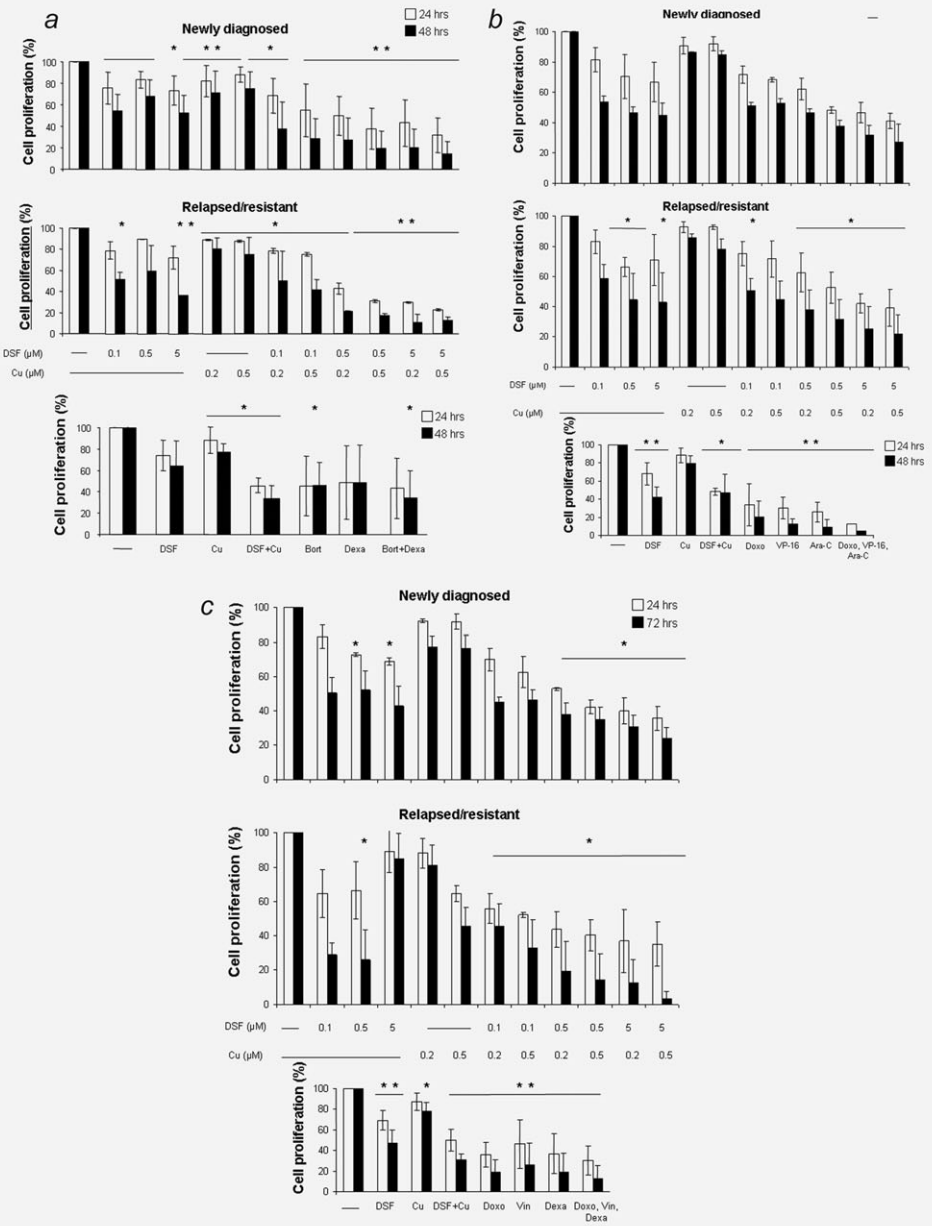


Figure 1. Apoptotic effects of DSF-copper (DSF-Cu) mixture on MM, AML and ALL cells. (*a*) Primary myeloma cells from 19 patients with MM at diagnosis and from 3 patients with relapsed/resistant MM (upper panels) were treated with different concentrations of DSF and Cu. After 24 and 48 hr, cell proliferation was evaluated by the MTS assay. The results represent the mean ± SD of 19 and 3 independent experiments in triplicate. Percentage of cell proliferation of MM primary cells treated for 24 and 48 hr with DSF (0.5 μM) or Cu (0.5 μM) alone or in combination, Bortezomib (1 μM, Bort), dexamethasone (100 μg/ml, Dexa) alone or in combination (lower panel). The percentage of cell proliferation cells was determined using the MTS assay. The results show the mean ± SD of 14 independent experiments in triplicate. (*b*) Acute leukemia blasts from eight AML patients at diagnosis (upper panel) and 4 relapsed/resistant AML (lower panel) patients of different subtypes were treated with different concentrations of DSF and Cu. After 24 and 72 hr, cell proliferation was evaluated by the MTS assay. The results represent the mean ± SD of eight and four independent experiments in triplicate. Percentage of cell proliferation of AML primary cells treated for 24 and 48 hr with DSF (0.5 μM) and Cu (0.5 μM) alone or in combination or etoposide (0.5 μg/ml, VP-16), cytarabine (2.5 μg/ml, Ara-C) and doxorubicine (5μM, Doxo) alone or in combination (lower panel). The results show the mean ± SD of eight independent experiments in triplicate. (*c*) Acute leukemia blasts from 4 ALL patients at diagnosis (upper panel) and 7 relapsed/resistant ALL (lower panel) patients of different subtypes were treated with different concentrations of DSF and Cu. After 24 and 72 hr, cell proliferation was evaluated by the MTS assay. The results represent the mean ± SD of four and seven independent experiments in triplicate. Percentage of cell proliferation of ALL primary cells treated for 24 and 48 hr with DSF (0.5 μM) and Cu (0.5 μM) alone or in combination, or doxorubicine (5 μM, Doxo), vincristine (1 μM, Vin) and dexamethasone (100 μM, Dexa) alone or in combination (lower panel). The percentage of cell proliferation cells was determined using the MTS assay. The results show the mean ± SD of six independent experiments in triplicate. (*d*) Analysis of apoptosis of cells treated with DSF-Cu complex. Primary cells from 4 MM patients and 6 AML patients were treated with 0.5 μM DSF, 0.5 μM Cu or DSF plus Cu for 24 hr and analyzed by flow cytometry with AV and PI as described in Material and Methods section. One representative experiment of MM and AML sample is shown. (*e*) Loss of MMP by flow cytometry analysis in cells from 6 MM primary cells, 2 AML and 2 ALL samples after 0.5 μM DSF, 0.5 μM Cu or DSF plus Cu treatment for 24 hr. Mitochondrial depolarization is indicated by the increase in green fluorescence after treatment. A representative experiment of one MM, AML and ALL sample is shown.

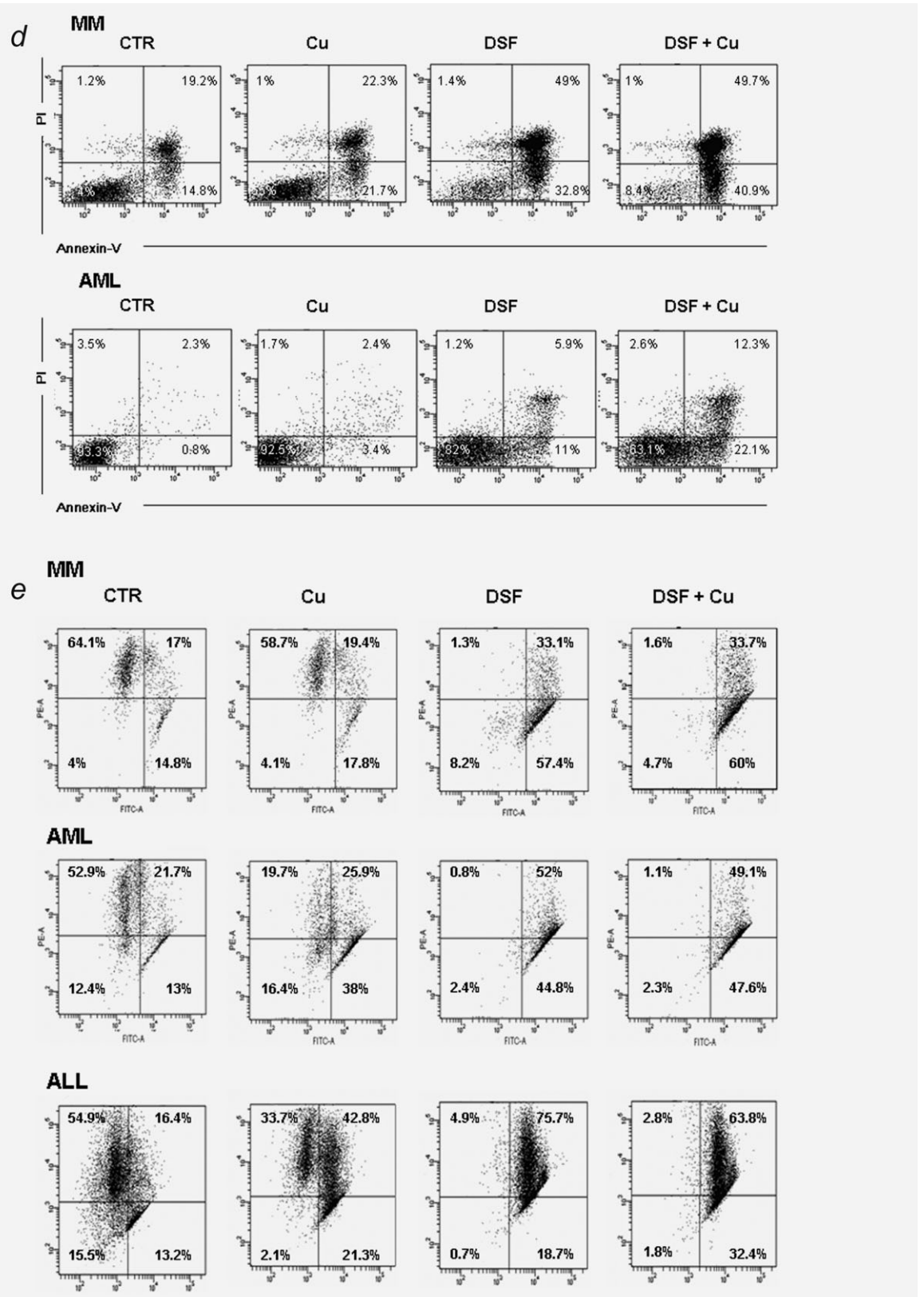
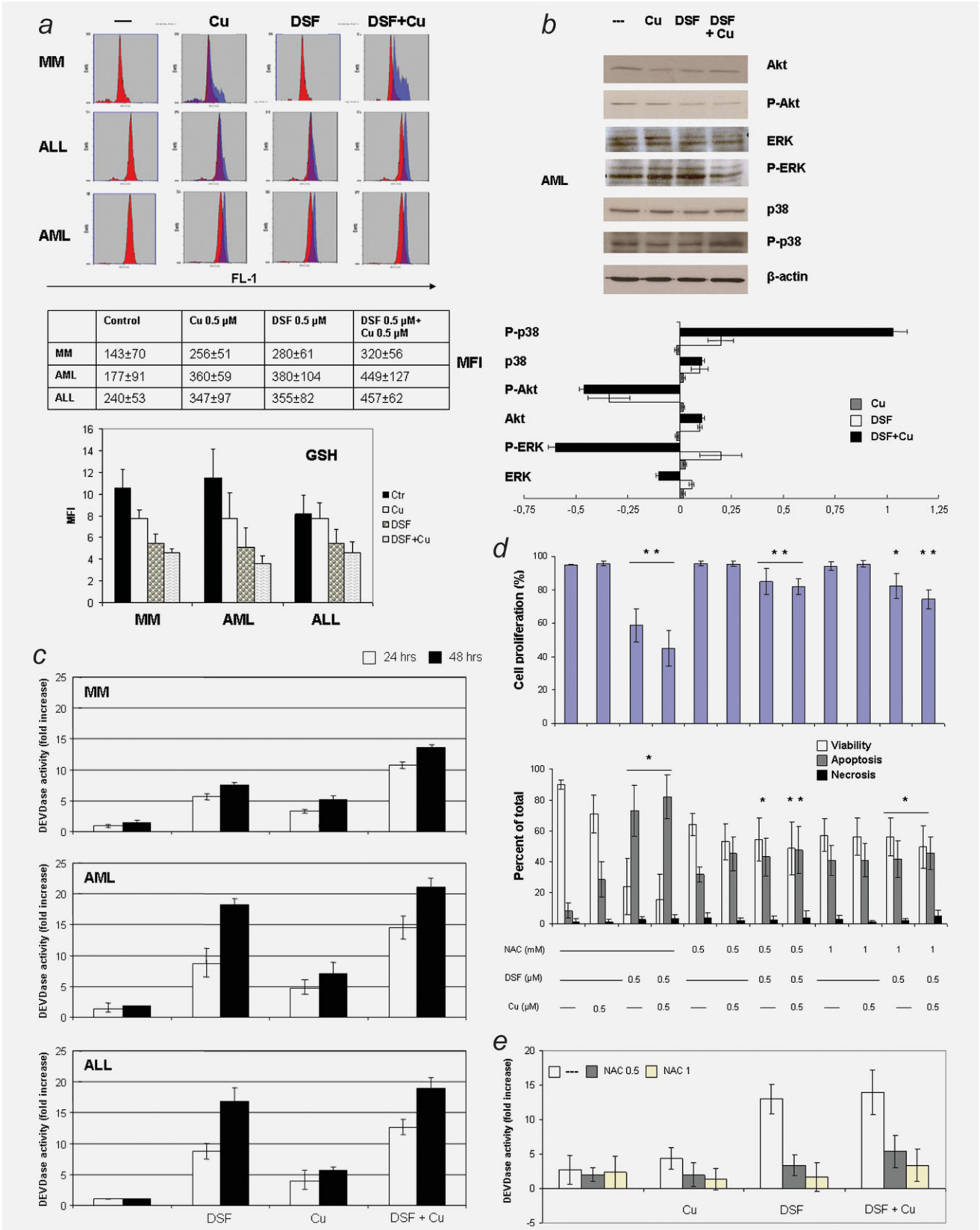


Figure 1. (Continued).



	Control	Cu 0.5 μM	DSF 0.5 μM	DSF 0.5 μM+ Cu 0.5 μM
MM	143±70	256±51	280±61	320±56
AML	177±91	360±59	380±104	449±127
ALL	240±53	347±97	355±82	457±62

MFI

Figure 2.

cell lines and MM PCs and AL blasts before and after DSF and DSF-copper treatment. We have not found any direct activity of DSF alone or in combination with copper against the proteasome or any changes in NF-kB status (data not shown). In fact, NF-kB is constitutively expressed in the majority of the nuclei of AL samples, but its localization is not affected by DSF or DSF-copper treatment. Conversely, in MM PCs, we have observed that NF-kB is cytoplasmic, and its localization is not affected by DSF treatment. This observation is in line with our previous research.[14,15]

In any case, in contrast with previous studies showing that DSF-copper combination mediates direct inhibition of the proteasome or of transcription factors such as NF-kB or ATF/CREB *in vitro*, these mechanisms do not seem to be important in DSF-copper-mediated toxicity of MM PCs and AML and ALL blasts. We can hypothesize that the effects of DSF rely on its ability to negatively affect constitutive levels of proteasome-degraded proteins rather than apoptosis-related proteins.

A potent mechanism of cell death can be dysregulation of cellular redox status, and generation of ROS may cause initiation of intracellular apoptosis signal.[16]

Several reports have attributed the cytotoxic activity of DSF to proapoptotic redox-related mitochondrial membrane permealization and cellular redox changes.[2,4,5,7] DSF binds to proteins *via* formation of mixed disulfide bonds with free thiol groups of various proteins,[17] affecting MMP and generating ROS.[4] Moreover, drug-induced ROS stress triggers a cascade of redox-dependent signaling events at different cellular levels, including MAPK and Akt signal transduction cascades.[18] This eventually culminates in mitochondria-dependent apoptosis.[16–19]

We have found that a mixture of DSF with Cu generates ROS and decreases GSH levels (Fig. 2*a*). This could be just a consequence of proteasome inhibition. Indeed, it has been recently described how bortezomib-induced proteasome inhibition exerts its toxicity against cancer cells through the proapoptotic protein NOXA that induces caspase cleavage in the context of reactive oxidative stress-induced cell death.[20] In addition, treatment with the mixture of DSF with Cu is associated with downregulation of Akt and MAPK/Erk expression levels and, as expected, upregulation of phospho-p38 MAPK (Fig. 2*b*, Supporting Information Fig. S6) and activation of caspase (Fig. 2*c*). PI3K/Akt, Raf/MEK/ERK and p38/MAPK-signaling pathways have been involved in the regulation of survival decisions in several neoplastic cell types.[21–24] Activation of Akt occurs in response to oxidative damage to attenuate lethality,[21] while activation of the Raf/MEK/ERK cascade limits free-radical production and mitochondrial dysfunction.[25–27]

It is noteworthy that phosphorylation of Bcl-2 by activated p38/MAPK in mitochondrial compartment leads to cytochrome c release, caspase activation and apoptosis. These processes are absent in p38/MAPK-deficient cells.[28]

It is therefore conceivable that inactivation of the Akt and Raf/MEK/ERK cascade and activation of p38 in DSF-Cu-treated cells serve to amplify oxidative injury and, by extension, the apoptotic response.

To assess directly the role of ROS production in DSF-Cu-induced apoptosis, we exposed primary cells to free-radical scavenger NAC that blocks ROS generation, before treatment with the mixture. NAC abolished the induction of apoptosis (Fig. 2*d*) and caspase activation (Fig. 2*e*), thus suggesting that cellular redox status has a pivotal role in DSF-Cu-induced apoptosis. However, we cannot completely exclude that the binding of NAC with copper prevents the formation of DSF-Cu complex.

We can therefore hypothesize that the DSF-Cu mixture induces cellular stress that alters MMP and causes ROS production. This ends up in the death of the apoptotic cell. In conclusion, our experiments demonstrate that the mixture

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Figure 2. Disulfiram-Cu complex induces ROS production. (*a*) Flow cytometry analysis of reactive oxygen species (ROS) in primary cells from 6 MM patients, 5 AML patients and 6 ALL patients. Cells were treated with 0.5 μM DSF, 0.5 μM Cu or DSF-Cu complex for 24 hr and then washed, stained for ROS and GSH and analyzed by flow cytometry as described in Material and Methods section. Fluorescence distribution histogram and mean fluorescence intensity (MFI) are shown. Control untreated cells are shown as well (grey histograms). (*b*) Immunoblot analysis of expression of Erk, P-Erk, Akt, P-Akt, p38 and P-p38 in 3 AML samples untreated or treated with 0.5 μM DSF, 0.5 μM Cu or DSF-Cu complex for 24 hr. One representative experiment of three performed is shown. Densitometric analysis of p38, P-p38, Akt, P-Akt, Erk and P-Erk levels in AML cells treated with DSF, Cu or DSF-Cu complex for 24 hr. Protein levels shown are the mean ± SD of 3 independent experiments. Comparable results were obtained with KMS-18, KMS-27 and ARH-77 cell lines and two MM primary samples (Supporting Information Fig. S6). (*c*) Activation of caspases in DSF-Cu complex treated cells. Cells derived from one MM patients (sample no. 4, Table I, Supporting Information figures), 3 AML (samples nos. 2, 3 and 7, Table II, Supporting Information figures) and 3 ALL (sample nos. 2, 6 and 9, Table II, Supporting Information figures) patients were incubated in the presence of 0.5 μM DSF, 0.5 μM Cu or DSF-Cu complex for 24 and 48 hr. Caspase activity was evaluated by cleavage of the fluorogenic substrate Ac-DEVD-AMC using a fluorimetric caspase assay. The data show the mean ± SD of three independent experiments (one MM sample, 3 AML sample and 3 ALL samples). (*d*) NAC is able to inhibit DSF-induced apoptosis in AML cells. Cells derived from 7 AML patients were incubated in the presence or absence of NAC (0.5 or 1 mM) for 1 hr, followed by treatment with 0.5 μM DSF, 0.5 μM Cu or DSF-Cu complex for 24 hr. Cells were then evaluated by the MTS assay for cell proliferation (upper panel) and analyzed by flow cytometry with AV and PI (lower panel). Results show mean ± SD of seven and three independent experiments in triplicate, respectively, for MTS and AV-PI assays. (*e*) Oxidative stress is required for the apoptosis induced by DSF-copper combination. Cells derived from three 3 AML patients were incubated with or without NAC (0.5 or 1 mM) for 1 hr and treated with 0.5 μM DSF, 0.5 μM Cu or DSF-copper combination for 24 hr. Caspase activity was evaluated by cleavage of the fluorogenic substrate Ac-DEVD-AMC using a fluorimetric caspase assay. Data show the mean ± SD of three independent experiments in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of DSF with Cu exerts its cytotoxic effect in hematological malignant cells by activating the mitochondrial apoptotic pathway.

Antabuse (DSF) was just proposed as a pilot case of "non-profit" drug against refractory solid tumors[29]: it is an inexpensive substance with negligible adverse effects in comparison with classical chemotherapy, and Cu is an essential trace element of diet. We therefore encourage future clinical studies with these two compounds in patients with refractory hematological malignancies.

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