Biochemical Pharmacology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Disulfiram-induced cytotoxicity and endo-lysosomal sequestration of zinc in breast cancer cells

Helen L. Wiggins, Jennifer M. Wymant, Francesca Solfa, Stephen E. Hiscox, Kathryn M. Taylor, Andrew D. Westwell, Arwyn T. Jones *

Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, Cardiff, Wales CF10 3NB, UK

ARTICLE INFO

Article history:
Received 31 October 2014
Accepted 23 December 2014
Available online xxx

Keywords:
Breast cancer
Disulfiram
Lysosomes
Zinc
Fluozin-3

ABSTRACT

Disulfiram, a clinically used alcohol-deterrent has gained prominence as a potential anti-cancer agent due to its impact on copper-dependent processes. Few studies have investigated zinc effects on disulfiram action, despite it having high affinity for this metal. Here we studied the cytotoxic effects of disulfiram in breast cancer cells, and its relationship with both intra and extracellular zinc. MCF-7 and BT474 cancer cell lines gave a striking time-dependent biphasic cytotoxic response between 0.01 and 10 μM disulfiram. Co-incubation of disulfiram with low-level zinc removed this effect, suggesting that availability of extracellular zinc significantly influences disulfiram efficacy. Live-cell confocal microscopy using fluorescent endocytic probes and the zinc dye Fluozin-3 revealed that disulfiram selectively and rapidly increased zinc levels in endo-lysosomes. Disulfiram also caused spatial disorganization of late endosomes and lysosomes, suggesting they are novel targets for this drug. This relationship between disulfiram toxicity and ionophore activity was consolidated via synthesis of a new disulfiram analog and overall we demonstrate a novel mechanism of disulfiram-cytotoxicity with significant clinical implications for future use as a cancer therapeutic.

1. Introduction

Many current cancer therapies are limited by the severity and frequency of adverse side effects and there is high demand for non-toxic alternatives. One source of new therapies may be through repurposing of clinically approved drugs, where safety in patients has already been demonstrated. Disulfiram has a long medical history as an alcohol deterrent, however more recently has demonstrated anti-cancer effects in a range of solid and hematological malignancies [1]. The biological activity of disulfiram is attributed to its ability to bind divalent cations and consequently disrupt metal dependent processes, particularly those involving copper and zinc [2,3]. Observations that both these metal ions are involved in oncogenic development have led to increased interest in the anti-cancer potential of this drug [4]. As part of a copper complex, disulfiram has been reported to induce apoptosis in both cultured breast cancer cells and xenografts through proteasomal inhibition [5–7]. These complexes have also been shown to stabilize the NFκB inhibitor protein, IκB, thus re-sensitizing gemcitabine resistant tumors with enhanced NFκB signaling [8]. In a case study of a patient with stage IV ocular melanoma with liver metastases, combination therapy involving disulfiram and zinc gluconate was able to induce remission with almost no side effects [9]. These observations have led to its introduction to clinical trials, including one involving patients with hepatic malignancies treated with disulfiram and copper gluconate (NCT00742911, University of Utah). Additionally, disulfiram treatment has been reported to remove essential copper and zinc ions from enzymes that regulate extracellular matrix degradation and oxygen metabolism resulting in suppression of cancer invasion and angiogenesis in vitro and in vivo [2,3].

Much of the current literature surrounding disulfiram focuses on its capacity to bind copper ions, via two metal binding regions in its structure (Fig. 1A). Relatively little has been done to determine the role of zinc in its anti-cancer properties despite the fact that it also has high affinity for this metal [3]. Studies have highlighted the role of zinc in the etiology of breast cancer where high expression of zinc transporter proteins such as ZIP7 and ZIP10, in breast cancer cell models increases intracellular zinc levels and is associated with endocrine therapy resistance and increased invasiveness [10,11]. Additionally, zinc has been reported to increase pro-survival signaling [12] and inhibit caspases [13] in

* Corresponding author.
E-mail address: jonesat@cardiff.ac.uk (A.T. Jones).

http://dx.doi.org/10.1016/j.bcp.2014.12.014

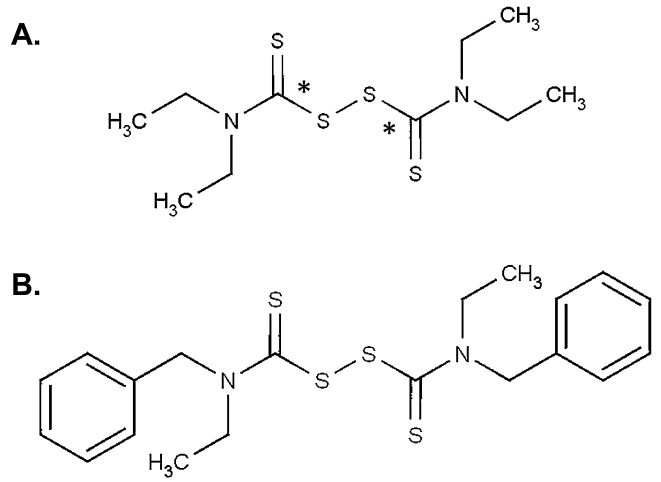

Fig. 1. Structure of disulfiram and the disulfiram analog FS03EB. (A) *Indicates metal binding regions within the structure. (B) ^{1}H NMR (500 MHz, $CDCl_3$) d 1.30 (3H, bs, CH_3), 1.47 (3H, s, CH_3), 4.05 (4H, bs, CH_2CH_3), 5.26 (2H, s, CH_2Ph), 5.41 (2H, s, CH_2Ph), 7.39 (10H, m, ArH); ^{13}C NMR (125 MHz, $CDCl_3$) d 11.12 (CH_3), 13.20 (CH_3), 47.18 (CH_2), 52.04 (CH_2), 55.80 (CH_2), 59.54 (CH_2), 127.48 (ArCH), 127.72 (ArCH), 128.20 (ArCH), 128.49 (ArCH), 128.79 (ArCH), 128.99 (ArCH), 134.57 (ArC), 135.24 (ArC), 198.82 (C=S), 195.33 (C=S); MS (EI^+) *m/z* 420.08 (M^+); HR-MS (ESI^+) *m/z* $[M+H]^+$ calculated 421.0895, found 421.0896.

vitro. Taken together these reports suggest that high zinc levels promote cancer cell survival. Paradoxically, high intracellular zinc is also associated with oxidative toxicity, implying that the cell maintains tight homeostatic control of this metal and that drugs which dysregulate this fine balance may induce toxicity [14]. As the concentration of zinc is higher in cancerous compared to non-cancerous breast tissue [15] it is possible that drugs which alter intracellular zinc levels would be selectively toxic to cancer cells.

In this study we investigate the role of both intra and extracellular zinc in the anti-cancer activity of disulfiram. We demonstrate the effect of zinc and copper on the cytotoxicity of the drug across a panel of cancerous and non-cancerous breast cell lines. We describe a novel mechanism of action for disulfiram, via its ability to rapidly increase intracellular zinc levels in endo-lysosomal compartments and alter the subcellular localization specifically of late lysosomal structures. Both these effects potentially impact on lysosome function. Interestingly, zinc levels in a non-cancerous breast cell line remain unaltered by disulfiram treatment and taken in the context of the literature surrounding zinc dysregulation in breast cancer, our results demonstrate a selective effect of disulfiram that may have significant clinical implications for its future clinical use.

2. Materials and methods

2.1. Chemicals and reagents

Disulfiram, diethyldithiocarbamate (DDC), sodium pyrithione, DMSO, Na-HEPES, NH_4Cl, Triton X-100, BSA, $ZnCl_2$, $CuCl_2$, cholera toxin, insulin, epidermal growth factor, and hydrocortisone were obtained from Sigma–Aldrich (Dorset, UK). Disulfiram, DDC, and sodium pyrithione were dissolved in DMSO to produce a stock concentration of 10 mM and stored at −20 °C. CellTiter blue viability reagent was purchased from Promega (Southampton, UK). Anti-EEA1 (#6104490), anti-LAMP2 (#H4B4), and anti-LC3B (#2775) antibodies were obtained from BD Bioscience (Oxford, UK), Developmental Studies Hybridoma Bank (Iowa, USA) and Cell Signaling Technology (MA, USA) respectively. RPMI, FBS, DMEM/F12, horse serum, Fluozin-3, Hoechst 33342, dextran-Alexa 647 (10 kDa), Alexa-488 (A-11001) and Alexa-546 (A-11010) conjugated anti-mouse and anti-rabbit antibodies were from Life Technologies (Paisley, UK).

2.2. Synthesis of disulfiram analog

FS03EB (bis(N-benzylethylthiocarbamoyl)disulphide; Fig. 1B) was synthesized according to the method of Liang et al. [16]. Briefly, N-benzylethylamine and carbon disulphide (2:1 molar ratio) were mixed together in the presence of carbon tetrabromide (one equivalent) in dimethylformamide as solvent at room temperature. Following purification by column chromatography, the identity and purity of the product was confirmed using NMR spectroscopy and mass spectrometry [17]. FS03EB was then dissolved in DMSO to produce a stock concentration of 10 mM.

2.3. Cell culture

MDA-MB-231, MCF-7, T47D, and BT474 were maintained in RPMI 1640 supplemented with 10% FBS. MCF-10A cells were maintained in DMEM/F12 supplemented with 5% horse serum, 100 ng/ml cholera toxin, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, and 500 ng/ml hydrocortisone [18]. Herein these are respectively termed complete media. All cell lines were obtained from ATCC and routinely tested for mycoplasma infection.

2.4. Viability assays

To account for different growth rates, cells were seeded in black 96-well plates at densities that provided 70% confluency after 72 h. After a minimum of 24 h, cells were treated with disulfiram, disulfiram metabolite DDC, FS03EB or DMSO ± copper or zinc supplements for the indicated time points. Viability studies were conducted using the CellTiter Blue assay according to manufacturer's protocol. All viability studies were conducted in complete media.

2.5. Live cell imaging of intracellular zinc

Microscopy analysis was conducted on a Leica SP5 confocal inverted microscope equipped with a 488 nm laser and 40× objective using Leica LAS AF software. For this, cells were preloaded with 5 μM Fluozin-3 diluted in cell imaging media (phenol red free RPMI media supplemented with 10% FBS and 50 mM Na-HEPES pH 7.4) for 30 min, before being washed thrice with PBS which was then replaced with 1 ml cell imaging media. In live cells representative region of interest was captured before and subsequent to addition of a 1 ml solution of 10 μM disulfiram, sodium pyrithione (positive control) or diluent control. Images are displayed as a multiple projection of 10 *z*-planes through the cells.

2.6. Flow cytometry

Cells were preloaded with Fluozin-3 for 30 min as above and treated with disulfiram, DDC, FS03EB, sodium pyrithione or diluent control in cell imaging media or serum free imaging media (phenol red free RPMI supplemented with 50 mM Na-HEPES pH 7.4) ±20 μM zinc or copper for 10 min. Following trypsinization, cells were resuspended in PBS, and centrifuged three times at 150 × *g*. Cells were then resuspended in media, and 10,000 events were analyzed via flow cytometry using a BD Biosciences FACSVerse system equipped with a 488 nm laser.

2.7. Comparative localization of intracellular zinc with endocytic probes in disulfiram treated cells

To label the entire fluid-phase endocytic network, MCF-7 cells were incubated for 4 h with 2.5 mg/ml dextran-Alexa 647 diluted