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SHORT REPORT

Inhibition of proteasome activity, nuclear factor-KB translocation and cell survival by the antialcoholism drug disulfiram

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The proteasome pathway is an important target for anticancer drug development. Here, we identify the antialcoholism drug disulfiram and its analogue pyrrolidine dithiocarbamate (PDTC) as inhibitors of the 26S proteasome activity in a cell-based screening assay. As expected for proteasome inhibitors, these compounds also inhibited TNF-α-induced nuclear factor-KB (NF-KB) translocation and were cytotoxic. Disulfiram was more cytotoxic against chronic lymphocytic leukemia cells compared to peripheral blood mononuclear cells (PBMC) at clinically achievable concentrations. Proteasome and NF-KB inhibition were achieved with a potency in the same range as that of the clinically used proteasome inhibitor bortezomib. Disulfiram was also able to induce accumulation of $p27^{Kip1}$ and to prolong the half-life of c-Myc, both targets for proteasome-dependent degradation. It is concluded that the previously observed antitumoral and NF-KB inhibiting activity of disulfiram and PDTC could be attributed to their inhibition of the 26S proteasome.

Key words: disulfiram; proteasome inhibition; nuclear factor-KB; cytotoxicity

The thiocarbamate drug disulfiram has been used extensively in the treatment of alcoholism.[1] Disulfiram has also been shown to induce apoptosis in a number of tumor cell lines[2–4] as well as to reduce the growth of glioma, lung carcinoma[5] and melanoma tumors in mice.[6] However, the mechanism underlying the cytotoxic and antitumoral activity of disulfiram has not been conclusively established. It has been proposed that the induction of cell death involves generation of reactive oxygen species.[5] Additionally, disulfiram has been shown to inhibit nuclear factor-KB (NF-KB) in hepatoma[4] and colorectal cancer cell lines.[2] The NF-KB-inhibiting activity of the disulfiram analogue pyrrolidine dithiocarbamate (PDTC) has been attributed to its antioxidative effect.[7] In this work, we propose a novel mechanism for the antitumoral and NF-KB-inhibiting activities of disulfiram. Screening of a diverse pharmacological library of compounds identified disulfiram as an inhibitor of 26S proteasome activity. Our findings suggest that the antitumor effect of disulfiram could, at least partly, be attributed to its proteasome-inhibitory action, stabilizing proteasome-degraded proteins, *e.g.*, IKB, $p27^{Kip1}$ and c-Myc, subsequently resulting in the inhibition of cell-cycle progression and induction of apoptosis.

Materials and methods

Chemicals

Compounds used for screening were part of the LOPAC 1280™ library (Sigma Aldrich, St. Louis, MO). The library is a collection of pharmacologically active compounds with a diverse range of proposed target receptors and enzymes. The proteasome-inhibiting substance bortezomib was provided by Millennium Pharmaceuticals (Cambridge, MA). All other chemicals were from Sigma Aldrich.

Cell lines

The stably transfected human embryo kidney cell line HEK 293 expressing the ZsProSensor-1 fusion protein (HEK 293 ZsGreen, BD Biosciences, San Jose, CA) was used for screening and evaluation of proteasome inhibitors. The ZsProSensor-1 consists of the green fluorescent protein ZsGreen fused to mouse ornithine decarboxylase (mODC) and is degraded by the 26S proteasome.[8] Inhibition of the proteasome leads to accumulation of the fluorescent protein. HEK 293 ZsGreen was grown in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin (Sigma Aldrich). The antibiotic G418 was included in the culture medium at a concentration of 0.2 mg/ml to select for stably transfected cells. The myeloma cell line RPMI8226 was grown in RPMI1640 supplemented as earlier.

Cellular proteasome inhibition assay

HEK 293 ZsGreen cells were seeded in collagen I-coated 96-well microtiter plates at a density of 8,000 cells per well and were incubated overnight to allow cell adherence. Following compound exposure for 12 hr to allow accumulation of detectable levels of ZsGreen, plates were washed and cells were fixated and nuclei were counterstained by adding a solution of 3.7% formaldehyde in PBS with 10 μM Hoechst 33342. Processed plates were loaded into the ArrayScan® HCS reader (Cellomics, Pittsburgh, PA) for automated image acquisition and analysis. The ArrayScan is an inverted fluorescence microscope with the ability to focus and acquire images of cells in microtiter plates. Images are automatically processed to identify cells based on their nuclear Hoechst 33342 staining and to quantify fluorescence intensity and localization based on the staining from other fluorochromes.

To quantify the degree of 26S proteasome inhibition, the intensity of ZsGreen fluorescence of each cell was measured. The average green cellular intensity of more than 500 cells was calculated for each well and the nuclear channel was used to address morphological changes.

NF-KB translocation assay

HEK 293 ZsGreen cells were plated as described earlier. Compounds were added at various concentrations and cells were incubated for 1 hr. After the incubation, cells were stimulated with 100 ng/ml of TNF-α (Sigma Aldrich) for 30 min. Negative control cells without TNF-α were used as a reference to quantify the extent of baseline NF-KB translocation in the cell line. The NF-KB translocation was visualized using the NF-KB Activation HCS HitKit™ reagents (Cellomics) according to manufacturer instructions. A primary antibody specific for the p65 subunit of NF-KB and a secondary antibody conjugated with the fluorophore Texas Red was used. Hoechst 33342 was added to label the nuclei of the cells.

Quantification of NF-KB activation was performed by measuring the spatial translocation of NF-KB from the cell cytoplasm to

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the nucleus, using the ArrayScan HCS reader (Cellomics) as described earlier.[9] The degree of translocation of about 500 cells per well was averaged. The translocation was presented as percentage NF-KB translocation of that in control wells with 100% being the maximum translocation of p65 achievable in cells exposed to TNF-α.

20S proteasome enzymatic activity

The enzymatic activity of the 20S proteasome was measured by adding test substances or known inhibitors to human erythrocyte 20S enzyme in an assay buffer of 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.05% NP-40 and 0.03% SDS (Plymouth Meeting, PA). For some experiments, the buffer was complemented with 10 μM $CuCl_2$ or $ZnSO_4$. After 10 min incubation to allow enzyme and substrate interaction, the fluorogenic proteasome substrate SUC-LLVY-AMC was added. The increase in fluorescence was measured every third minute during 1 hr. Monitoring the increase in fluorescence over time, allowed quantification of the enzymatic activity.

Cytotoxicity assay

Cytotoxic effect of disulfiram, PDTC and bortezomib was studied using the fluorometric microculture cytotoxicity assay (FMCA) previously described in detail.[10] Briefly, this assay measures the hydrolysis of fluorescein diacetate to fluorescent fluorescein in cells with intact plasma membrane. For experiments, cell suspension was added to drug-containing microtiter plates. After 72 hr incubation, the plates were washed, fluorescein diacetate was added and the generated fluorescence was measured after 40 min. Data are expressed as survival index (SI %), calculated by dividing the fluorescence in test wells with that of control wells, with blank values subtracted. Fluorescent signal from the ZsGreen protein accumulated in cells did not interfere with the analysis (not shown).

Western blot analysis

Exponentially growing RPMI8226 cells at 0.4×10^6 cells/ml were treated with 4 μM of disulfiram for 4 hr, followed by treatment with 10 μg/ml cycloheximide (Sigma), to inhibit new protein synthesis, and whole cell lysates were prepared at indicated times and subjected to western blot analysis as described previously.[11] Five micrograms of the extracts were fractionated on Novex NuPAGE (10%) precast gels, using the Novex electrophoresis and blotting system (Novex, San Diego, CA). Primary antibodies used were p27 (C-19), c-Myc (N-262) and β-actin (I-19) (Santa Cruz Biotechnology, Santa Cruz, CA). The bands were quantified by a Fujix Bio-Imaging Analyzer Base 2000 (Fuji, Stockholm, Sweden) and divided by actin band intensity, to correct for loading differences (data not shown).

Results

Proteasome activity and NFKB translocation in HEK 293 ZsGreen

In a cell-based high-content screen of 1,280 compounds (not shown), using HEK 293 ZsGreen cells expressing the ZsProSensor-1 fusion protein, we identified disulfiram and PDTC as potent inhibitors of the 26S proteasome activity. Images of HEK 293 ZsGreen exposed to 0.16 μM disulfiram or PDTC or 0.016 μM bortezomib are shown in Figure 1*a*. The pattern and localization of ZsGreen staining within the cells were similar between the thiocarbamate compounds, disulfiram, PDTC and bortezomib. Dose-response evaluation of ZsGreen accumulation induced by 12 hr incubation with disulfiram, PDTC and the clinically used proteasome inhibitor bortezomib are shown in Figure 1*b*. Disulfiram and PDTC potently inhibited the 26S proteasome activity in a dose-dependent manner.

All 3 compounds were also shown to inhibit NF-KB translocation. Analysis of the TNF-α-induced nuclear–cytoplasm translocation of the NF-KB subunit p65, after a 1 hr incubation with compounds, indicates an inverse relationship between the degree of translocation and proteasome inhibition (Fig. 1*b*). Studies using purified SDS-activated 20S proteasome, with or without Zn^{2+} or Cu^{2+}, did not indicate inhibitory activity of disulfiram or PDTC. Also, the main *in vivo* disulfiram metabolite diethyldithiocarbamate (DEDTC) potently inhibited cellular proteasome activity as well as NF-KB translocation but had no effect on the 20S enzymatic activity (not shown).

Cytotoxic activity of disulfiram and PDTC

The cytotoxic activity of the compounds in the HEK 293 ZsGreen cell line indicated a positive relationship between proteasome inhibition and cytotoxicity. Dose–response evaluation after 72 hr incubation with compounds resulted in IC_{50} values for disulfiram, PDTC and bortezomib of 13, 86 and 11 nM, respectively (Fig. 1*c*). These IC_{50} values are close to the concentrations causing proteasome inhibition. DEDTC was also cytotoxic when tested on the HEK 293 ZsGreen cell line (not shown). Cytotoxic activity of disulfiram, PDTC and bortezomib in the myeloma cell line RPMI8226 is shown in Figure 1*d* with IC_{50} values of 94, 290 and 260 nM, respectively.

The cytotoxic effect of disulfiram at 0.1 μM was tested in peripheral blood mononuclear cells (PBMC) from 4 healthy donors and chronic lymphocytic leukemia (CLL) cells from 8 patients. The average SI of 39.5% for PBMC was significantly higher than the 5.8% for the CLL samples ($p < 0.01$).

Effect of disulfiram on proteasome target proteins

Exposing the myeloma cell line RPMI8226 to 4 μM disulfiram for 4 hr induced a strong accumulation of $p27^{Kip1}$ protein. Chase analysis of $p27^{Kip1}$ in cells after inhibition of protein synthesis showed stable levels of $p27^{Kip1}$, suggesting that the increase in $p27^{Kip1}$ was the result of inhibited proteasomal degradation. The constitutive level of c-Myc expression in RPMI8226 cells was high and did not show an increase after exposure to disulfiram. However, chase analysis indicated a clear stabilization of the c-Myc protein in disulfiram-exposed cells (Fig. 2).

Discussion

In this article, we describe, for the first time, the 26S proteasome inhibiting activity of disulfiram, a drug mainly known for its effects in treating alcoholism. The proteasome controls the turnover of critical components of the cell cycle and apoptosis regulatory pathways, often found to be dysfunctional in malignant cells. The regulation of the proteasome pathway is, therefore, an important target for anticancer drug development, and the first proteasome inhibitor approved for clinical use, bortezomib, has shown promising activity in myeloma patients.[12] Disulfiram has previously attracted some interest as an inducer of apoptosis in tumor cells both *in vitro* and *in vivo*. However, the mechanism for the apoptosis-inducing effect is not clear. A number of different mechanisms have been proposed, among them is the redox-related processes involving copper and zinc.[3,4,6]

The 26S proteasome is a multi-enzyme complex consisting of the 20S catalytically active unit and two 19S regulatory units. In this work, using a cell-based assay, we were able to show that disulfiram, at clinically achievable concentrations,[13] inhibits the 26S proteasome activity with potencies in the range of that of the clinically used proteasome inhibitor bortezomib. This was also observed for PDTC, a widely used NF-KB inhibitor and DEDTC, the main *in vivo* disulfiram metabolite.[14] Recently, it has been shown that PDTC combined with Zn^{2+} is able to inhibit proteasome-dependent proteolysis in HeLa cells,[15] and the combination of PDTC and Cu^{2+} was shown to inhibit the proteasome in a prostate cancer cell line.[16] In our cell-based model system, PDTC itself inhibits the proteasome activity and this could provide an alternative explanation for the NF-KB-inhibiting activity of PDTC usually attributed to its antioxidant effect.[7]

Accumulation of ZsGreen was not observed in cells exposed to a number of generally toxic and antitumoral compounds in the original screening, strengthening the conclusion that the accumulation is not due to unspecific toxic effect on cells. However, when

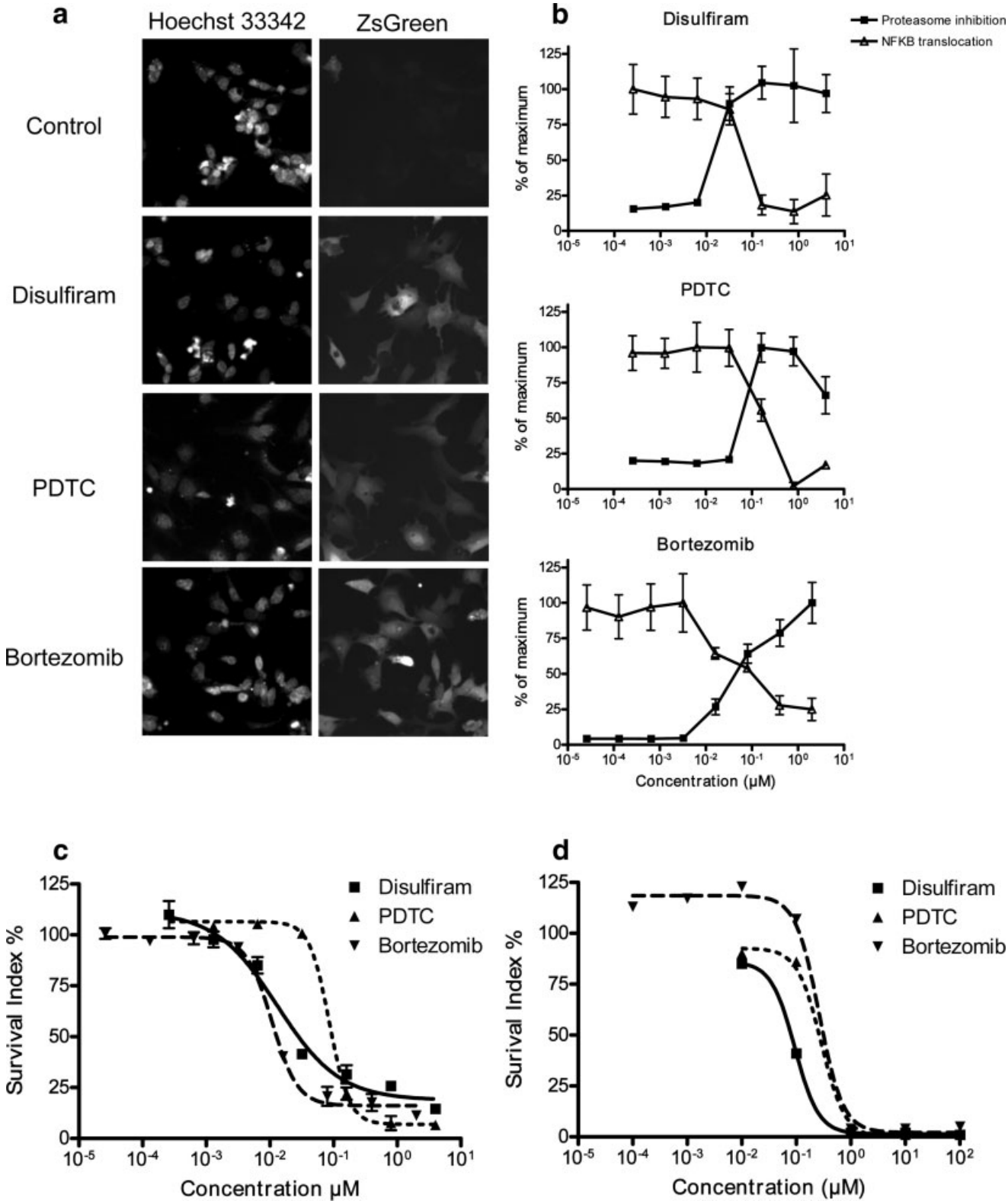

Figure 1 – (*a*) Images of HEK 293 ZsGreen cells acquired using the ArrayScan HCS reader. Cells were exposed to 0.16 μM disulfiram or PDTC or 0.016 μM bortezomib for 12 hr. Cells exposed to vehicle are included as control. High levels of accumulated ZsGreen protein could be seen for all compounds, without changes in nuclear morphology. Note the bright, similar staining pattern induced by all 3 compounds. (*b*) The inhibitory effect of disulfiram, PDTC and bortezomib was analyzed in HEK 293 ZsGreen cells exposed for 12 hr. Cellular green fluorescence was measured using the ArrayScan HCS reader and was averaged for at least 500 cells per well. Data presented as percent of maximum achievable proteasome inhibition, mean ± SEM of triplicate experiments. NF-KB translocation of cells exposed to compounds for 1 hr is plotted in the same graph. Using the ArrayScan HCS reader and immunostaining of p65, the nuclear–cytoplasm intensity difference was calculated for each cell and averaged for at least 500 cells per well. Data are presented as percent of maximum TNF-α-induced translocation, mean ± SEM of triplicate experiments. Cytotoxic activity of disulfiram, PDTC and bortezomib in HEK 293 ZsGreen cells (*c*) and RPMI8226 myeloma cell line (*d*). Cells were exposed to the compounds for 72 hr followed by analysis of cytotoxicity, using the FMCA method.

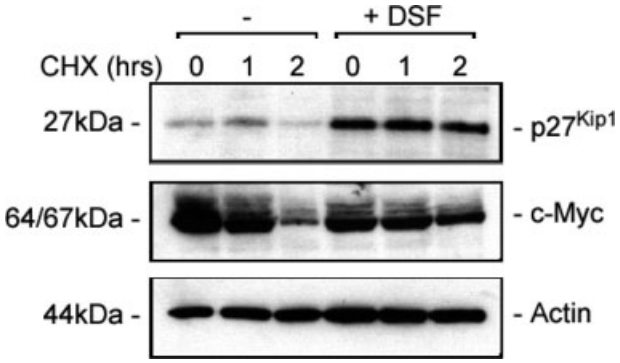

FIGURE 2 – $p27^{Kip1}$ strongly accumulated in RPMI8226 cells exposed to 4 μM disulfiram (DSF) for 4 hr. Chase analysis at 1 and 2 hr after addition of 10 μg/ml cycloheximide (CHX) showed stable levels of $p27^{Kip1}$. RPMI8226 cells exhibited high constitutive levels of c-Myc and addition of disulfiram did not induce accumulation but increased the half-life of c-Myc. Actin is included as a loading reference.

tested in a cell-free system, disulfiram and PDTC were not able to inhibit the 20S activity. Addition of Cu^{2+} or Zn^{2+} did not cause inhibition of the enzyme in the cell free system. This indicates that the thiocarbamate compounds require intracellular metabolism or that they are interfering with the regulatory subunits of the 26S proteasome. These results are in accordance with a recent study demonstrating an indirect inhibition of the ubiquitin-proteasome pathway by PDTC and Cu^{2+}/ Zn^{2+} combination.[17] The mODC-ZsGreen proteasomal substrate expressed in the HEK 293 ZsGreen cell line does not require ubiquitin to be degraded by the proteasome, excluding the interference of disulfiram and PDTC with the complex system of ubiquitin-conjugating enzymes.[12] Thus, the exact mechanism for proteasome inhibition by the dithiocarbamate compounds disulfiram and PDTC, and how it differs from that of bortezomib that is known to inhibit the 20S proteasome, needs to be further explored.

To confirm the proteasome-inhibiting properties of disulfiram and PTDC and to study targets downstream of the proteasome, we tested their effect on NF-KB translocation. A short incubation with the compounds prior to stimulation with TNF-α inhibited the nuclear translocation of p65. The relationship between proteasome inhibition and inhibition of p65 translocation indicate that this effect is mediated by reduced degradation of IKB, the inhibitory subunit of NF-KB that, upon phosphorylation, is degraded by the proteasome.[12] Disulfiram has previously been shown to inhibit drug-induced and constitutive NF-KB translocation and activity in hepatoma and colorectal cancer cell lines.[2,4] Our results also show that disulfiram and PDTC are able to induce cytotoxic effects in HEK 293 ZsGreen cells at concentrations inhibiting the proteasome and the NF-KB translocation. Furthermore, the compounds are able to induce cytotoxic activity in the myeloma cell line RPMI8226.

To confirm and to further characterize the proteasome inhibiting activity of disulfiram, we tested whether the compound was able to affect the levels of 2 proteins known to be degraded by the proteasome. Two of these proteins are the cell-cycle regulatory protein $p27^{Kip1}$ and the transcription factor c-Myc. Disulfiram induced accumulation of $p27^{Kip1}$ already after 4 hr of exposure. The accumulation of $p27^{Kip1}$ was accompanied by a stabilization of the protein, in accordance with previous studies of proteasome inhibitors.[18] As with $p27^{Kip1}$, the stability of c-Myc was increased, indicating inhibition of its proteasomal degradation. However, disulfiram did not further increase the constitutively high levels of c-Myc. This could be due to the complex regulation of its expression through other transcription factors dependent on proteasome activity.

Proteasome inhibitors has gained large interest in the clinical treatment of both hematological and solid tumors.[12] Disulfiram was shown to be more active against CLL cells compared to PBMC, indicating a potential for tumor cell selectivity. A single case where disulfiram combined with zinc gluconate induced clinical remission in a stage IV metastatic ocular melanoma has been reported.[6] Pharmacokinetic data on disulfiram are limited due to complex routes of metabolism. However, studies indicate that micromolar concentrations of disulfiram, well above the levels used in our study to inhibit the proteasome, can be achieved in man.[13] Together with the relatively mild side effects and the large clinical experience of the drug, clinical studies of disulfiram as a candidate proteasome inhibitor in a variety of hematological and solid tumors seem warranted.

References

1. Anton RF. Pharmacologic approaches to the management of alcoholism. J Clin Psychiatry 2001;62 (Suppl 20):11–7.
2. Wang W, McLeod HL, Cassidy J. Disulfiram-mediated inhibition of NF-κB activity enhances cytotoxicity of 5-fluorouracil in human colorectal cancer cell lines. Int J Cancer 2003;104:504–11.
3. Cen D, Gonzalez RI, Buckmeier JA, Kahlon RS, Tohidian NB, Meyskens FL, Jr. Disulfiram induces apoptosis in human melanoma cells: a redox-related process. Mol Cancer Ther 2002;1:197–204.
4. Liu GY, Frank N, Bartsch H, Lin JK. Induction of apoptosis by thiuramdisulfides, the reactive metabolites of dithiocarbamates, through coordinative modulation of NFκB, c-fos/c-jun, and p53 proteins. Mol Carcinog 1998;22:235–46.
5. Marikovsky M, Nevo N, Vadai E, Harris-Cerruti C. Cu/Zn superoxide dismutase plays a role in angiogenesis. Int J Cancer 2002;97:34–41.
6. Brar SS, Grigg C, Wilson KS, Holder WD, Jr, Dreau D, Austin C, Foster M, Ghio AJ, Whorton AR, Stowell GW, Whittall LB, Whittle RR, et al. Disulfiram inhibits activating transcription factor/cyclic AMP-responsive element binding protein and human melanoma growth in a metal-dependent manner in vitro, in mice and in a patient with metastatic disease. Mol Cancer Ther 2004;3:1049–60.
7. Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor κ B activation in intact cells. J Exp Med 1992;175:1181–94.
8. Murakami Y, Matsufuji S, Kameji T, Hayashi S, Igarashi K, Tamura T, Tanaka K, Ichihara A. Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature 1992;360:597–9.
9. Ding GJ, Fischer PA, Boltz RC, Schmidt JA, Colaianne JJ, Gough A, Rubin RA, Miller DK. Characterization and quantitation of NF-κB nuclear translocation induced by interleukin-1 and tumor necrosis factor-α. Development and use of a high capacity fluorescence cytometric system. J Biol Chem 1998;273:28897–905.
10. Larsson R, Nygren P. A rapid fluorometric method for semiautomated determination of cytotoxicity and cellular proliferation of human tumor cell lines in microculture. Anticancer Res 1989;9:1111–9.
11. Dimberg A, Bahram F, Karlberg I, Larsson LG, Nilsson K, Oberg F. Retinoic acid-induced cell cycle arrest of human myeloid cell lines is associated with sequential down-regulation of c-Myc and cyclin E and posttranscriptional up-regulation of p27(Kip1). Blood 2002;99:2199–206.
12. Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer 2004;4:349–60.
13. Faiman MD, Dodd DE, Minor SS, Hanzlik R. Radioactive and nonradioactive methods for the in vivo determination of disulfiram, diethyldithiocarbamate, and diethyldithiocarbamate-methyl ester. Alcohol Clin Exp Res 1978;2:366–9.
14. Brien JF, Loomis CW. Disposition and pharmacokinetics of disulfiram and calcium carbimide (calcium cyanamide). Drug Metab Rev 1983;14:113–26.
15. Kim I, Kim CH, Kim JH, Lee J, Choi JJ, Chen ZA, Lee MG, Chung KC, Hsu CY, Ahn YS. Pyrrolidine dithiocarbamate and zinc inhibit proteasome-dependent proteolysis. Exp Cell Res 2004;298:229–38.
16. Chen D, Peng F, Cui QC, Daniel KG, Orlu S, Liu J, Dou QP. Inhibition of prostate cancer cellular proteasome activity by a pyrrolidine dithiocarbamate–copper complex is associated with suppression of proliferation and induction of apoptosis. Front Biosci 2005;10:2932–39.
17. Si X, McManus BM, Zhang J, Yuan J, Cheung C, Esfandiarei M, Suarez A, Morgan A, Luo H. Pyrrolidine dithiocarbamate reduces coxsackievirus B3 replication through inhibition of the ubiquitin-proteasome pathway. J Virol 2005;79:8014–23.
18. Almond JB, Cohen GM. The proteasome: a novel target for cancer chemotherapy. Leukemia 2002;16:433–43.