Diethyldithiocarbamate complex with copper: the mechanism of action in cancer cells

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Abstract: The idea of "repurposing" of existing drugs provides an effective way to develop and identify new therapies. Disulfiram (Antabuse), a drug commonly used for the treatment of alcoholism, shows promising anticancer activity in both preclinical and clinical studies. In the human body, disulfiram is rapidly converted to its reduced metabolite, diethyldithiocarbamate. If copper ions are available, a bis(diethyldithiocarbamate)-copper(II) complex is formed. Disulfiram´s selective anticancer activity is attributed to the copper(II) complex´s ability to inhibit the cellular proteasome. It is assumed that the complex inhibits the proteasome by a mechanism that is distinct to the clinically used drug bortezomib, targeting the 19S rather than the 20S proteasome. This difference could be explained by inhibition of the JAMM domain of the POH1 subunit within the lid of the 19S proteasome.

Keywords: Breast cancer, Copper, Disulfiram, JAMM domain, Proteasome.

INTRODUCTION

Disulfiram (Antabuse, tetraethylthiuram disulfide) has been used worldwide for alcohol aversion therapy since the 1950s [1], although the reason for its efficacy, i.e., aldehyde dehydrogenase inhibition, was not fully understood until many years later [2]. Dr. E.F. Lewison discovered another clinically important application of disulfiram in 1977 [3]. He observed complete remission of a metastatic breast cancer tumor in a severely alcoholic patient taking disulfiram. The anticancer activity of disulfiram has been confirmed in further studies; it was shown to suppress the progression of chemically induced cancers of the forestomach, large intestine [4] and urinary bladder [5, 6] in rats. Additionally, in a phase II placebo-controlled clinical trial, the main metabolite of disulfiram, diethyldithiocarbamate (EtDTC), known as ditiocarb, was successfully used as an adjuvant therapy for non-metastatic, high-risk breast cancer [7]. There are many possible explanations for disulfiram´s effectiveness as a cancer therapeutic [8]. First, disulfiram, and consequently EtDTC, are potent inhibitors of nuclear-factor κB (NF-κB), a key pro-survival factor in cancer cells [9, 10]. In fact, disulfiram-mediated inhibition of NF-κB led to increased cytotoxicity of 5-fluorouracil in colorectal cancer cell lines [11]. Second, disulfiram can overcome multidrug resistance by directly targeting P-glycoprotein and its maturation [12, 13]. Moreover, disulfiram is able to attenuate key phenomena that are closely related to tumor progression, including both angiogenesis and cell invasion [14, 15].

Interestingly, recent studies [16, 17] have shown that the anticancer activity of disulfiram increases considerably in the presence of copper or zinc ions *in vitro* as well as *in vivo* [18]. The simultaneous oral administration of zinc gluconate and disulfiram induced significant reduction in hepatic metastasis and clinical remission in patient with metastatic ocular melanoma [18]. Likewise, the addition of cupric ions potentiated a chemosensitizing effect of disulfiram on conventional chemotherapeutic agents in breast, colon [19] and leukemia cancer cell lines [20]. The potent antitumor activity is attributed to the complex [17, 21] formed from the reaction between disulfiram (or EtDTC) and bivalent copper or zinc [17, 21]. However, the exact mechanism of action of the complex that is involved in its toxicity against cancer cells is unknown. Many published studies aiming to elucidate the mechanism of action of the complex rely on various mixtures of EtDTC or pyrrolidine dithiocarbamate (PyDTC) and metal ions [22-27]. The use of mixtures with undefined stoichiometry instead of synthetic and thoroughly characterized complexes provides an extra challange and makes difficult the assessment of results.

COMPLEX FORMATION IN THE HUMAN BODY

Following ingestion, disulfiram is rapidly converted to its reduced metabolite EtDTC in the acidic environment of the stomach or in the bloodstream by the glutathione reductase system of erythrocytes [28]. It is well established that EtDTC is able to form coordination complexes with various transitions metal ions, including copper(II) and zinc(II) [29]. If cupric ions are available, the bis(diethyldithiocarbamate)-copper complex ($Cu(EtDTC)_2$) is formed in the human body (Fig. **1**) [28]. Moreover, EtDTC is able to sequester copper ions from the active site of superoxide dismutase, forming the $Cu(EtDTC)_2$ complex [30, 31]. Similarly, disulfiram added to fresh blood and plasma reacts with cupric ions bound to plasma proteins to form the $Cu(EtDTC)_2$ complex. The *in vivo* formation of the complex in patients taking

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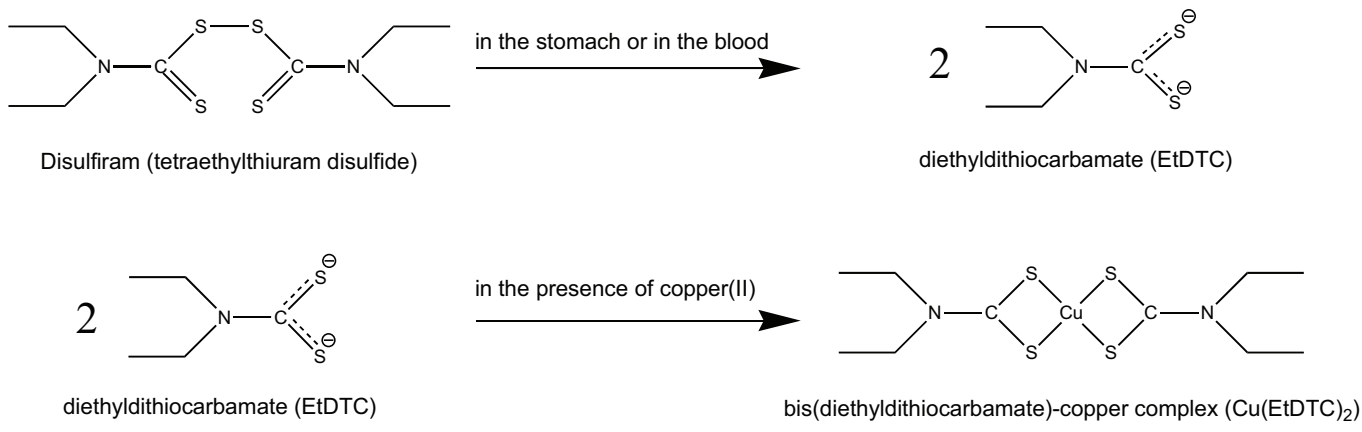

Fig. (1). Formation of the disulfiram-copper complex in the human body.

disulfiram has also been observed. Low concentrations of the complex in the plasma obtained from patients treated with disulfiram may be due to the lipophilic character of the complex resulting in its distribution through the cell membrane or its decomposition into tissue [32]. Because $Cu(EtDTC)_2$ is a lipophilic and stable compound, it is the most absorbed form of disulfiram [33]. In addition, 30 min after intraperitoneal injection of EtDTC (500 mg/kg), EtDTC was shown to chelate copper ions *in vivo*, form the $Cu(EtDTC)_2$ complex and permeate the blood-brain barrier in rats [34]. Consequently, administration of EtDTC or disulfiram in animals resulted in copper accumulation in several tissues, including the nervous system [35-37]. In addition, disulfiram markedly increased the *in vitro* intracellular copper content in primary astrocytes [23] and melanoma cells, which thereby induced apoptosis in the melanoma cells but not in normal melanocytes [16]. It is suggested that $Cu(EtDTC)_2$ selective toxicity to cancer cells is caused by its ability to inhibit the cellular proteasome [17, 21]. Nevertheless, further experiments using synthetic $Cu(EtDTC)_2$ rather than mixtures of disulfiram or EtDTC and metals are needed to uncover the exact mechanism involved in its antineoplastic and apoptosis-inducing effect.

BIOLOGICAL EFFECTS OF A MIXTURE OF DIETHYLDITHIOCARBAMATE AND COPPER(II)

Although it is more suitable to utilize a discrete and thoroughly characterized substance [38], mixtures of ligand and metal ions have been employed in several studies [23-27]. This practice leads to difficulty in determining what causes the observed results (the complex, the ligand or the metal and products of their reactions with other chemicals in the medium), especially when nonstoichiometric amounts that deviate from the optimal (i.e., 2:1 ligand to copper) molar ratio are used [23, 24, 27]. Importantly, both EtDTC (or disulfiram) and copper ions are highly reactive compounds with pleiotropic effects on cell metabolism and survival [39, 40]. In accordance with a disruption in the redox balance, which has been observed after applying a EtDTC-Cu(II) mixture [22, 23] or a PyDTC-Cu(II) mixture [24], both dithiocarbamates and the metal alone exhibit strong oxidative activity [41, 42]. Furthermore, it is well known that EtDTC and disulfiram are potent inhibitors of the essential antioxidant enzyme, CuZn superoxide dismutase (SOD-1) [30, 31, 43-45], which markedly increases the level of reactive oxygen species (ROS), namely, superoxide radical ($O_2^{-\bullet}$), in the cell [46, 47]. An elevation of the cellular superoxide concentration induced by EtDTC has been reported to be responsible for stimulating pro-apoptotic bax mRNA expression, causing subsequent apoptosis in rat cardiac myocytes [48] and triggering apoptosis in vascular smooth muscle cells [49]. Consequently, SOD inhibition caused free radical-mediated damage to mitochondrial membranes, the release of cytochrome c and apoptosis of cancer cells; thus, utilizing SOD as a target for the selective killing of cancer cells has already been proposed [45, 50].

Another important guard of cell redox homeostasis, with which EtDTC interacts, is the glutathione system. As the most abundant non-protein thiol, glutathione (GSH) has important roles in redox regulation and apoptosis [51]. Under oxidizing conditions, GSH is oxidized to its disulphide form, GSSH, and the GSH/GSSH ratio decreases. GSSH can be reduced back to GSH by glutathione reductase (GR) [52]. According to published studies, both the EtDTC-Cu(II) and PyDTC-Cu(II) mixtures depleted GSH content, thereby increasing free radical production in primary astrocytes and HL-60 cells [23, 24]. Importantly, the studies report that dithiocarbamates *per se* have similar effects. Disulfiram was found to rapidly decrease GSH levels *in vitro* [53], and disulfiram administration in rat brains caused GSH decline with a concomitant increase in GSSH levels [54, 55], formation of disulfiram-glutathione conjugates [56] and inhibition of GR activity [54, 55]. PyDTC has shown an analogous impact on GSH content *in vivo* [57]. In contrast, another study [58] showed that a high dose of disulfiram (200 μM) significantly increases GR activity in a cell-free system, and despite their pro-oxidative properties, dithiocarbamates have long been used as antioxidants [59-61]. These evident discrepancies on the impact of disulfiram on GR activity are most likely caused by the distinct conditions of each experiment because GR could be inhibited by some metabolite of disulfiram, which is unlikely in a cell-free system [32]. Furthermore, the effect of

dithiocarbamates has been shown to be dependent on cell type [62], concentration [63], medium [64, 65], presence of Cu(II) or Zn(II) [62] and even the density of the cell cultures [62, 64].

Apart from dithiocarbamates, copper ions also interact with GSH to form a redox-active complex [66-68], and even low concentrations (10 μM) of copper(II) can decrease the GSH content [69] of the cell. GSH depletion is often considered a byproduct of oxidative stress during the process of cell death; recent discoveries suggest that GHS depletion is a critical regulator of apoptosis [70], and GSH depletion was observed independently of ROS during apoptosis [71]. Thus, a proper explanation of the above-mentioned results cannot be based only on the decrease of GSH levels, and is likely more complex than oxidative stress. Furthermore, co-treatment of the cell with an EtDTC-Cu(II) mixture or a PyDTC-Cu(II) mixture and N-acetylcysteine (NAC), a precursor of GSH, was less sufficient at inducing apoptosis [23, 24]; however NAC has also been shown to block intracellular accumulation of copper [64] that potentiates the toxicity of dithiocarbamates [16, 22, 23, 72]. Despite acting as a potent antioxidant, it is possible that NAC directly modifies certain signaling proteins and promotes cell growth and survival pathways [73]. Hence, the importance of oxidative stress in the process of apoptosis based on the role of GSH and NAC might be more complex.

The exact mechanism involved in the production of ROS in response to an EtDTC-Cu(II) mixture remains a largely open question. It is well established that copper(II) ions are able to induce ROS formation via Fenton reactions [74]. In the presence of superoxide or cellular reductants, the cupric ion can be reduced to cuprous ion, which is capable of catalyzing the generation of hydroxyl radicals [74]. However, EtDTC has been shown to inhibit significantly copper-catalyzed ROS generation via the Fenton reaction in human cerebrospinal fluid [75]. In addition, because it is highly redox active, any intracellular free copper is likely limited to far less than one free copper ion per cell, suggesting extensive copper-chelating overcapacity in the cell [76].

We propose yet another possible explanation for the oxidative stress observed in cells treated with a mixture of EtDTC-Cu(II). The oxidative stress may be just one of the consequences of inhibition of the cellular proteasome, as both the EtDTC-Cu(II) mixture and $Cu(EtDTC)_2$ complex are potent inhibitors of the proteasome [17, 21].

PROTEASOME AND OXIDATIVE STRESS

Prior to degradation, proteins are usually tagged with multiple ubiquitin molecules through a series of three enzymatic activities by ubiquitin activating (E1), ubiquitin conjugating (E2) and ubiquitin ligase (E3) enzymes [77]. Proteins attached by a polyubiquitin chain are recognized by the 26S proteasome and subsequently hydrolyzed (Fig. **2**). The 26S proteasome is composed of a one or two 19S regulatory particles (RP) bound to a 20S core particle (CP). The 19S RPs are responsible for substrate recognition, unfolding and deubiquitination [78], whereas the 20S CP facilitates degradation of proteins translocated from 19S. CP has a barrel-shaped structure and contains two pairs of three proteolytic subunits: β1, β2 and β5 with caspase-like (C-like), trypsin-like (T-like) and chymotrypsin-like (CT-like) activities, respectively [79]. The overall machinery of ubiquitination, as well as deubiquitination and proteasomal degradation, is known as the ubiquitin-proteasome system (UPS) [80].

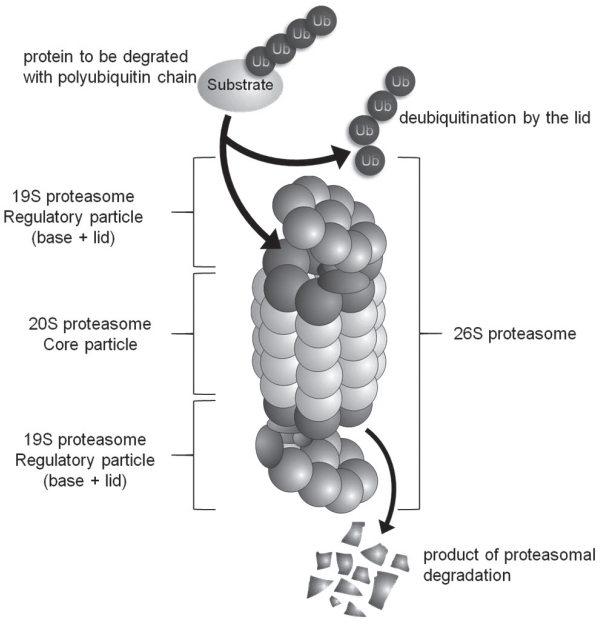

Fig. (2). Structure and function of the proteasome.

Protein degradation by the proteasome is a fundamental process in maintaining the viability and homeostasis of the cell. In addition to the degradation of short-lived regulatory proteins, the proteasome prevents the accumulation of non-functional, damaged or misfolded, and potentially toxic proteins. This process is particularly important in protecting the cell against unfavorable conditions, including oxidative stress [81]. Just as the ubiquitin-proteasome system (UPS) regulates cellular redox status, it is also affected by redox imbalance in the cell. Mild oxidative stress can impair the proteasome, and it appears that the 26S proteasome is more susceptible than the 20S proteasome [82], as oxidative stress causes the 26S proteasome to dissociate into 19S and 20S [83], which is responsible for degrading oxidatively damaged proteins [84]. Importantly, both inhibition of purified 20S proteasome by EtDTC-Cu(II) [17] and PyDTC-Cu(II) mixtures [85] and the results of a study in which ROS scavengers were insufficient to overcome the proteasome-inhibitory activity of a neocuproine-Cu mixture [86], suggest that copper complexes are able to inhibit the cellular proteasome directly, independently of ROS [86].

However, inhibition of the proteasome by various compounds could lead to oxidative stress. This result was reported for several cell lines and multiple of proteasome inhibitors, including bortezomib [87-92], MG132 [93-95], lactacystin [95-97], aLLN [95], MG262 [95], carbobenzoxy-

Leu-Leu-Leu-aldehyde [97] and *N*-benzyloxycarbonyl-Ile-Glu (O-*t*-butyl)-Ala-leucinal [98]. ROS generation by proteasome inhibitors induces c-Jun N-terminal kinase (JNK) [92] and activator protein-1 (AP-1) activation [95], loss of mitochondrial membrane potential ($\Delta\Psi m$) [87, 91-94, 97, 98], cytochrome-c release [87, 90-92, 97, 98], caspase-3 activation [87-93, 97] and subsequent apoptosis. Similar events (e.g., JNK and AP-1 activation, decreased $\Delta\Psi m$ and caspase-3 activation) were observed in cells treated with mixtures of EtDTC-Cu(II) [23] or PyDTC-Cu(II) [24]. However, bortezomib also did not increase ROS generation, and several antioxidants failed to protect cells from bortezomib-induced apoptosis [99], suggesting the tissue- and cell line-specific involvement of ROS in apoptosis is caused by the inhibition of the proteasome.

Because proteasomes eliminate misfolded or unassembled proteins in a process known as endoplasmic reticulum (ER)-associated protein degradation (ERAD) [100], they protect cells from ER stress leading to unfolded protein response (UPR) [101], which can trigger apoptosis if unmitigated [102, 103]. Not surprisingly, inhibition of the proteasome resulted in UPR-associated cell death in several cell lines [104-110]. Interestingly, involvement of UPR in apoptosis has also been reported in cells treated with a PyDTC-Cu(II) [27] or disulfiram-Cu(II) mixture [111] and other copper compounds [111-113].

MECHANISM OF PROTEASOME INHIBITION BY THE CU(ETDTC)$_2$ COMPLEX

Targeting UPS in cancer therapy is a new and promising approach used by the first-in-class drug bortezomib (Velcade), which reversibly inhibits the active sites in the 20S proteasome [114]. The obscurity of the antitumor and chemosensitizing activity of disulfiram has been uncovered in a study [17] in which disulfiram was shown to inhibit the cellular proteasome in the presence of copper. In contrast to disulfiram alone, a disulfiram-Cu(II) mixture potently inhibited the CT-like activity of the purified 20S proteasome (with an IC_{50} value of ~ 7.5 μM). Similarly, inhibition of the CT-like activity of the cellular 26S proteasome was observed in malignant MDA-MB-231 and MCF10DCIS.com breast cells treated with the disulfiram-Cu(II) mixture, whereas inhibition was not seen in normal immortalized MCF-10A cells. Importantly, inhibition of the cellular proteasome preceded induction of apoptosis and resulted in the accumulation of tumor suppressor proteins p27 and bax, which are natural proteasome substrates, indicating that the apoptosis was induced by proteasome inhibition. The proteasome-inhibitory, apoptosis-inducing and antitumor activity of disulfiram has been confirmed *in vivo* in human breast tumor xenografts containing higher levels of copper [17].

Interestingly, in sharp contrast to the disulfiram-Cu(II) mixture, a synthetic Cu(EtDTC)$_2$ complex was not able to inhibit CT-like activity of the purified 20S proteasome, yielding an IC_{50} value of >50 μM [21]. However, even at a much lower concentration (20 μM), the Cu(EtDTC)$_2$ complex inhibited ~ 90% of cellular 26S proteasome activity in MDA-MB-231 cells [21]. One explanation for this antagonic behavior on 20S by the disulfiram-Cu(II) mixture versus the synthetic Cu(EtDTC)$_2$ complex could be the ability of copper (II) ions to independently inhibit the 20S proteasome, as demonstrated elsewhere [17, 85, 86]. Likewise, while 50 μM of a PyDTC-Cu(II) mixture inhibited the CT-like activity of the purified proteasome by ~ 90%, the same concentration of Cu(PyDTC)$_2$ complex caused only partial (40%) inhibition [85]. Furthermore, by increasing the concentration of PyDTC to match a fixed concentration of Cu(II) ions, the potency of these mixtures to inhibit purified 20S proteasome significantly decreased [85].

These results indicate that, unlike bortezomib, the target of the Cu(EtDTC)$_2$ complex is the 26S proteasome rather than the 20S. We have previously reported that a possible target could be the JAMM domain of the POH1 subunit (RPN11 in yeast) within the lid of the 19S proteasome [39, 115], and a mechanism was proposed elsewhere [21]. POH1, a member of the JAMM (JAB1/MPN/Mov34 metalloenzyme) domain deubiquitinases, is responsible for releasing the polyubiquitin chain attached to a substrate before its translocation and degradation in 20S CP; thus it is necessary for activity of the 26S proteasome [116, 117] and viability of the cell [118, 119]. Moreover, the JAMM domain motif is located in the CSN5 subunit of the COP9 signalosome complex, [120] which has an emerging role in cancer [121] and is a negative regulator of many tumor suppressors, including p27 [122]. Furthermore, CSN5 isopeptidase activity is essential for breast epithelial transformation and progression, suggesting it may be a potential breast cancer oncogene and therapeutic target [123].

Although only 20S proteasome inhibitors are being tested in ongoing clinical trials, UPS provides many promising opportunities to treat cancer [124], and targeting deubiquitinases is undoubtedly one of them [125]. Several members of the JAMM class, including POH1 or CSN5, might make good drug targets. In particular, targeting the POH1 subunit introduces a new approach to proteasome inhibition [126]. This idea has been supported by a recent study [127] where inhibition of another two deubiquitinases associated with the 26S proteasome, USP14 and UCH37, induced tumor cell apoptosis and *in vivo* inhibition of tumor progression.

CONCLUSION

Finally, EtDTC shows promising activity against solid tumors that has been confirmed in a phase II clinical trial with high-risk breast cancer patients. Moreover, a combination of oral zinc gluconate and disulfiram induced clinical remission of hepatic metastasis in patient with ocular melanoma. These achievements led to an ongoing clinical trial at the Huntsman Cancer Institute in Utah (ClinicalTrials.gov Identifier NCT00742911) that elucidated the effect of simultaneous administration of disulfiram and copper gluconate on cancers involving the liver. Consequently, the toxicity of disulfiram or EtDTC (summarized in Fig. **3**) considerably increases in the presence of copper, suggesting that the active compound is actually the Cu(EtDTC)$_2$ complex, and it acts as a potent inhibitor of the cellular proteasome. In addition, disulfiram´s targets, multidrug resistance, angiogenesis, invasion and

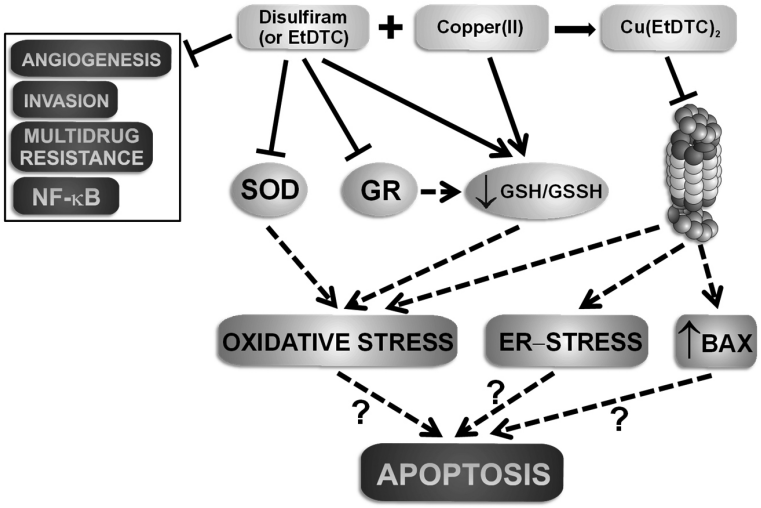

Fig. (3). Suggested simplified mechanism of observed activity of disulfiram and its metabolites.

anticancer activity as a DNA demethylating agent [128] or inhibitor of RING-Finger E3 ubiquitin ligases have been described [129]. Nevertheless, further studies and larger clinical trials are required to utilize disulfiram in clinics as an anticancer therapy. The term "repurposing" refers to the identification of new uses for existing drugs, and it has been proposed to provide an effective way to develop new chemotherapeutics [130, 131]. According to Francis S. Collins, the director of the NIH (National Institutes of Health), drug rescue and repurposing will be an important focus of the NIH´s proposed National Center for Advancing Translational Sciences [132]. Importantly, disulfiram is a generic and unpatentable drug; therefore, the research and trials will be financed by a government, non-profit organization or charity (such as GlobalCures), leading to approval of disulfiram as the first non-profit drug against cancer [133].

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This work was financed by project OP VK CZ.1.07/2.3.00/20.0062 "An inexpensive drug Antabuse as anticancer remedy: mechanism of action and clinical trials" from resources of European Union and the Czech Republic.

ABBREVIATIONS

ΔΨm	=	Mitochondrial membrane potential
AP-1	=	Activator protein 1
C-like	=	Caspase-like
CP	=	Core particle of the proteasome
CT-like	=	Chymotrypsin-like
$Cu(EtDTC)_2$	=	Bis(diethyldithiocarbamate)-copper complex
DNA	=	Deoxyribonucleic acid
ER	=	Endoplasmic reticulum
ERAD	=	ER-associated protein degradation
EtDTC	=	Diethyldithiocarbamate
GR	=	Glutathione reductase
GSH	=	Glutathione (reduced form)
GSSH	=	Glutathione (oxidized form)
IC_{50}	=	Half maximal inhibitory concentration
JAMM	=	JAB1/MPN/Mov34 metalloenzyme
JNK	=	c-Jun N-terminal kinase
NAC	=	N-acetylcysteine
NF-κB	=	Nuclear-factor κB
NIH	=	National Institutes of Health
$O_2^{-\bullet}$	=	Superoxide radical
P-glycoprotein	=	Permeability glycoprotein
PyDTC	=	Pyrrolidine dithiocarbamate
RP	=	Regulatory particle of the proteasome
ROS	=	Reactive oxygen species
SOD-1	=	CuZn superoxide dismutase
T-like	=	Trypsin-like
UPR	=	Unfolded protein response
UPS	=	Ubiquitin-proteasome system

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Received: December 14, 2011 Revised: March 02, 2012 Accepted: March 06, 2012