International Journal of Antimicrobial Agents 54 (2019) 85–88

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Disulfiram, an alcohol dependence therapy, can inhibit the in vitro growth of *Francisella tularensis*

Karleigh A. Hamblin[a], Helen Flick-Smith[a,*], Kay B. Barnes[a], José B. Pereira-Leal[b,c], Jaroslaw Surkont[b], Richard Hampson[d], Helen S. Atkins[a,e], Sarah V. Harding[a]

[a] *CBR Division, Defence Science and Technology Laboratory, Porton Down, Salisbury, UK*
[b] *Instituto Gulbenkian de Ciência, Oeiras, Portugal*
[c] *Ophiomics – Precision Medicine, Lisbon, Portugal*
[d] *Thelial Technologies S.A., Lisbon, Portugal*
[e] *Biosciences, University of Exeter, Exeter, UK*

ARTICLE INFO

Article history:
Received 8 January 2019
Accepted 6 April 2019

Keywords:
Disulfiram
Francisella tularensis
Drug repurposing
Bioinformatics
MIC

ABSTRACT

Disulfiram (DSF) can help treat alcohol dependency by inhibiting aldehyde dehydrogenase (ALDH). Genomic analysis revealed that *Francisella tularensis*, the causative agent of tularemia, has lost all but one ALDH-like domain and that this domain retains the target of DSF. In this study, minimum inhibitory concentration (MIC) assays demonstrated that both DSF and its primary metabolite diethyldithiocarbamate (DDC) have strong antimicrobial activity against *F. tularensis* strain SCHU S4, with the MIC of DSF determined as 2 μg/mL in comparison with 8 μg/mL for DDC. The activity of DSF was further confirmed using an in vitro human macrophage infection assay. *Francisella tularensis* bacteria in DSF-treated cells were reduced in comparison with untreated and DDC-treated cells, comparable with that observed in doxycycline-treated cells. This suggests that DSF may be suitable for further investigation as an in vivo therapy for tularemia.

1. Introduction

Francisella tularensis, a Gram-negative, facultative, intracellular bacterium, is the causative agent of tularemia, a potentially fatal disease in humans. Although cases of human tularemia are relatively rare, sporadic outbreaks still occur in the USA, Asia and Scandinavia where it is transmitted primarily via arthropod vectors. It has a low infectious dose particularly via the aerosol route (approximately 10 CFU) and this has led to concerns about its potential use as a biowarfare agent. There is no licenced vaccine for tularemia and, although there are a number of effective antibiotics currently available, new approaches to finding antimicrobial agents effective as post-exposure therapies are ongoing [1]. Attempting to identify and license new antibiotics is a costly and time-consuming process, with conservative estimates of ≥10 years and US$800 million [2]. Furthermore, considering current pricing policies for antibiotics, there is little incentive for pharmaceutical companies to develop new therapies for diseases such as tularemia where natural occurrence is relatively low (approximately 200 cases per year in the USA [3]). Repurposing licensed drugs provides significant advantages over de novo discovery as the pharmacology and toxicology of these drugs is well established, reducing the cost and effort required for drug development. Well-known examples of repurposing include teicoplanin (from Gram-positive bacterial infections to Ebola virus therapy) and azithromycin (from bacterial infections to Zika virus therapy). There has been a considerable effort to review 'old' antibiotics to determine whether modern advances in medicine can enable these drugs to be used safely [4], but perhaps other licensed therapies also have an unknown antimicrobial effect and could be used to treat tularemia.

One drug that has received some attention for potential antimicrobial use is disulfiram (DSF), which was discovered accidently in the 1940s [5]. DSF (tetraethylthiuram disulfide) given via the oral route has been used for many years in the management of alcohol dependence. DSF and its metabolites [e.g. diethyldithiocarbamate (DCC)] blocks the metabolism of alcohol by inhibiting aldehyde dehydrogenase (ALDH) and therefore results in extreme 'hangover' symptoms when consumed with alcohol, hence its use as an anti-alcoholism drug. There are some restricted claims in the literature

* Corresponding author. Tel.: +44 1980 951688.
E-mail address: hcfsmith@dstl.gov.uk (H. Flick-Smith).

https://doi.org/10.1016/j.ijantimicag.2019.04.002

of the antimicrobial action of DSF and its metabolites against certain bacteria, specifically in vitro against *Staphylococcus aureus* [6,7] and in vitro and in vivo against *Mycobacterium tuberculosis* [8,9].

Intracellular parasites are well known to suffer extensive gene loss [10–12]. One of the authors (JBPL) has previously formulated the hypothesis that in addition to the loss of whole pathways and biosynthetic capabilities, intracellular parasites lose genetic redundancy, i.e. the ability to perform the same function using multiple related versions of the same gene, be they paralogues or xenologues [13]. In that work, it was noted that *F. tularensis* had a specific case of predicted loss of genetic redundancy that could have pharmacological implications. i.e. the loss of paralogues of the ALDH-like family. In humans, this protein is the substrate and mediator of the toxicity of DSF.

Hence, in the current study, we tested whether DSF, and its metabolite DCC, is an antimicrobial with anti-tularemia activity.

2. Materials and methods

2.1. Bioinformatics

The SUPERFAMILY definition of domain ALDH-like superfamily was used (accession no. **SSF53720**), based on release 1.75 [14]. All of the proteins for the species described above that contained at least one instance of the ALDH-like superfamily domain were extracted. The total number of genes for each species was extracted from GenBank, and the taxonomic tree from NCBI taxonomy [15]. Protein sequences were aligned using MAFFT v.7.305 [16] with default parameters. Mapping of secondary structure elements was based on the crystal structure of human ALDH2 (PDB: 5L13) [16].

2.2. Reagents

All reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. Before use, DSF was dissolved in dimethyl sulfoxide (DMSO), whereas its metabolite DDC and doxycycline were dissolved in sterile distilled water prior to dilution in modified cysteine partial hydrolysate (MCPH) broth or Leibovitz (L)-15 medium (Gibco, Loughborough, UK).

2.3. Bacterial strains and culture

Francisella tularensis subsp. *tularensis* strain SCHU S4 and *F. tularensis* subsp. *holarctica* strain HN63 were obtained from the US Army Medical Research Institute of Infectious Diseases (Frederick, MD). Both strains were cultured on blood–cysteine–glucose agar (BCGA) and in MCPH broth [17]. Bacterial numbers were determined by enumeration of serially diluted bacteria on agar plates. All experiments with *F. tularensis* were carried out in a Class III microbiological safety cabinet complying with British Standard 5726. *Staphylococcus aureus* strain ATCC 29213, grown on Luria–Bertani agar, was used for quality control.

2.4. Minimum inhibitory concentration (MIC) assays

MICs for DSF and DDC were determined by the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [18], using MCPH broth as this better supported *F. tularensis* growth (data not shown). Owing to the low solubility of DSF in water, the highest concentration that could be used in MIC assays was 32 μg/mL with 5% DMSO. Plates were incubated at 37°C for 24 h and were read using a Multiskan® EX plate reader (Thermo Fisher Scientific) at 620 nm. The MIC was the lowest concentration of antibiotic resulting in <10% growth compared with MCPH broth containing no antibiotic. Doxycycline was used as a control, with the *S. aureus* MICs for doxycycline within the acceptable quality control limits.

2.5. In vitro cell infection assay

The non-adherent human monocytic cell line THP-1 [European Collection of Authenticated Cell Cultures (ECACC), Public Health England, Porton Down, UK] was grown in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO_2. Prior to infection, THP-1 cells were centrifuged ($300 \times g$; 15 min) and were resuspended in RPMI 1640 medium, counted and re-adjusted to the required cell concentration of 1×10^6 cells/mL. Cells were activated with phorbol 12-myristate 13-acetate (PMA) at a final concentration of 100 ng/mL, were plated into 24-well plates at 1 mL/well and were incubated overnight to produce an adherent monolayer.

A loop of *F. tularensis* SCHU S4, grown overnight on BCGA plates, was suspended in L-15 medium to give an optical density at 590 nm (OD_{590}) of 0.2 (approximately 1×10^9 CFU/mL) and then 10-fold dilutions were made in L-15 medium to prepare the inoculum. RPMI medium was removed from the THP-1 cells and was replaced with 1 mL of approximately 1×10^6 CFU/mL *F. tularensis* SCHU S4 to give a multiplicity of infection (MOI) of 1. Following incubation for 45 min at 37°C, bacteria were removed, the cells were washed with 1 mL of sterile phosphate-buffered saline (PBS) and then 1 mL of antibiotic or L-15 medium was added per well. DSF was tested at 8 μg/mL (27 μM) and 4 μg/mL (13.5 μM), and DDC was tested at 32 μg/mL (144 μM) and 16 μg/mL (72 μM) (equating to $4 \times$ and $2 \times$ MIC, respectively) in comparison with doxycycline added at 1 μg/mL (2.25 μM, equating to $4 \times$ MIC). Cells were incubated overnight at 37°C (except for the 0 h time point) and were then lysed by addition of 1 mL of distilled water for 5 min. Ten-fold dilutions of the lysed cell suspensions were made in PBS and then 100 μL of lysate was plated onto BCGA plates in triplicate. Following overnight incubation, the antibiotic/control-treated cells were lysed with water and dilutions were plated onto BCGA plates. Plates were incubated at 37°C for 5 days and the bacteria were enumerated. All treatments were conducted in triplicate within assays, and assays were conducted on three separate occasions, except for doxycycline which was evaluated in the second and third assays only.

2.6. Statistical analysis

Graphs were constructed and statistical analysis was performed using GraphPad Prism v.6.0 (GraphPad Software Inc., La Jolla, CA). Intracellular assays were analysed using one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparison. Differences with a P-value of <0.05 were considered statistically significant.

3. Results

3.1. Bioinformatics

To confirm the hypothesis that there is a loss of copies in the ALDH-like domain-containing proteins in the genome of *F. tularensis* relative to related species, a list of representative species was compiled and the total number of proteins containing that domain as well as the complete number of protein-coding genes in that genome was calculated. *Francisella tularensis* has a single protein containing the ALDH-like domain, whereas all other genomes contain multiple proteins with the domain (Fig. 1A). The observation that, in a genome that suffers extensive gene loss, one copy of this domain is retained argues for its importance for the organism,

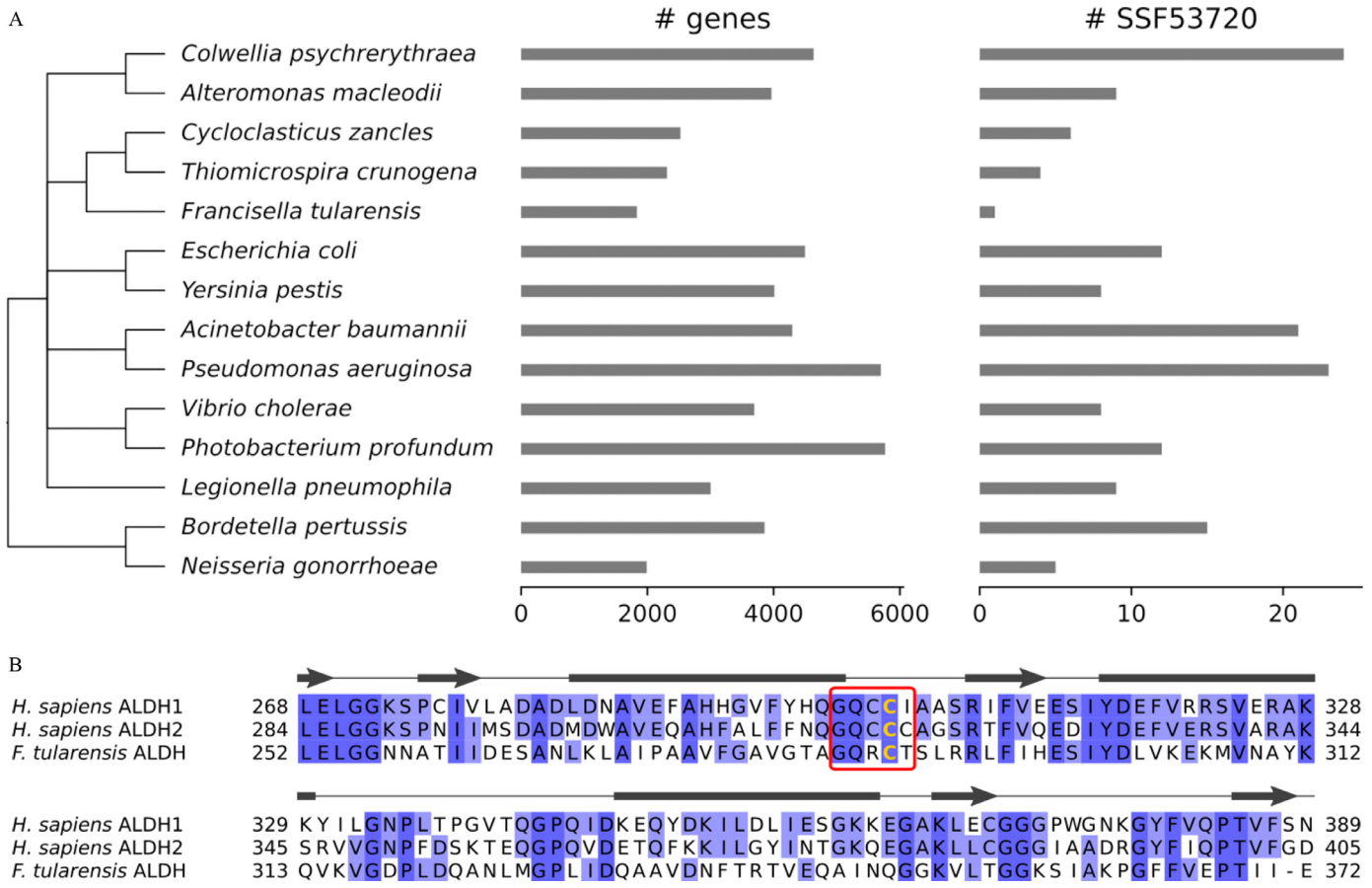

Fig. 1. (A) Plots of the total number of proteins (centre) and proteins containing the aldehyde dehydrogenase (ALDH)-like domain (#SSF3720, right) in bacteria phylogenetically close to *Francisella tularensis*, and ordered according to their taxonomic relationship (left). (B) Multiple sequence alignment of human ALDH1 and ALDH2 as well as ALDH from *F. tularensis*. Conserved positions are highlighted with a dark background. The active site is shown in the red frame with the catalytic cysteine in yellow. Secondary structure elements are denoted by bars (α-helices) and arrows (β-sheets). The catalytic cysteine is conserved in all species of *Francisella* found in GenBank (see Supplementary Fig. S1).

further suggesting that targeting this function can be deleterious for its survival [13].

Next we investigated whether the remaining copy of the domain could be a substrate for DSF binding. The action of DSF on human mitochondrial ALDH is mediated by a characteristic sequence FNQGQC(301)C(302)C(303), derived from the enzyme active site region, with modifications at Cys(302) [19]. The *F. tularensis* ALDH-like protein (Fig. 1B) has conserved the target cysteine, suggesting that DSF will be able to bind to inhibit the function of ALDH-like protein in *F. tularensis*.

3.2. Minimum inhibitory concentration assays

DSF had antimicrobial activity against the two strains of *F. tularensis* tested in the study, with MICs of 2 μg/mL and 0.5 μg/mL for strains SCHU S4 and HN63, respectively. Similar MICs were obtained with doxycycline (1 μg/mL and 0.25 μg/mL for strains SCHU S4 and HN63, respectively). The DSF metabolite DDC also had antimicrobial activity, with MICs of 8 μg/mL and 2 μg/mL for strains SCHU S4 and HN63, respectively.

3.3. In vitro cell infection assay

Prior to starting the cell infection assays, it was necessary to identify a suitable macrophage cell line as these drugs have been reported to be cytotoxic to some cell lines [20]. A cytotoxic effect of DSF and DDC was observed on three murine cell lines tested (J774A.1, RAW264.7 and MH-S). Specifically, cells were observed to be detached from the plate surface following overnight incubation in the presence of the drugs and, when tested for viability by trypan blue exclusion, showed a higher proportion of dead cells in comparison with the untreated control cells (data not shown). In comparison, human-derived THP-1 cells did not show any visible cytotoxic effects following overnight incubation with the drugs and were therefore selected for use in the cell infection assay.

A significant reduction ($P \leq 0.05$) in the number of intracellular bacteria present in THP-1 cells was seen in all treatment groups compared with the untreated control group, except for the 2 × MIC DSF-treated group. Treatment with 4 × MIC DDC was the most effective treatment ($P \leq 0.01$) and reduced the level of bacteria comparable with that seen in doxycycline-treated cells (Fig. 2).

4. Discussion

Evolutionary reasoning and comparative genomics suggested that *F. tularensis* would be susceptible to the antimicrobial action of DSF, an anti-alcoholism drug. To test this hypothesis, the in vitro activity of DSF was evaluated using MIC and intracellular assays. MIC assays indicated that the in vitro activity of DSF against *F. tularensis* is comparable with doxycycline, a recommended prophylactic antibiotic for *F. tularensis* exposure and used to treat tularemia. *Francisella tularensis* HN63 strain was more susceptible to DSF and doxycycline than SCHU S4 strain. The reasons for this are unclear as this key cysteine region is fully conserved in

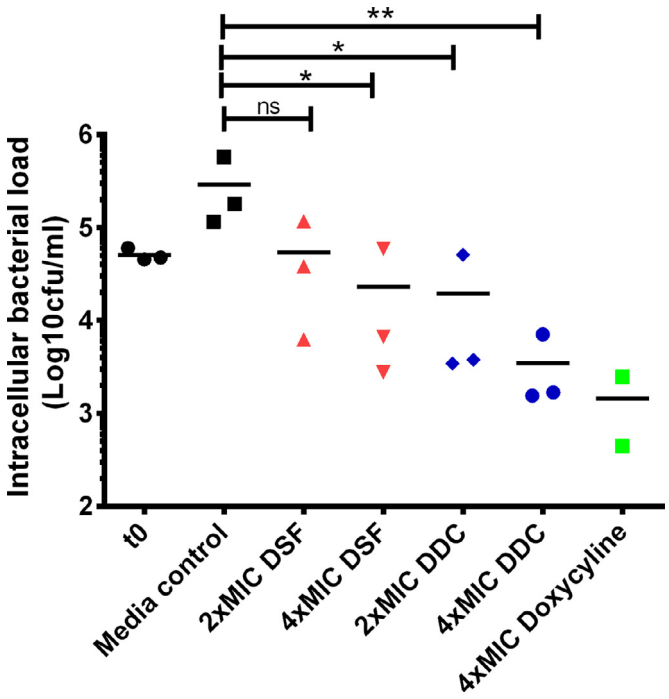

Fig. 2. Antibacterial activity of disulfiram (DSF) and its primary metabolite diethyldithiocarbamate (DDC) on the intracellular growth of *Francisella tularensis*. THP-1 cells were infected with *F. tularensis* at a multiplicity of infection (MOI) of 1 and were incubated with DSF or DDC at 2 × or 4 × MIC for 24 h. Intracellular growth within untreated THP-1 cells is shown from time zero ($t = 0$) to 24 h ($t = 24$) (medium control). Doxycycline at 4 × MIC was used as a control. Asterisks indicate significant differences: * $P \leq 0.05$; ** $P \leq 0.01$. ns, no significant difference ($P > 0.05$). MIC, minimum inhibitory concentration.

all *F. tularensis* strains available in GenBank. Intracellular assays demonstrated that DSF and its metabolite DDC could decrease the bacterial load within *F. tularensis*-infected THP-1 cells following overnight incubation in comparison with untreated control cells. The relatively low *F. tularensis* MICs and the intracellular activity demonstrated in this study suggest that DSF should be further investigated as a putative tularemia therapy.

Acknowledgments

The authors thank Claas Junghans and Adam Levy for their input.

Funding

This work was funded by the UK Ministry of Defence (United Kingdom) and the Instituto Gulbenkian de Ciência (Oeiras, Portugal).

Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.04.002.

References

[1] Jones CL, Napier BA, Sampson TR, Llewellyn AC, Schroeder MR, Weiss DS. Subversion of host recognition and defense systems by *Francisella* spp. Microbiol Mol Biol Rev 2012;76:383–404.
[2] Conly J, Johnston B. Where are all the new antibiotics? The new antibiotic paradox. Can J Infect Dis Med Microbiol 2005;16:159–60.
[3] Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Tularemia as a biological weapon: medical and public health management. JAMA 2001;285:2763–73.
[4] Falagas ME, Grammatikos AP, Michalopoulos A. Potential of old-generation antibiotics to address current need for new antibiotics. Expert Rev Anti Infect Ther 2008;6:593–600.
[5] Pal A, Pattanayak RD, Sagar R. Tracing the journey of disulfiram: from an unintended discovery to a treatment option for alcoholism. J Mental Health Human Behav 2015;20:41–3.
[6] Phillips M, Malloy G, Nedunchezian D, Lukrec A, Howard RG. Disulfiram inhibits the in vitro growth of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 1991;35:785–7.
[7] Long TE. Repurposing thiram and disulfiram as antibacterial agents for multidrug-resistant *Staphylococcus aureus* infections. Antimicrob Agents Chemother 2017;61 e00898-17.
[8] Byrne ST, Gu P, Zhou J, Denkin SM, Chong C, Sullivan D, et al. Pyrrolidine dithiocarbamate and diethyldithiocarbamate are active against growing and nongrowing persister *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2007;51:4495–7.
[9] Horita Y, Takii T, Yagi T, Ogawa K, Fujiwara N, Inagaki E, et al. Antitubercular activity of disulfiram, an antialcoholism drug, against multidrug- and extensively drug-resistant *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother 2012;56:4140–5.
[10] Georgiades K. Genomics of epidemic pathogens. Clin Microbiol Infect 2012;18:213–17.
[11] Wernegreen JJ. Genome evolution in bacterial endosymbionts of insects. Nat Rev Genet 2002;3:850–61.
[12] Merhej V, Royer-Carenzi M, Pontarotti P, Raoult D. Massive comparative genomic analysis reveals convergent evolution of specialized bacteria. Biol Direct 2009;4:13.
[13] Mendonça AG, Alves RJ, Pereira-Leal JB. Loss of genetic redundancy in reductive genome evolution. PLoS Comput Biol 2011;7:e1001082.
[14] Wilson D, Pethica R, Zhou Y, Talbot C, Vogel C, Madera M, et al. SUPERFAMILY—sophisticated comparative genomics, data mining, visualization and phylogeny. Nucleic Acids Res 2008;37:D380–6.
[15] Sayers EW, Barrett T, Benson DA, Bolton E, Bryant SH, Canese K, et al. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 2012;40:D13–25.
[16] Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 2013;30:772–80.
[17] Hamblin KA, Armstrong SJ, Barnes KB, Davies C, Wong JP, Blanchard JD, et al. Liposome-encapsulation of ciprofloxacin improves protection against highly virulent *Francisella tularensis* Schu S4 strain. Antimicrob Agents Chemother 2014;58:3053–9.
[18] Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—tenth edition, Wayne, PA: CLSI; 2012. Document M07-A10.
[19] Lipsky JJ, Shen ML, Naylor S. In vivo inhibition of aldehyde dehydrogenase by disulfiram. Chem Biol Interact 2001;130:93–102.
[20] Kanno S, Matsukawa E, Miura A, Shouji A, Asou K, Ishikawa M. Diethyldithiocarbamate-induced cytotoxicity and apoptosis in leukemia cell lines. Biol Pharm Bull 2003;26:964–8.