Biochemical Pharmacology 182 (2020) 114267

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Mechanism of cystathionine-β-synthase inhibition by disulfiram: The role of bis(N,N-diethyldithiocarbamate)-copper(II)

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ARTICLE INFO

Keywords:
H_2S
Gasotransmitters
Disulfiram
Mitochondria
Bioenergetics
Cancer
Down syndrome

ABSTRACT

Background: Hydrogen sulfide (H_2S) is an endogenous mammalian gasotransmitter. Cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) are the principal enzymes responsible for its biogenesis. A recent yeast screen suggested that disulfiram (a well-known inhibitor of aldehyde dehydrogenase and a clinically used drug in the treatment of alcoholism) may inhibit CBS in a cell-based environment. However, prior studies have not observed any direct inhibition of CBS by disulfiram. We investigated the potential role of bioconversion of disulfiram to bis(N,N-diethyldithiocarbamate)-copper(II) complex (CuDDC) in the inhibitory effect of disulfiram on H_2S production and assessed its effect in two human cell types with high CBS expression: HCT116 colon cancer cells and Down syndrome (DS) fibroblasts.

Methods: H_2S production from recombinant human CBS, CSE and 3-MST was measured using the fluorescent H_2S probe AzMC. Mouse liver homogenate (a rich source of CBS) was also employed to measure H_2S biosynthesis. The interaction of copper with accessible protein cysteine residues was evaluated using the DTNB method. Cell proliferation and viability were measured using the BrdU and MTT methods. Cellular bioenergetics was evaluated by Extracellular Flux Analysis.

Results: While disulfiram did not exert any significant direct inhibitory effect on any of the H_2S-producing enzymes, its metabolite, CuDDC was a potent inhibitor of CBS and CSE. The mode of its action is likely related to the complexed copper molecule. In cell-based systems, the effects of disulfiram were variable. In colon cancer cells, no significant effect of disulfiram was observed on H_2S production or proliferation or viability. In contrast, in DS fibroblasts, disulfiram inhibited H_2S production and improved proliferation and viability. Copper, on its own, failed to have any effects on either cell type, likely due to its low cell penetration. CuDDC inhibited H_2S production in both cell types studied and exerted the functional effects that would be expected from a CBS inhibitor: inhibition of cell proliferation of cancer cells and a bell-shaped effect (stimulation of proliferation at low concentration and inhibition of these responses at higher concentration) in DS cells. Control experiments using a chemical H_2S donor showed that, in addition to inhibiting CBS and CSE, part of the biological effects of CuDDC relates to a direct reaction with H_2S, which occurs through its complexed copper.

Conclusions: Disulfiram, via its metabolite CuDDC acts as an inhibitor of CBS and a scavenger of H_2S, which, in turn, potently suppresses H_2S levels in various cell types. Inhibition of H_2S biosynthesis may explain some of the previously reported actions of disulfiram and CuDDC *in vitro* and *in vivo*. Disulfiram or CuDDC may be considered

Abbreviations: XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; 3-MP, 3-mercaptopyruvate; 3-MST, 3-mercaptopyruvate sulfurtransferase; BrdU, 5-bromo-2′-deoxyuridine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); AzMc, 7-azido-4-methylcoumarin; ALDH, aldehyde dehydrogenase; AOAA, aminooxyacetic acid; AMA, antimycin A; CuDDC, bis(N,N-diethyldithiocarbamate)-copper(II) complex; BSA, bovine serum albumin; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; CBS, cystathionine beta-synthase; CSE, cystathionine gamma-lyase; Cys, cysteine; CcOX, cytochrome *c* oxidase; DDC, diethyldithiocarbamate; DMSO, dimethyl sulfoxide; DS, Down's syndrome; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; H_2S, hydrogen sulfide; Oligo, oligomycin; PLP, pyridoxal 5'-phosphate; ROS, reactive oxygen species; Rot, rotenone; SAM, S-(5′-adenosyl)-L-methionine; SQR, sulfide:quinone oxidoreductase; MTT, thiazolyl blue tetrazolium bromide; TS, transsulfuration pathway; TCEP, tris(2-carboxyethyl)phosphine; ZnDDC, zinc diethyldithiocarbamate.

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https://doi.org/10.1016/j.bcp.2020.114267
Received 27 August 2020; Received in revised form 1 October 2020; Accepted 1 October 2020
Available online 7 October 2020

as potential agents for the experimental therapy of various pathophysiological conditions associated with H_2S overproduction.

1. Introduction

Hydrogen sulfide (H_2S) is now viewed as an important endogenous mammalian gasotransmitter, which regulates a variety of physiological and pathophysiological processes in health and disease. The expression of various H_2S-producing enzymes and, consequently, the levels of H_2S are increased in various diseases, including local and systemic inflammation, critical illness, several forms of cancer and in Down syndrome [DS]) [55,53,58].

Three principal mammalian H_2S-producing enzymes are known: cystathionine β -synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) (reviewed in [18,22,45,53]. Both CBS and CSE lead to H_2S generation using cysteine as substrate, while 3-MST catalyzes the conversion of 3-mercaptopyruvate (3-MP) to pyruvate and an enzyme-bound persulfide which is then released in the presence of a sulfur acceptor. Physiological modulation of the activity of the three enzymes has been shown to occur via interaction with allosteric effectors and/or changes of the oxidation state of redox sensitive centers [46,17]. In various cancers, and in DS, the increased biological H_2S levels are the result of the upregulation of CBS as well as 3-MST, with opposite biological outcomes. On the one hand, cancer cells are equipped with a sophisticated sulfide detoxifying enzymatic machinery which, through the inner mitochondrial membrane-associated sulfide: quinone oxidoreductase (SQR), injects H_2S-derived electrons along the electron transport chain, thus supporting proliferation and bioenergetics [50,3,16,58]. On the other hand, sulfide oxidizing metabolism is significatively less efficient in fibroblasts and neurons [26]. In DS fibroblasts, where H_2S levels are elevated as compared to healthy tissues, cellular bioenergetics and proliferation is impaired because of H_2S-mediated inhibition of cytochrome *C* oxidase [39,40,52]. Pharmacological inhibition of CBS has recently emerged as a potential experimental therapeutic strategy for cancer and DS [58], and it is expected to induce opposite effects according to the disease model, namely inhibition or stimulation of cellular viability, respectively. However, the number of selective pharmacological tools and, especially, potentially clinically translatable CBS inhibitors is very limited.

A recent screen, on recombinant CBS, has identified a few potentially repurposable inhibitors, including benserazide (which is clinically used in many countries as one component of a two-component anti-Parkinson drug, Madopar) [12]. Another screen, conducted in a cell-based system (yeast) have discovered that disulfiram (a multifunctional pharmacological agent traditionally seen as an inhibitor of aldehyde dehydrogenase (ALDH) and a clinically used drug in the treatment of alcoholism), has cellular actions that are consistent with a CBS inhibitory effect in yeast model systems as well as in rodent DS models. In addition, disulfiram was able to suppress cognitive phenotypes in a mouse model of Down syndrome (Tg(CBS) mice) [34]. However, in a prior campaign, where all of the clinically used drugs – including disulfiram – were screened, disulfiram did not emerge as a significant direct inhibitor of the recombinant CBS enzyme [12]. The current study was designed to resolve this paradox. In particular, we have explored the possibility that potential bioconversion of disulfiram is necessary for its CBS inhibitory effect. The data presented in the current article are consistent with the hypothesis that disulfiram inhibits CBS in cellular environment via conversion to bis(N,N-diethyldithiocarbamate)-copper(II) complex (CuDDC), and the active principle of its action is, in fact, related to the complexed copper in this molecule. Accordingly, the data demonstrate that disulfiram and CuDDC exert cellular actions in cancer cells and DS cells that are similar to the effect of CBS deficiency and other, previously tested CBS inhibitors. Although disulfiram is not a direct CBS inhibitor, the current data suggest that disulfiram, CuDDC or disulfiram/copper combinations may be future options for experimental therapeutic CBS inhibition *in vivo*.

2. Materials and methods

2.1. Materials

7-Azido-4-methylcoumarin (AzMC), bovine serum albumin (BSA), copper chloride ($CuCl_2$), disulfiram, 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), homocysteine, lactalbumin hydrolysate, S-(5′-adenosyl)-L-methionine (SAM), sodium diethyldithiocarbamate (DDC) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma/Aldrich Chemie Gmbh (Munich, Germany). Pyridoxal 5′-phosphate (PLP) and cysteine (Cys) were obtained from Applichem (Darmstadt, Germany). Copper diethyldithiocarbamate (CuDDC) and zinc diethyldithiocarbamate (ZnDDC) were purchased from Tokyo Chemical Industry (Tokyo, Japan). GYY4137 from Cayman Chemical (Ann Arbor, Michigan, USA). Cell proliferation kit II [2,3-Bis-(2-methoxy-4-nitro-5-sulphophenyl)–2H-tetrazolium-5-carboxanilide (XTT)] and cell proliferation ELISA 5-bromo-2′-deoxyuridine (BrdU) kit were purchased from Roche Diagnostics (Sigma–Aldrich Chemie Gmbh: Munich, Germany). All other materials and reagents for cell culture were obtained from Thermo Fisher Scientific (Basel, Switzerland).

2.2. Cell culture

The human colorectal carcinoma cell line HCT116 (ATCC®, CCL-247™) was cultured in McCoy's 5A medium while Detroit 551 and Detroit 539 (human dermal fibroblasts from healthy and DS subjects, respectively) (LGC Standards, Wesel, Germany) were cultured in Advanced Dulbecco's Modified Eagle Medium/nutrient mixture F-12 (DMEM / F-12, 1:1; 1X), supplemented with 2 mM Glutamax™, and 0.1% lactalbumin hydrolysate. All the culture media were supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37 °C and 5% CO_2 atmosphere.

2.3. Recombinant human CBS and CSE expression and purification

Recombinant full-length human CBS, CSE and 3-MST were purchased from GenScript (Piscataway, NJ, USA). Both CBS and CSE storage buffer contained 0.5 mM TCEP, thus favoring their reduced state.

2.4. Mouse liver homogenate

Female Balb/c mice were anesthetized under isoflurane flux and euthanized. All animal-related procedures performed were approved by the Swiss Federal Food Safety and Veterinary Office (license no. FR_2018_11). Liver tissue was homogenized in RIPA buffer supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail using Fisherbrand™ Bead Mill 4 Homogenizer set 120 s at 3 m/s. The resulting mixture was stirred overnight at 4 °C. Supernatant was collected after 15 min centrifugation at 17,000×*g* and 4 °C, and protein concentration was quantified with Pierce™ Coomassie Plus (Bradford) Assay Reagent, against BSA protein standard. The resulting absorbance was detected with an Infinite M200 Pro microplate reader (Tecan, Männedof, Switzerland). All the reagents were purchased from Thermo Fisher (Basel, Switzerland).

2.5. Compound preparation

All compounds were freshly dissolved in DMSO (disulfiram and CuDDC) or MilliQ water ($CuCl_2$ and DDC) to produce a stock solution 100 mM, which was used to prepare serial dilutions of each compound. Before cell treatments, compounds were diluted in the culture medium to yield a final concentration ranging from 0.1 μM to 100 μM (0.1% DMSO).

2.6. Detection of H_2S generation in mouse liver homogenate and recombinantly expressed human enzyme CBS, CSE and 3-MST

H_2S enzymatic generation from mouse liver homogenate, recombinant CBS, CSE or 3-MST were conducted as described [12,4]. Activity assays were carried out using black 96-well plate with flat bottom. For H_2S detection from tissue homogenate, the reaction mixture, in TRIS-HCl buffer 50 mM pH 8.0, contained 13.5 μg/well mouse liver homogenate, 5 μM PLP, 500 μM SAM and 10 μM AzMC. Each well received 2 μl of test compounds at increasing concentration, in a total assay volume of 200 μl. The reaction was triggered by addition of 2 mM cysteine and 500 μM homocysteine (final concentration). Enzymatic activity of the human recombinant full-length CBS was measured in the presence of 100 μM SAM (1 μg/well) or CSE (5 μg/well) (without SAM), in otherwise identical assay conditions. For 3-MST activity, 5 μg/well of recombinant 3-MST was incubated with the 3-MST substrate 3-mercaptopyruvate at final concentration of 1 mM; AzMC was used at the final concentration of 10 μM. For all enzymatic assays, fluorescence (an index of H_2S production) was measured in kinetic mode for 2 h at 37 °C with an Infinite M200 Pro plate reader (Tecan, Männedof, Switzerland), at $\lambda_{excitation}$ = 365 nm and $\lambda_{emission}$ = 450 nm, respectively. Control experiments aiming to evaluate the possible H_2S-scavenging effect of the test compounds were performed by employing the H_2S donor GYY4137 (2 mM final concentration) instead of the recombinant enzyme, using AzMC-aided H_2S detection as described above. Data were analyzed in Excel (Microsoft Corporation, Redmond, Washington, USA) and activity was estimated from the initial slope of the fluorescence over the time.

2.7. Determination of number of free thiol group in recombinantly expressed human enzyme CBS, CSE and 3-MST

Free protein thiols were quantitated with the DTNB method. Briefly, 900 μl of enzyme sample (5 μM in 100 mM phosphate buffer, pH 8.0) was dispensed in Eppendorf tubes and each supplemented with increasing concentrations of $CuCl_2$ (0–50 μM), followed by 30 min incubation at room temperature (RT) in the absence of light. Next, samples received 100 μl DTNB 10 mM and were further incubated for 30 min at RT in the absence of light. Spectra were typically acquired between 250 and 500 nm using a quartz cuvette and an Infinite M200 Pro spectrophotometer (Tecan, Männedof, Switzerland). The 2-nitro-5-thiobenzoic acid anion formation was estimated by analysing absorbance values at 412 nm and using an extinction coefficient of 14,150 M^{-1} cm^{-1}, as recommended by the manufacturer. Spectra of DTNB alone (blank) and protein samples prior to DTNB addition (intrinsic protein absorption at 412 nm) were also acquired. Number of free thiols were determined according to the following equation:

$$freethiols = \frac{Abs_{protein+DTNB} - Abs_{protein} - Abs_{DTNB}}{14,150 \times [E]}$$

where [E] is the enzyme concentration in the cuvette determined at 280 nm.

2.8. Detection of $CuCl_2$ and CuDDC interference with Cys and 3-MP

Assays were carried out in flat-bottom 96-well plates, in 100 mM phosphate buffer pH 8.0, using the Infinite M200 Pro plate reader (Tecan, Männedof, Switzerland). Briefly, each well received 2 mM Cys or 3-MP and increasing concentration of $CuCl_2$ (0 – 100 μM). After gently resuspension of the assay mixture, the plate was incubated for 30 min at 37 °C in the absence of light, followed by the addition of 50 μl of 10 mM DTNB (2 mM final concentration), and further incubated for 10 min at RT and in the absence of light. The resulting mixture was diluted 1:5 in the same buffer in a final volume of 100 μl and unreacted thiols were detected spectrophotometrically at 412 nm. In the case of CuDDC, the presence of thiolic moieties within its structure (Fig. 1) raised an issue of incompatibility with the DTNB method. Therefore, in order to detect a possible instability of this complex in the presence of Cys or 3-MP, it was exploited its characteristic absorption at 450 nm [32]. Briefly, each well received 10 μM CuDDC and increasing concentrations of Cys or 3-MP (0 – 2 mM). After gently resuspension of the assay mixture, the plate was incubated for 30 min at 37 °C in the absence of light. Spectra were

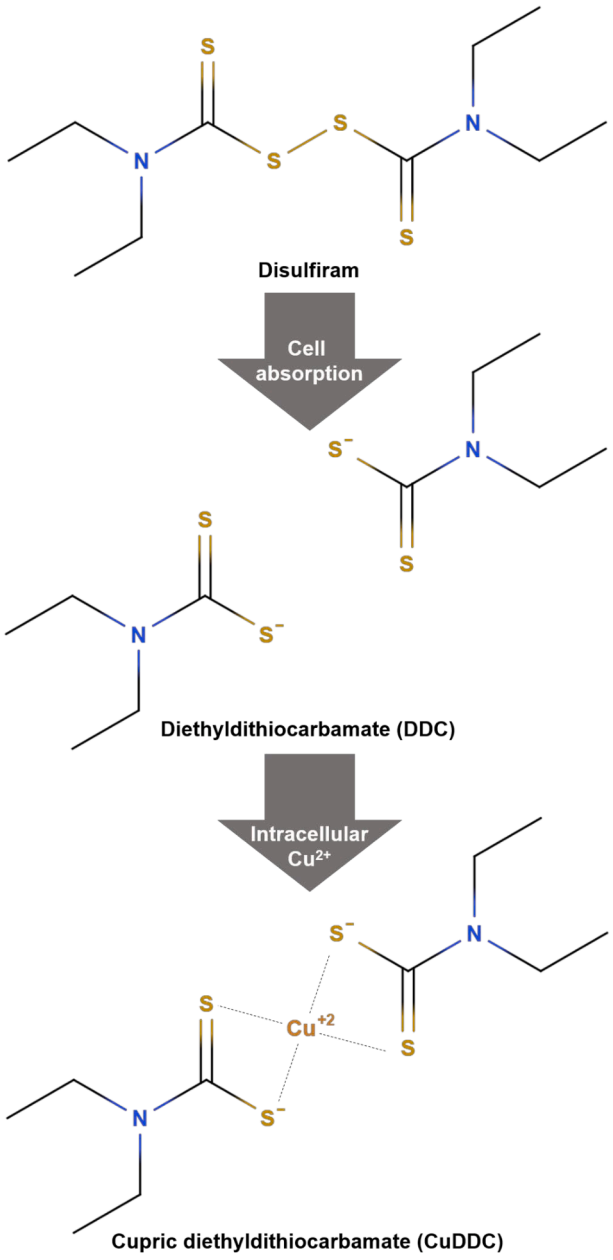

Fig. 1. Disulfiram (DSF) reaction with copper. The DSF's disulfide bond (S-S) is promptly reduced after cell intake thus producing diethyldithiocarbamate (DDC), which is a strong chelator of transition divalent metal ions, such as copper (II).

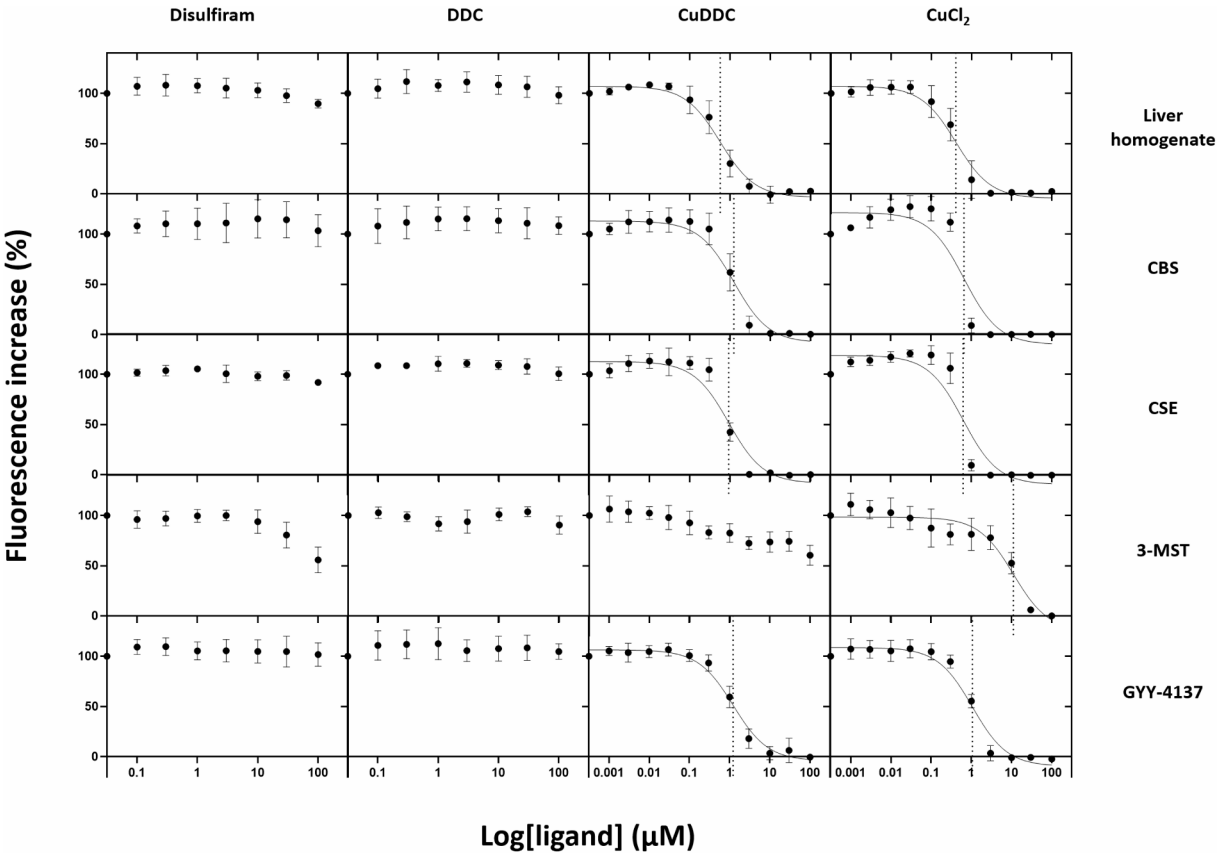

Fig. 2. Effect of disulfiram, CuDDC, $CuCl_2$ and DDC on AzMC signal. Assays were carried out on mouse liver homogenate, isolated full-length human CBS, isolated human CSE and isolated 3-MST. Compounds were counterscreened in the presence of the H_2S-donor GYY4137 for their ability to scavenge H_2S. Where available, a dashed line with the corresponding IC_{50} value is also shown. Data represent values of n = 5 independent experiments (mean ± SD).

recorded from 350 to 600 nm.

2.9. Measurement of cell proliferation

Cells were typically seeded in Corning® Costar® TC-treated flat-bottom 96-well plates in a final volume of 100 µl and incubated in a humidified incubator at 37 °C and 5% CO_2 atmosphere, unless otherwise stated. HCT116 cells were seeded at a cell density of 6,000 cells/well and incubated overnight. Detroit 551 and 539 cell lines were seeded at a density of 20,000 cells/well and incubated for 2 h. In both cases incubation was followed by a 24-h treatment with test compounds. Subsequently, cells were incubated with 10 µM BrdU labelling solution for 4 h at 37 °C and 5% CO_2. This assay relies on the ability of proliferating cells to incorporate BrdU, a pyridine analogue, in place of thymidine into the newly synthesized DNA. After removing the culture medium, DNA denaturation-fixation was performed, followed by incubation with the anti-BrdU antibody. Subsequently, the colorimetric substrate reaction was measured. Absorbance was measured at 450 nm with 690 nm as the reference wavelength using an Infinite M200 Pro plate reader (Tecan, Männedof, Switzerland). The absorbance values directly correlate to the amount of DNA synthesized, and therefore is function of the number of proliferating cells in the respective microcultures.

2.10. Measurement of cell viability

Following cell treatment (as described above), HCT116 cells were further incubated for 2 h with 0.5 mg/ml MTT. The converted formazan dye was dissolved in DMSO and the absorbance was measured at 570 nm and 690 nm (reference wavelength) with an Infinite 200 Pro plate reader (Tecan, Männedof, Switzerland). Detroit 551 and 539 cell viability were measured with XTT assay as described [40]. Each micro-well received 50 µl of XTT labelling reagent and 1 µl of electron coupling reagent, yielding final XTT concentration of 0.3 mg/ml. Cells were incubated with the XTT labelling mixture for 6 h at 37 °C. Plates were then gently shaken for 5 min and absorbance was measured at 492 and 690 nm (reference wavelength) using an Infinite M200 Pro plate reader (Tecan).

2.11. Detection of H_2S production in live cells

Cells were plated in in flat-bottomed Thermo Fisher Nunc® black 96-well plates and treated as described above. After the treatment, culture medium was replaced with HBSS buffer supplemented with AzMC 10 µM (Detroit 551 and Detroit 539) and further incubated for 1 h. The AzMC signal was acquired at $\lambda_{excitation}$ = 365 nm and $\lambda_{emission}$ = 450 nm an Infinite M200 Pro plate reader (Tecan, Männedof, Switzerland). Visualization of H_2S-generation in HCT116 living cells was performed as previously described [40,4]. Briefly, 30,000 HCT116 cells were seeded in a Lab-Tek II chamber coverglass system and incubated at 37 °C and 5% CO_2 for 48 h, followed by acute treatment with test compounds (2 h). After the treatment, culture medium was replaced with 100 µM AzMC in HBSS buffer and further incubated for 1 h. Cells were washed in HBSS buffer and dye's specific fluorescence was visualized using Olympus CKX53 inverted microscope and images were captured with Olympus SC50 digital camera and cellSens imaging software (Olympus, Volketswil, Switzerland). Images were then analyzed with the ImageJ software (v. 1.8.0; NIH, Bethesda, Maryland, USA).

2.12. Bioenergetic analysis in liver cells

The Seahorse XFe-24 flux analyzer (Agilent Technologies, Santa

Table 1
IC_{50} (μM) values of CuDDC and $CuCl_2$. Values were calculated using non-linear fitting equation using GraphPad Prism software. Mean ± SEM values (n = 5).

Compound	Mouse liver homogenate	CBS	CSE	3-MST	GYY4137
CuDDC	0.6 ± 0.2	1.2 ± 0.4	1.0 ± 0.2	>100	1.2 ± 0.4
$CuCl_2$	0.4 ± 0.2	0.6 ± 0.4	0.6 ± 0.2	11.2 ± 3.5	1.1 ± 0.3

Clara, California, USA) was used to estimate mitochondrial respiration of Detroit 551 and Detroit 539 cell lines as previously described [37,39]. Cells were seeded in Seahorse XFe-24 Cell Culture Microplates at a 20,000 cells/well density in a total volume of 200 μl, incubated for 2 h, followed by treatment for 24 h. Next, culture medium was replaced with Seahorse XF DMEM, supplemented with 20 μM glutamine, 10 μM pyruvate and 1 mM glucose (final concentration). The microplate was then incubated in a CO_2-free incubator at 37 °C for 1 h, to allow temperature and pH equilibration, as recommended by the manufacturer. The assay protocol consisted in two measurements of basal values of oxygen consumption rate (OCR), followed by the injection of 1 μM oligomycin, used to evaluate ATP generation rate. Subsequently, the mitochondrial oxidative phosphorylation uncoupler FCCP (2 μM), was employed to estimate maximal mitochondrial respiratory capacity. Eventually, 0.5 μM of rotenone and antimycin A were injected to inhibit the electron flux through the complex I and III, respectively, aiming to detect the extra-mitochondrial OCR. Data were analyzed with Wave (v. 2.6; Agilent Technologies, Santa Clara, California, USA) and graphed with GraphPad Prism 8 (GraphPad Software Inc.; San Diego, California, USA).

2.13. Statistical analysis

Data are shown as mean ± SEM of at least five independent experiments, unless otherwise stated. Differences among data are considered significant for p values < 0.05. Two-way ANOVA, followed by post-hoc Bonferroni's multiple-comparison test, was used to compare Detroit 551 to Detroit 539. Alternatively, ordinary one-way ANOVA, followed by post-hoc Bonferroni's multiple-comparison test, was employed to identify differences within HCT116 cell line as compared to non-treated control. Statistical calculations were performed using GraphPad Prism 8 (GraphPad Software Inc.; San Diego, California, USA).

3. Results

3.1. Effect of disulfiram, DDC, $CuCl_2$ and CuDDC on mammalian H_2S generation

In cellular milieu, disulfiram has been shown to be reduced to diethyldithiocarbamate (DDC), which is considered its major metabolite [20]. DDC is a very strong chelator of transition divalent metal ions, mainly copper(II) (Cu) and zinc. Intracellularly, DDC can therefore, rapidly react with copper to form a complex known as cupric diethyldithiocarbamate (CuDDC) [20,9,29,8] (Fig. 1). In order to test the effect of disulfiram, DDC, $CuCl_2$ and CuDDC on H_2S enzymatic generation from an intact tissue, were firstly carried out activity assays in a mouse liver homogenate, using the selective fluorescent H_2S probe AzMC. As shown in Fig. 2 and Table 1, disulfiram and DDC produced a weak inhibitory effect on H_2S generation (approx. 10% inhibition at the highest concentration, 100 μM tested), while CuDDC and $CuCl_2$ concentration-dependently and markedly reduced the AzMC signal with half maximal inhibitory concentrations (IC_{50}) of ~ 0.6 μM and ~ 0.4 μM, respectively.

To investigate whether this effect was restricted to CBS or involved also the others H_2S producing enzymes, H_2S detection assays were carried out in presence of recombinant CBS, CSE and 3-MST. For both CBS and CSE, neither disulfiram, nor DDC had any inhibitory effect on the AzMC signal, whereas the IC_{50} of CuDDC and $CuCl_2$ was 1 μM and 0.6 μM, respectively. In contrast, 3-MST-derived H_2S production was inhibited by $CuCl_2$ with an IC_{50} ~ 11 μM. The inhibitory effect of disulfiram and CuDDC on 3-MST-derived AzMC signal approached (but did not quite reach) 50% inhibition even at the highest concentration (100 μM) of the compounds tested (i.e. $IC_{50} > 100$ μM).

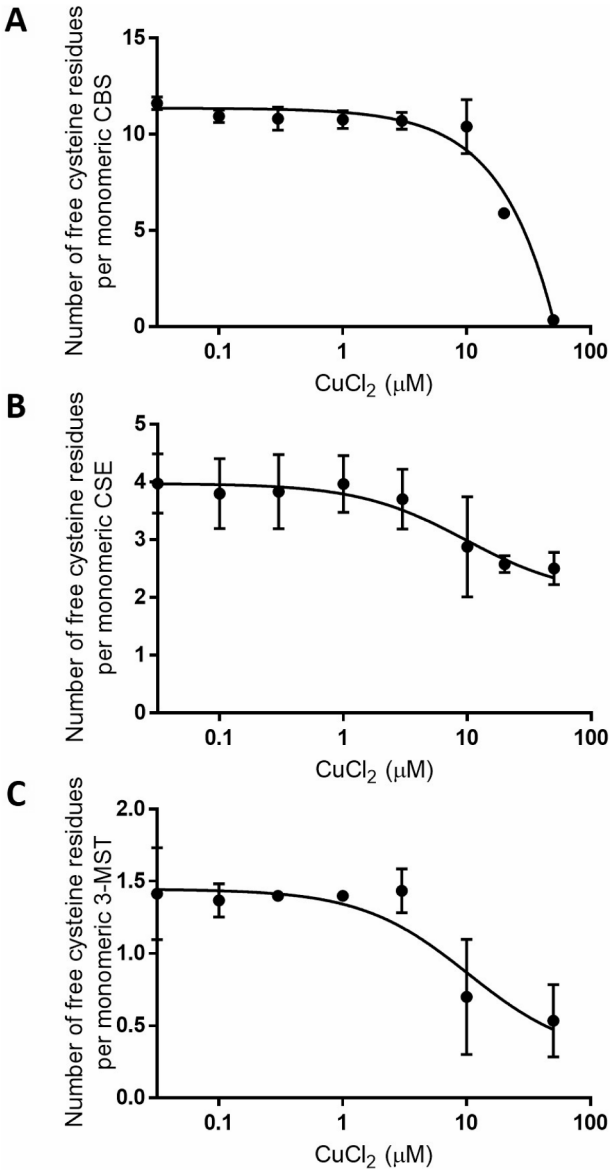

Fig. 3. Binding of $CuCl_2$ to cysteine residues of CBS, CSE and 3-MST. Assays were carried out on isolated full-length human CBS, isolated human CSE and isolated 3-MST in the presence of increasing concentration of $CuCl_2$. Data represented refers to at least n = 3 independent experiments (mean ± SD).

Since copper itself is not only known as a potent inhibitor of CBS and CSE [35,6,12] but it is also known to directly interact with various sulfur species [12,51], the question arose as to whether the observed AzMC signal reduction by CuDDC and/or $CuCl_2$ was due to a direct inhibition of the enzymatic activity or H_2S scavenging may have also contributed. The H_2S producing enzymes were therefore replaced with GYY4137, an organic H_2S donor that releases H_2S at a slow, steady rate [28] and the effect of the various compounds on the AzMC signal was recorded. While disulfiram and DCC exerted no inhibitory effect on the GYY4137-generated H_2S signal, both CuDDC and $CuCl_2$ significantly reduced the

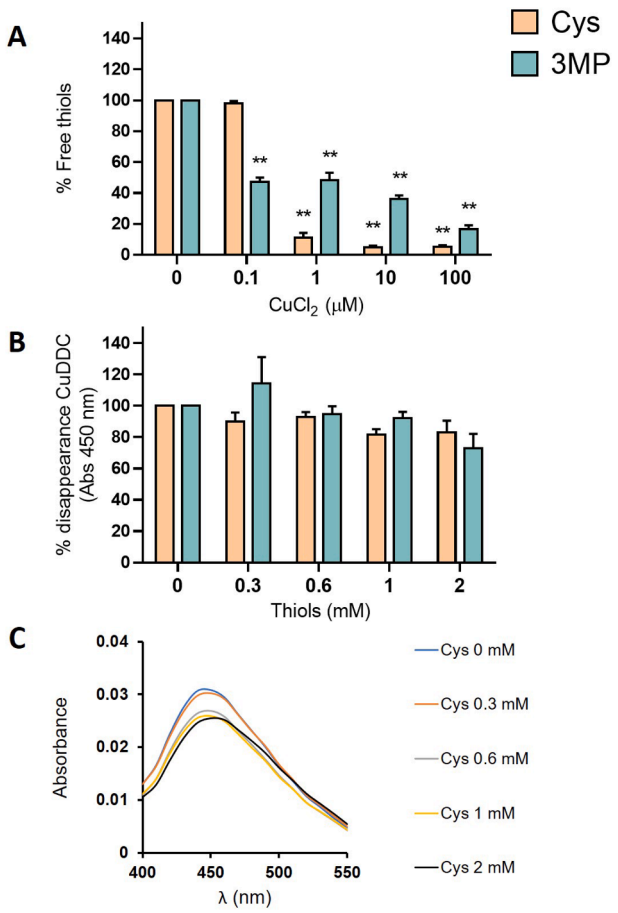

Fig. 4. Interference of $CuCl_2$ or CuDDC with enzyme substrates. A) Interaction of 2 mM cysteine or 3-MP with increasing concentrations of $CuCl_2$ by measuring free thiols with the DTNB method, B) interaction of 10 μM CuDDC at increasing concentration of cysteine or 3-MP, as evaluated by recording changes on CuDDC absorption at 450 nm. C) Representative experiment of CuDDC spectral changes in presence of increasing concentration of cysteine. Data represent values of n = 3 independent experiments (mean ± SEM). **p < 0.01 indicate significant differences compared to control.

signal, with IC_{50} values of ~ 1.2 μM and ~ 1.1 μM, respectively.

The enzymatic activity of the three H_2S producing enzymes is regulated by the presence of redox-sensitive cysteines [46]. This prompted us to investigate whether a direct interaction of copper with protein exposed cysteine residues could play a role in their enzymatic inhibition. Free/accessible thiols were determined for CBS, CSE and 3-MST with the DTNB method and protein-copper binding was assessed by evaluating any changes in the number of free cysteine residues. CBS was the only one having a significant propensity to bind copper at the lowest concentration tested (0.1 μM), while both CSE and MST showed a significant decrease in free thiols number only at 10 μM $CuCl_2$. Particularly, untreated CBS displayed 11.6 ± 0.4 free cysteine residues, and after treatment with 0.1 μM $CuCl_2$ 10.9 ± 0.3 residual free cysteine residues ($p < 0.05$) were detected, suggestive of the formation of one Cu-S bond (Fig. 3A). The susceptibility of protein free cysteines to be targeted by copper ions was evaluated only against $CuCl_2$, as in the case of CuDDC the presence of thiolic moieties would interfere with the DTNB method.

Given the marked reactivity showed by copper ions against thiolic species, we wondered whether this could be translated into an interference of the catalytic activity through a direct interaction with enzymes substrates, namely Cys and 3-MP. With the aim to address this question, 2 mM Cys or 3-MP were incubated with increasing concentrations of $CuCl_2$. As shown in Fig. 4A, free Cys levels were reduced by

Table 2
H_2S production in mouse liver homogenate in presence of increasing concentration of zinc complexed with diethyldithiocarbamate (ZnDDC) or free zinc ions. Activity is expressed as % of control. Mean ± SEM values (n = 5).

Compound	Activity (% of control)		
	1 μM	10 μM	100 μM
ZnDDC	100 ± 0.2	100 ± 0.2	100 ± 0.2
$ZnCl_2$	100 ± 0.2	100 ± 0.2	100 ± 0.2
1 μM $CuCl_2$ + [c] μM disulfiram	0	4 ± 0.3	56 ± 0.4
1 μM $CuCl_2$ + [c] μM DDC	0	50 ± 0.4	84 ± 0.2

copper in a concentration-dependent mode, overall accounting for an IC_{50} of ~ 0.3 μM. With respect to 3-MP, the interaction with copper exhibited a biphasic profile. Although the residual free thiol levels dropped down to ~50% already at 0.1 μM $CuCl_2$, higher concentrations of copper appeared to have a much smaller effect of 3-MP. With the aim to evaluate the interference of CuDDC with Cys and 3-MP a different experimental approach was adopted, as the thiolic moieties of CuDDC are reactive towards DTNB, thus possibly leading to artifacts. The complex of copper with DDC displays a yellow color with a characteristic absorption peak at 450 nm which is not exhibited by copper ions or DDC alone [32]. It was, therefore, exploited this feature to evaluate the stability of the complex at increasing concentration of Cys or 3-MP (Fig. 4C). As shown in Fig. 4B, CuDDC appeared to be relatively stable even at 2 mM Cys or 3-MP.

ZnDDC, another metal complex of DDC, exerted no inhibitory effect on enzymatic CBS generation (Table 2), indicating that it was the copper moiety in CuDDC that was responsible for its pharmacological effects described above. As expected, the inhibitory effect of $CuCl_2$ on CBS activity was reduced with increasing concentrations of disulfiram or DDC, suggestive of the formation of a copper complex molecule (Table 2).

From the above data, we can conclude that disulfiram is not a direct inhibitor of CBS, but its metabolite CuDDC is (and its potency is comparable to that of free copper (II) ions). Moreover, both CuDDC and copper also directly interact with H_2S to catalyze its decomposition. The complexed form CuDDC appears to be slightly less potent than free copper as an inhibitor of the CBS-derived AzMC signal. Indeed, copper, but not CuDDC, interacts with Cys and, to a less extent, with 3-MP, thus reducing enzymatic activity via direct interference with the reaction substrates. We hypothesize that this difference in potency is due to the fact that in CuDDC the copper is present in a complexed form and may be less reactive towards protein cysteine residues, as well as Cys substrate, thus CuDDC's effects relies on its ability to release copper ions *in situ*. It is conceivable that the slight inhibition by disulfiram of H_2S generation that we have noticed in the liver homogenates may be due to the formation of CuDDC in the cellular environment, which contains redox-active components as well as copper. Interestingly, free copper (II) ions exerted a ~ 20 fold weaker effect on 3-MST-derived AzMC signal than on CBS-derived AzMC signal, while CuDDC only exerted a weak inhibitory effect, thus suggesting a higher propensity of copper to interfere with the catalytic activity of CBS and CSE than 3-MST.

3.2. *CuDDC reduces intracellular H_2S levels and proliferation of HCT116 cells*

HCT116 cells express high levels of CBS (as well as 3-MST) and utilize H_2S to support their bioenergetics and proliferation [54,41]. As shown in Fig. 5, disulfiram did not affect H_2S generation in HCT116 cells, while $CuCl_2$ exerted a partial inhibitory effect at the highest concentration tested. On the other hand, CuDDC exerted a concentration-dependent (and, at 3 and 10 μM, near-complete) inhibitory effect on the AzMC signal (Fig. 5). From the technical point of view, data above referred were obtained after an incubation period of 2 h since prolonged exposure to CuDDC caused strong morphological changes even at low

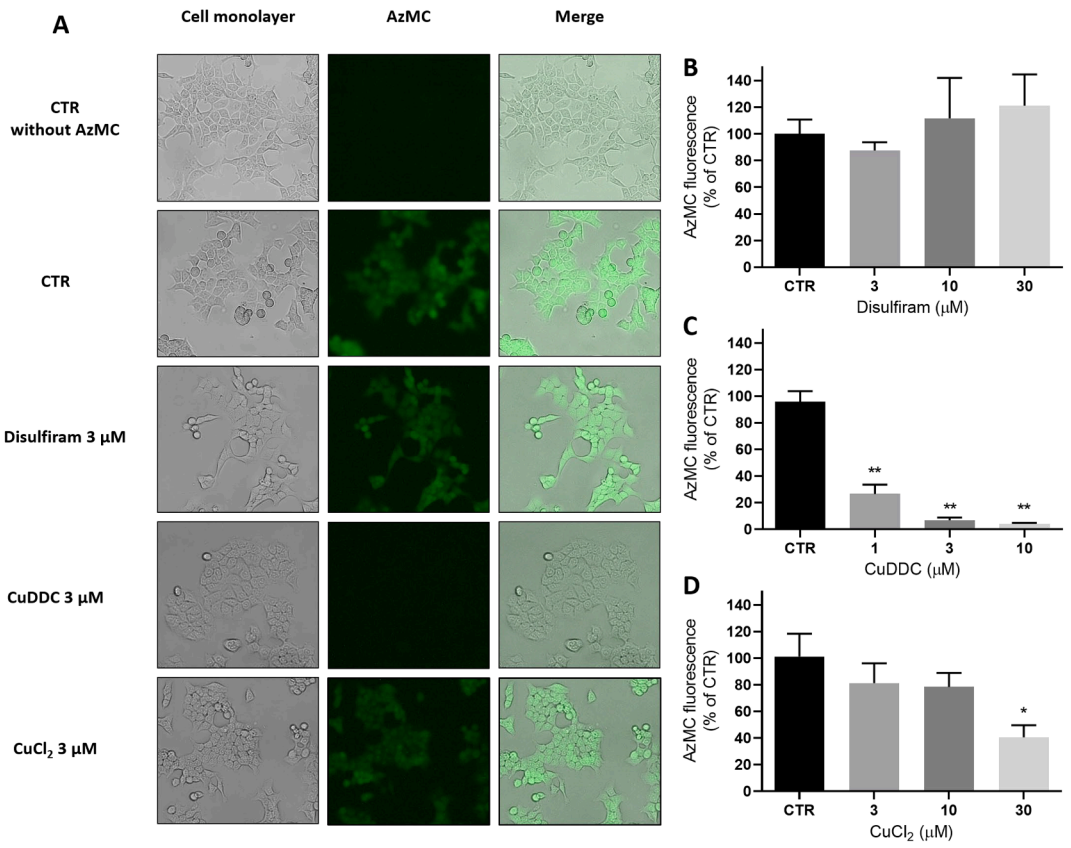

Fig. 5. H_2S detection on HCT 116 cells. A) Cells were treated with 3 μM Disulfiram, CuDDC or $CuCl_2$ for 2 h and intracellular H_2S was detected AzMC fluorescent probe. Control received only vehicle. B-D) Quantification of AzMC signal at increasing concentration of test compounds. Data represent values of n = 3 independent experiments (mean ± SEM). *p < 0.05 and **p < 0.01 indicate significant differences compared to control (non treated) cells.

concentration and thus live cell imaging was not performed.

While disulfiram did not have any significant effect on cell proliferation or viability, $CuCl_2$ decreased both of these parameters at the highest concentration used in this experiment (100 μM) (Fig. 6). A potent effect was observed with CuDDC, which exerted a concentration-dependent inhibitory effect both on cell proliferation and on viability with an IC_{50} of approximately 0.3 μM, possibly because of impairment of mitochondrial metabolism, as suggested by the MTT assay (Fig. 6).

These data indicate that CuDDC is a fairly potent inhibitor of cellular H_2S generation, and the inhibitory effect of cancer cell proliferation and viability may be consistent with the known role of H_2S in cancer cells. Nevertheless, additional pharmacological actions of CuDDC may also contribute to the observed effects (See: Discussion). The lack of disulfiram's effect may be attributed to either low cellular uptake of this compound into the tumor cell, lack of intracellular conversion to CuDDC in this cell type (which may be cell-type-dependent, see below), or lower content of endogenous copper. The lack of $CuCl_2$'s effect is most likely due to its low cell penetration.

3.3. *Disulfiram and CuDDC suppress intracellular H_2S levels in DS fibroblasts and restore their viability and proliferation rate*

DS fibroblasts exhibit a significant overproduction of H_2S, contributing to impaired viability, proliferation rate and bioenergetics [39,40]. As shown in Fig. 7A, cells were incubated for 24 h in the presence of the test compounds, at a concentration consistent with the increase of cell viability and proliferation rate (see below). Disulfiram, while not exerting any apparent effect on healthy control cells, significantly inhibited the AzMC signal in DS cells, already at the lowest concentration (0.1 μM) tested. CuDDC exerted a similarly potent inhibitory effect, while $CuCl_2$ was without any effect (Fig. 7).

Disulfiram exerted a bell shaped-effect on viability and proliferation rate in DS cells; in the submicromolar concentrations it improved these parameters so that the proliferation rate and the viability of these DS cells approximated those of the healthy control fibroblasts. However, the drug began to show inhibitory and cytotoxic effects in the higher concentration range tested (1–10 μM) (Fig. 8). Similar to the findings seen in HCT116 cells, copper alone did not display any effect in DS cells, whereas, CuDDC improved viability and proliferation of DS cells at 0.1–0.3 μM, but not at the higher concentration tested (1 μM) (Fig. 8).

In contrast to DS cells, in normal control fibroblasts, none of the compounds tested had any effect on the (low baseline) H_2S production; disulfiram (at 3–10 μM) and CuDDC (at 1 μM) suppressed cell viability and proliferation, probably by affecting pathways and cellular targets other than CBS or H_2S (see: Discussion).

The increase in cell viability, as assayed with XTT method, accounts for an increased mitochondrial metabolism (Fig. 8). With the aim to clarify this aspect, bioenergetic measurements were carried out on cells treated with test compounds at a concentration in line with cell viability and proliferation rate increase. Data shown in Fig. 9 indicate that disulfiram improves the various parameters of mitochondrial respiration and cellular oxygen consumption in DS cells, without affecting control cells, while neither CuDDC nor $CuCl_2$ exerted any significant effect on these parameters.

The data presented in the prior section indicate that in DS cells, disulfiram and CuDDC exert potent pharmacological effects. Already at low concentrations, disulfiram and CuDDC both inhibit H_2S production and improve cell viability. We have also noticed previously that other inhibitors of H_2S biosynthesis (e.g. the CBS inhibitor aminooxyacetate and the 3-MST inhibitor HMPSNE) are very potent in DS cells [39,40]; it is thus possible that this cell type has a high permeability for various pharmacological agents. The effects of disulfiram and CuDDC are similar

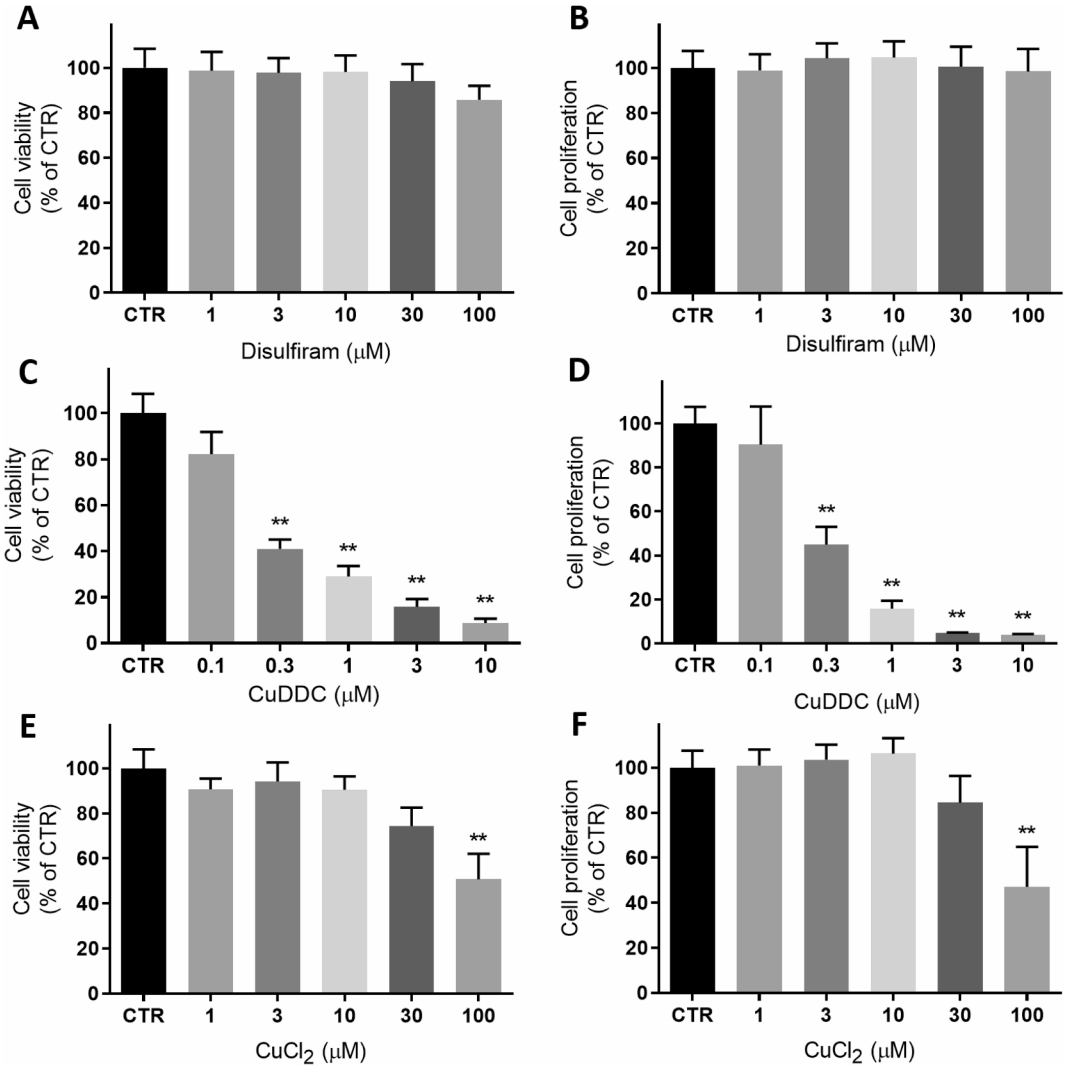

Fig. 6. Effect of disulfiram, CuDDC and $CuCl_2$ on HCT 116 cells viability and proliferation. Cells were treated over 24 h at increasing concentration of test compounds and cellular viability and proliferation were evaluated with MTT and BrdU method, respectively. Data represent values of n = 5 independent experiments (mean ± SEM). **p < 0.01 indicates significant differences as compared to control (non treated) cells.

to the above-mentioned inhibitors, in that at low concentrations they enhance cell viability and proliferation, while at higher concentrations an inhibitory effect becomes evident [39,40]. We interpret the current findings as follows: in DS fibroblasts (which exhibit defects in their mitochondrial bioenergetic function and have difficulties to maintain cellular functions due to their impaired cellular bioenergetic status), take up disulfiram from the extracellular space, which, in turn, inhibits intracellular H_2S production and, consequently improves cellular bioenergetics and proliferation. However, unexpectedly, in this cell type administration of CuDDC failed to improve cellular bioenergetic parameters. As expected, copper *per se* (similar to the situation in HCT116 cells) does not enter the DS cells (or the control fibroblasts) and does not exert detectable effects on H_2S production or cell viability or cell function.

4. Discussion

The main findings of the current report can be summarized as follows: (1) disulfiram does not exert any significant direct inhibitory effect on any of the recombinant H_2S-producing enzymes in cell-free conditions; (2) disulfiram exerts a slight inhibitory effect on H_2S generation in liver homogenates, presumably due to its conversion to active metabolites; (3) disulfiram does not significantly inhibit H_2S production in HCT116 colon cancer cells, but it does inhibit H_2S production in DS fibroblasts (presumably illustrating a cell-type-dependent uptake and/or metabolism); (4) accordingly, disulfiram does not have any functional effects in HCT116 cells, but it exerts a bell-shaped effect, a stimulatory effect at low concentrations and an inhibitory effect at higher concentrations on the viability and proliferation of DS cells (an effect, that is characteristic of inhibitors of H_2S biosynthesis in this cell type); (5) the main metabolite of disulfiram, DDC does not inhibit any of the H_2S producing enzymes and neither does it scavenge H_2S; (6) the copper complex of DDC (CuDDC) is a potent inhibitor of CBS and CSE, as well as a scavenger of H_2S; (7) CuDDC inhibits H_2S production both in HCT116 cells and in DS fibroblasts and exerts the expected functional effects (inhibitory effect in HCT116 cells and bell-shaped effects in DS cells on proliferation and viability); (8) the inhibitory effect of CuDDC on H_2S-related parameters is due to its complexed copper atom; another metal complex (ZnDDC) does not inhibit the H_2S/AzMC signal; (9) in line with prior findings, the current experiments confirm that free copper (II) is a potent inhibitor of the CBS and CSE-derived AzMC signal (and a weaker inhibitor of 3-MST-derived AzMC signal) and it is also able to directly interact with enzyme substrates Cys and 3-MP, as well as with chemically generated H_2S; (10) copper (II) binds CBS via formation of a Cu-S bond with a solvent accessible cysteine residue and possibly playing a role in CBS inhibition mechanism; (11) the *in vitro* effects of free copper

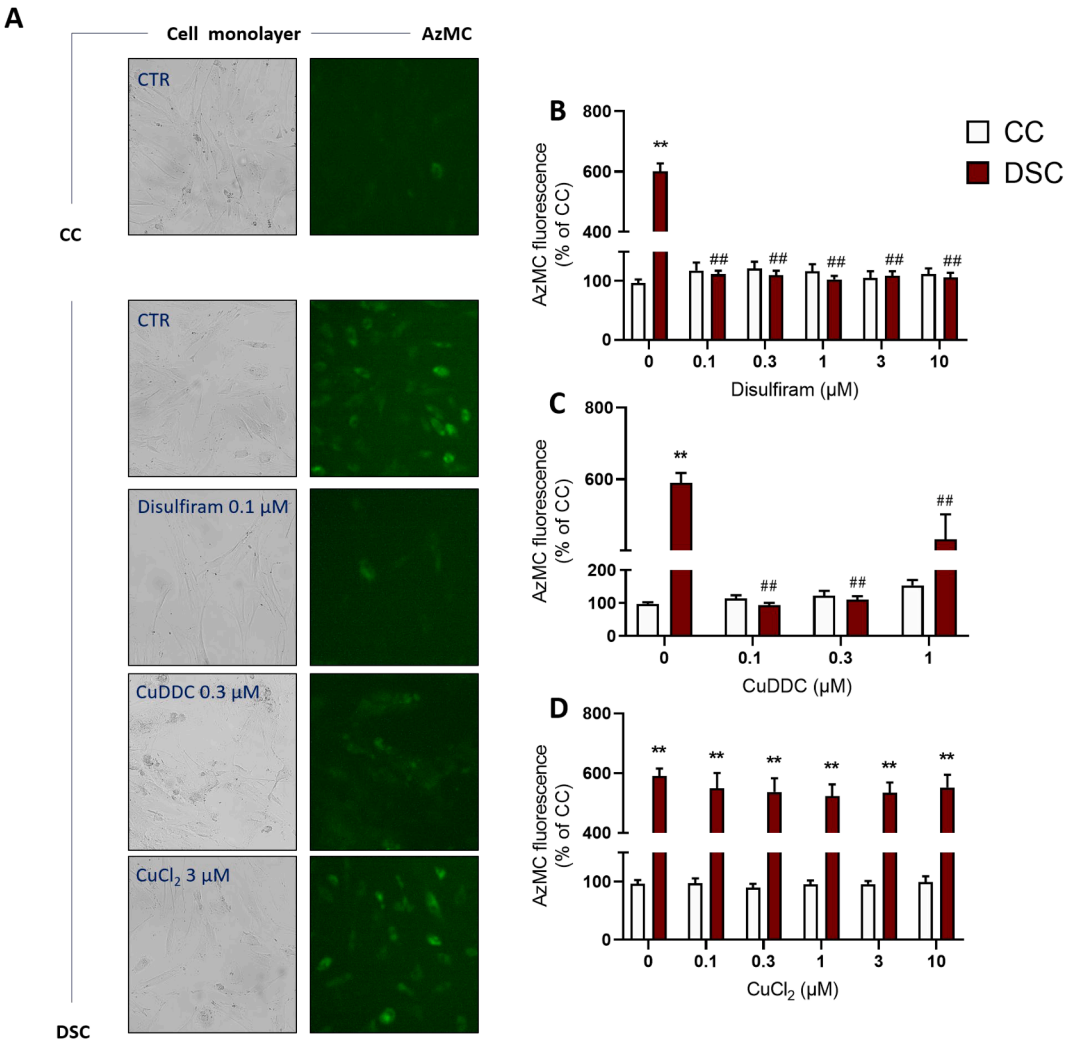

Fig. 7. H_2S detection on Detroit 551 (CC) and Detroit 539 (DSC) cells. A) Cells were treated with 0.1 µM disulfiram, 0.3 µM CuDCC or 3 µM $CuCl_2$ for 24 h and intracellular H_2S was detected AzMC fluorescent probe. Control received only vehicle. B-D) Quantification of AzMC signal upon treatment with increasing concentration of disulfiram, CuDDC or $CuCl_2$ for 24 h. Data represent values of n = 5 independent experiments (mean ± SEM). $^{**}p < 0.01$ indicates a significant increase in fluorescence in DSCs *vs.* CCs; $^{\#\#}p < 0.01$ indicates a significant inhibitory effect of the test compound on cellular H_2S levels in treated *vs.* non-treated DSCs.

on H_2S homeostasis cannot be recapitulated in cultured cells, presumably due to its low cellular uptake; and, finally, (12) CuDDC, unexpectedly (and in contrast to disulfiram), does not improve cellular bioenergetics in DS cells, even though it improves their viability and proliferation. The explanation for this last finding and discrepancy is currently unclear.

In mammals, the reverse transsulfuration pathway (TS) is a crucial pathway regulating the generation of cysteine from homocysteine, through two sequential reactions catalyzed by the cytosolic pyridoxal 5′-phosphate (PLP)-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), both producing hydrogen sulfide (H_2S) as side product [47,53]. One of the possible metabolic routes of the resulting cysteine is its bioconversion into 3-mercaptopyruvate (3-MP), a substrate for the cytosolic and mitochondrial enzyme 3-MST, which, in turn, generates pyruvate and H_2S as end-products [56,23]. Dysregulation of sulfur metabolism has deep implications in human pathophysiology, which so far have been broadly documented in cancer biology, as witnessed by the massive body of literature associating CBS upregulation with oncological diseases [50,3,16,58]. Increased H_2S levels has been found to stimulate cellular bioenergetics, migration, proliferation and invasion, first shown in HCT116 colon cancer cell line, which constitutively express elevated CBS levels [54] and subsequently expanded into many cell types [50,58]. Conversely, in the brain, where sulfide detoxification enzymatic machinery is less active [26], H_2S-overproduction may result in neuronal toxicity. Accordingly, high CBS and 3-MST expression in DS has been postulated to contribute to some of the clinical features of DS [39,40,21,52]. Indeed, brain tissues of DS patients were shown to be enriched in CBS content, and particularly, in older individuals it co-localizes with senile plaques [19]. Recently, working on fibroblasts of DS patients (Detroit 539) *vs* healthy control (Detroit 551), we have reported that DS cells display a decreased electron flux along the electron transport chain and this correlated with high H_2S levels, thus suggesting that a H_2S-induced inhibitory effect on cytochrome *C* oxidase may play a role in DS-associated mitochondrial impairment [39]. Interestingly, DS cells proliferation and mitochondrial function was significantly improved after CBS gene silencing or its pharmacological inhibition with aminooxyacetic acid (AOAA), thus identifying CBS as a potential target for therapy [39]. However, despite several medium-throughput academic screening campaigns conducted over the years, a potent and selective CBS inhibitor suitable for pharmacological application is not yet available [58].

Recently, Marechal et al. carried out a cell-based high-throughput drug screening on a *S. cerevisiae* model overexpressing *CYS4*, the yeast homolog of CBS, and identified disulfiram, as an inhibitor of the transsulfuration pathway [34]. A follow-up *in vivo* study in DS mice showed significant behavioral improvements after treatment with disulfiram

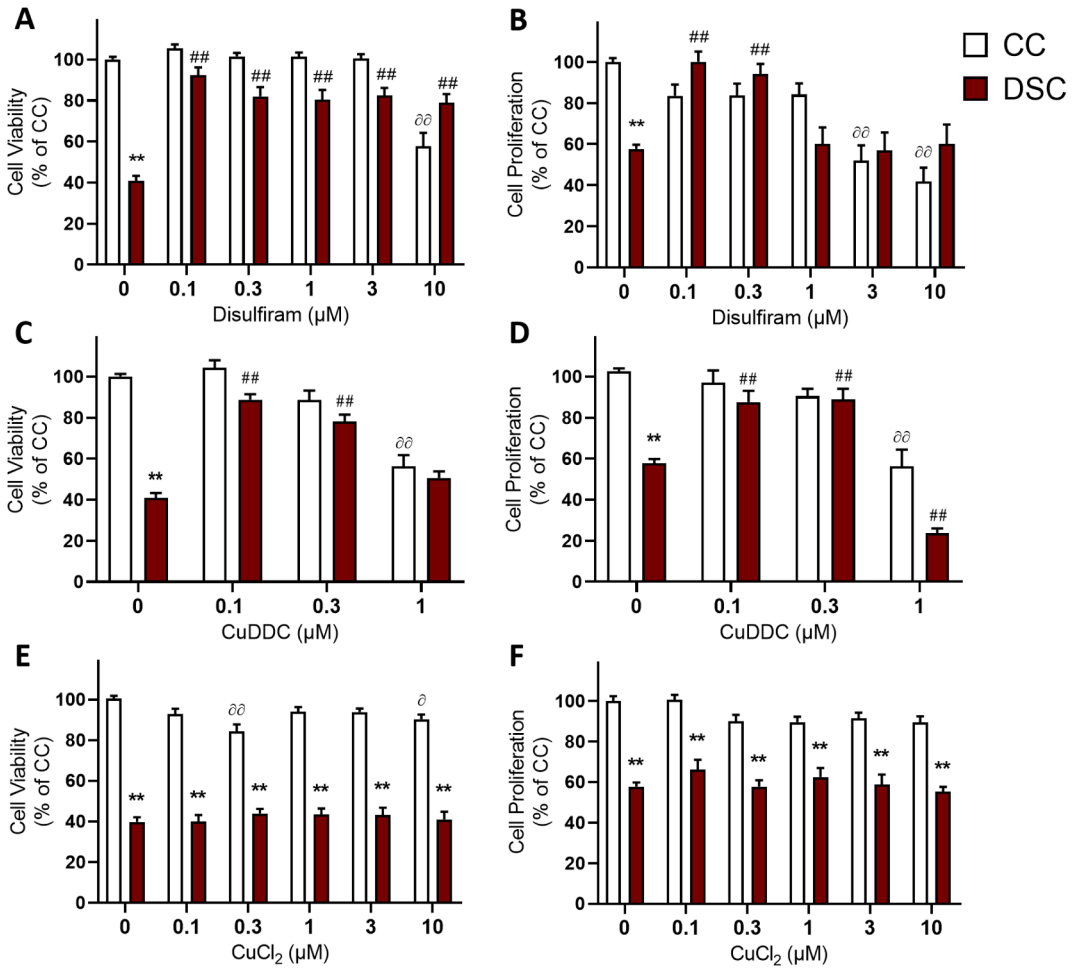

Fig. 8. Effect of disulfiram, CuDDC and $CuCl_2$ on Detroit 551 (CC) and Detroit 539 (DSC) viability and proliferation. Cells were treated over 24 h at increasing concentration of test compounds and cellular viability and proliferation were evaluated with XTT and BrdU method, respectively. Data represent values of n = 5 independent experiments (mean ± SEM). **p < 0.01 indicates a significant decrease in cell viability or proliferation rate of DSCs *vs.* CCs; $^{\#\#}$p < 0.01 indicates a significant increase of viability or proliferation rate of treated *vs* non-treated DSCs. ∂p < 0.05 and ∂∂p < 0.01 indicate the significant increase of viability or proliferation rate of treated *vs* non treated CCs.

[34]. Although these data supported the hypothesis that disulfiram, in some way interferes with CBS activity, a direct inhibition was unlikely due to two issues (1) after cellular absorption disulfiram is rapidly bioconverted to its thiolic cognate (DDC) and to other metabolites [21,25] and (2) disulfiram is not a direct inhibitor of recombinant CBS [12]. These findings and considerations stimulated the current experiments, in order to explore the potential underlying biochemical mechanisms of disulfiram's action on H_2S homeostasis. As shown in the Results section and summarized in the first paragraph of the Discussion section, the most likely explanation for disulfiram's inhibitory action on H_2S production is that the drug is converted to CuDDC, which, in turn, inhibits H_2S generation. In mammals, intracellular labile pools of bioavailable copper are stored in endosomes and lysosomes and their export to the cytosol is regulated by the membrane protein Cu transporter protein (Ctr) [44,2]. Particularly, it has been postulated that the biological effect of disulfiram (and/or DDC) can be at least in part explained by its ability to induce Ctr-independent copper (exogenous and endogenous) migration across biological membranes [1]. Here we show that the effects of disulfiram treatment are tissue dependent, likely because of different efficiency in cellular uptake and/or bioconversion to DDC. Moreover, it is conceivable that the result on endogenous copper mobilization is translated in a more pronounced biological response in tissues where copper content is higher, namely liver and brain [2]. We report that one of the possible consequences of intracellular copper mobilization is the inhibition of CBS and CSE thereby suppressing intracellular H_2S levels and exerting biological effects that are consistent with (and are expected from) the suppression of cellular H_2S levels.

Mechanistically, the copper-dependent inhibition of CBS and CSE could be viewed as a sum of three factors: a) direct interaction with the enzyme, b) direct scavenging of H_2S, and c) depletion of the substrate Cys by free copper ions. Among the three enzymes examined only CBS exhibited propensity to directly binding copper at a concentration below the IC_{50}. Niu et al. have recently shown that the CXXC motif situated in CBS central domain is a redox sensitive center and its reduction induces ~3-fold increase of the enzymatic activity [38]. Intriguingly, many copper-dependent enzymes display a conserved CXXC motif for metal binding [7], hence it may be speculated that copper binding to CBS involves the CXXC motif. By contrast, we did not detect any interaction below the IC_{50} value between copper and CSE, which shows four exposed cysteine residues involved two CXXC motifs and to which no regulatory function has been yet attributed [38,46].

Given the pronounced propensity of copper (and CuDDC) to direct interact with H_2S, the weak inhibitory effect towards 3-MST-dependent H_2S generation was somewhat unexpected. This apparent discrepancy is partly reconciled by the lack of direct interaction of copper with 3-MST (at low concentrations) together with the inability of copper to fully deplete 3-MP, by contrast to Cys. Moreover, differently from CBS and CSE, 3-MST's canonical reaction takes place in the presence of a sulfur

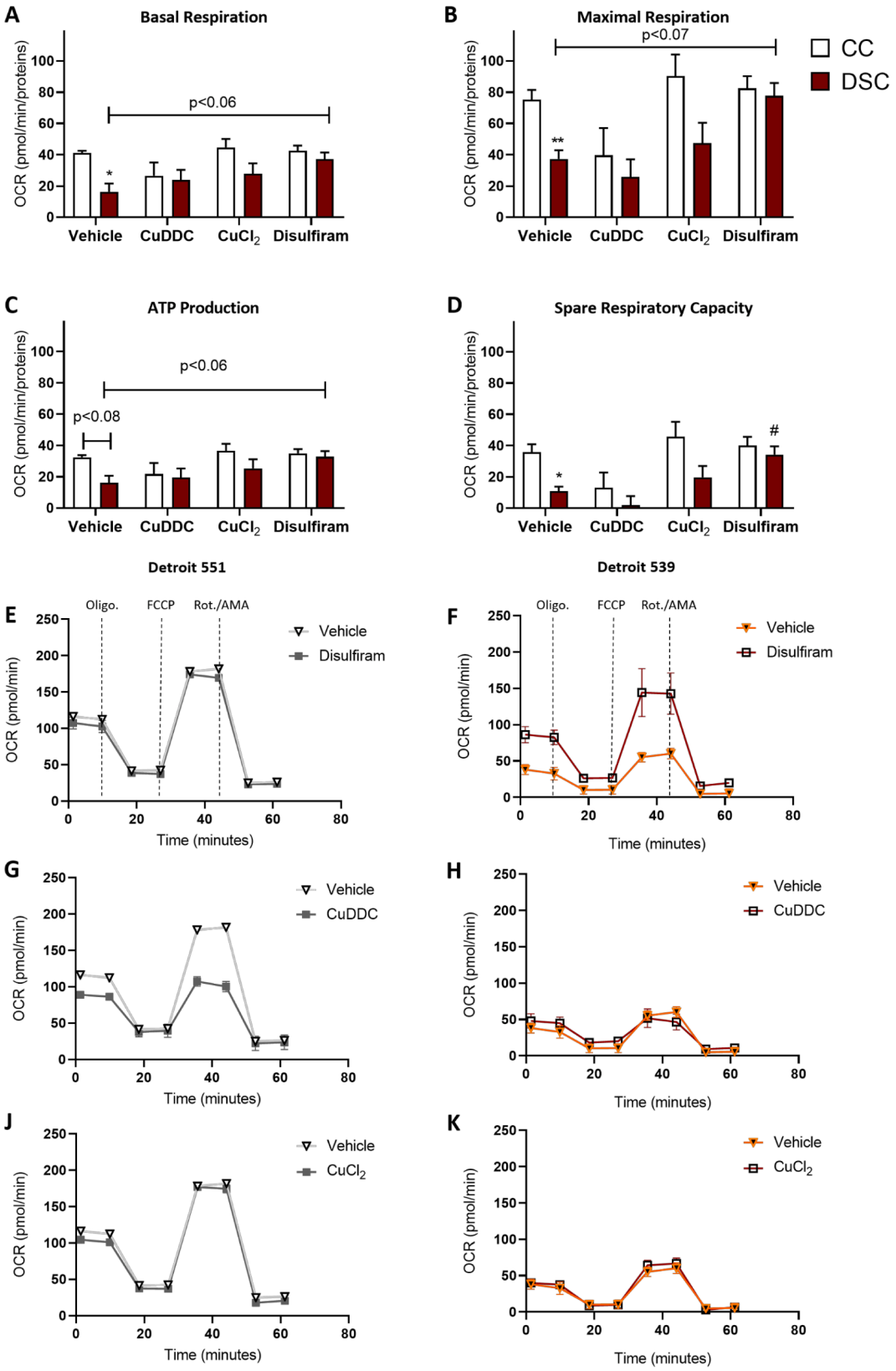

Fig. 9. Effect of disulfiram, CuDDC and $CuCl_2$ on Detroit 551 (CC) and Detroit 539 (DSC) mitochondrial respiration. Cells were treated over 24 h with 0.1 μM disulfiram, 0.3 μM CuDDC or 3 μM $CuCl_2$. A) Basal respiration; B) Maximal respiration; C) ATP production; D) Spare Respiratory Capacity; E-K) Representative experiments showing mitochondrial respiration profile of Detroit 551 cells and Detroit 539 upon treatment with DSF, CuDDC and $CuCl_2$ as compared with non-treated cells. Data represent values of n = 5 independent experiments (mean ± SEM). *$p < 0.05$ and **$p < 0.01$ indicate significant decreases in cell viability or proliferation rate of DSCs *vs.* CCs; #$p < 0.05$ indicates a significant increase of cell viability or proliferation rate of treated DSCs *vs.* non-treated DSCs.

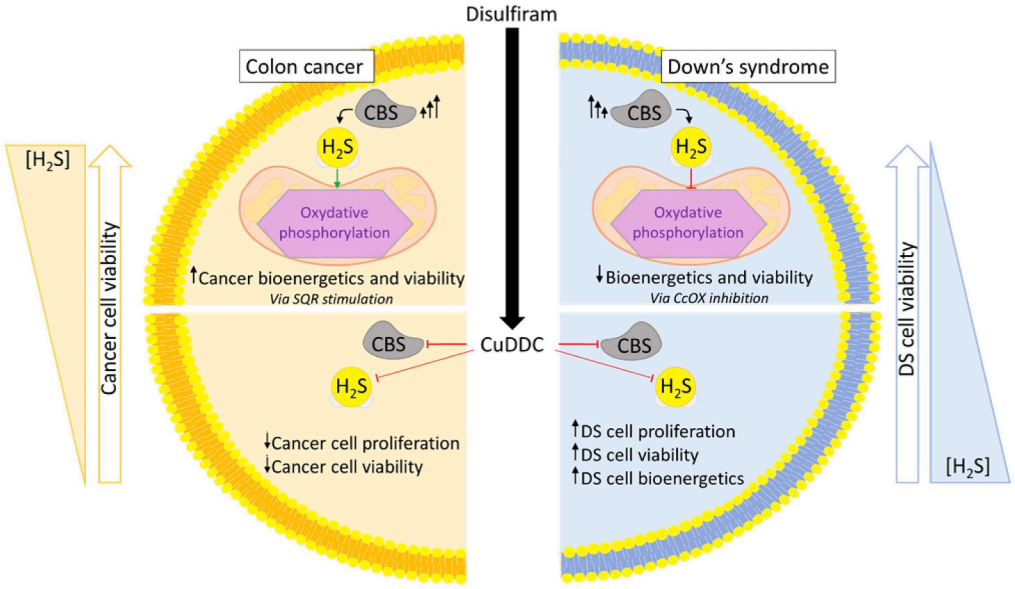

Fig. 10. Effect of disulfiram and its metabolite CuDDC on the H_2S system in cancer and Down's syndrome cell models. In colon cancer cells, the excess of H_2S supports cellular bioenergetics. Here, sulfide:quinone oxidoreductase (SQR) couples sulfide detoxification with energy production. *Vice versa*, in fibroblasts, where the sulfide oxidizing system is less represented, an increment H_2S levels may result in mitochondrial impairment because of cytochrome *c* oxidase (CcOX) inhibition. Disulfiram, through its metabolite CuDDC lowers intracellular H_2S levels through inhibition of cystathionine β-synthase and direct H_2S scavenging. This results in reduction or increase of cell viability and proliferation in colon cancer and DS fibroblasts, respectively. Disulfiram, through its metabolite CuDDC, lowers intracellular H_2S levels through inhibition of its biosynthesis, thus resulting in decrease or increase of cell viability in cancer and Down's syndrome fibroblasts, respectively.

acceptor such as cysteine, which leads to the formation of a persulfidated intermediate (Cys-SSH) instead of H_2S (which is then released through Cys-SSH non-enzymatic degradation) [59]. Herein, 3-MST activity was performed in the absence of a sulfur acceptor, a condition in which 3-MP itself likely works as a sulfur acceptor, thus producing 3-MP-SSH. The lower affinity of copper for 3-MP and, by extension 3-MP-SSH, as compared to H_2S may represent a further explanation of the weak effect of copper towards 3-MST activity.

The *in vitro* findings in DS fibroblasts presented in the current article are completely in line with the recently reported beneficial effects of disulfiram in DS rodent models [34]. However, potential pharmacological effects of disulfiram (or its metabolites) on other molecular targets cannot be excluded (see below).

Likewise, the findings with CuDDC in HCT116 cells are consistent with the role of H_2S in the maintenance of cancer cell viability and proliferation; a molecule that is a potent inhibitor of CBS and/or is a potent scavenger of H_2S would be expected to reduce cancer cell viability; similar effects were previously seen with other inhibitors of various H_2S producing enzymes in various cancer cells (reviewed in [50,3,58]). In fact, the antitumor effects of disulfiram and/or CuDDC are well known in the literature, although the mechanisms which have been previously attributed to these effects are diverse (and, up to this point, have not included the H_2S system). According to one line of thoughts, the effects of disulfiram are due to the *chelation of copper*, because when cellular copper levels are reduced, the cells have a defect in maintaining Cu-dependent chaperone proteins, Cu-binding ceruloplasmin, and various Cu-modulated enzymes like superoxide dismutases (SOD), which, in turn, diminishes their viability and makes them more susceptible to anticancer chemotherapeutic agents (reviewed in [30,44]). According to another line of thinking, the anticancer effects of disulfiram are dependent on the *presence of copper* in the cell, at least in part due to disulfiram's ability to chelate copper as CuDDC complex [49,1]. In fact, a recent study shows that the anticancer effect of disulfiram can be prevented by pretreatment of the cells with the copper chelator tetrathiomolybdate [10]. For instance, CuDDC has been shown to promote reactive oxygen species (ROS) generation; in addition, it was found to inhibit the ubiquitin–proteasome system, the P-glycoprotein multidrug efflux pump (thereby improving cancer multidrug resistance), suppressing the activation of NF-κB (thereby affecting pro-inflammatory and proliferative signalling), ultimately not only affecting the primary tumor but also cancer cell stemness and cancer metastasis [11,24,13,57]. It is clear that these effects are multiple, and this is not entirely unexpected, if we view CuDDC as a "ballistic missile with a copper 'warhead'" that is able to penetrate the target cell or may be even directly formed inside the target cell. If the principal mode of CuDDC' action involves copper chemistry, then we can expect that many enzymes will be susceptible to inhibition, and this is, indeed the case. CuDDC may owe its anticancer effect due to interaction with ALDH, which has been shown to be upregulated in different tumor types, being involved in the stimulation of cancer metabolism and drug resistance, possibly through the biosynthesis of retinoic acid [42]. Copper itself has been shown to play a role in tumor angiogenesis [31], and patients affected by different types of cancer (including colon cancer) displayed increased serum copper content [33]. As a result, copper chelation has been suggested to be a possible adjuvant in cancer therapy, to be associated with traditional chemotherapy. Indeed, in addition to CuDDC, other copper chelating agents may also exert beneficial effects in cancer treatment [15,5], thus possibly recapitulating a similar effect on CBS and CSE. Though having a larger dataset including intracellular copper levels and mobilization of cellular copper stores would offer a further insight on the mechanism of action of disulfiram and CuDDC. Certainly, disulfiram and/or CuDDC mode of action is the result of the interference with different metabolic pathways, with overlapping effects, eventually culminating with tissue-dependent outcome. In many cancer cell types H_2S has been shown to act as a metabolic fuel [50], therefore the anticancer effect of CuDDC could be summarized by reduction of H_2S bioavailability. Conversely, in fibroblasts, where the sulfide detoxifying machinery is less represented, high H_2S levels may represent a threat for cellular homeostasis, and the inhibition of H_2S-synthesizing enzymes may prove beneficial for cell viability (Fig. 10).

Although the mode of disulfiram's effect is not entirely clear, and it is clearly a drug with multiple modes of action, the drug (alone or in combination with copper) is currently evaluated as a candidate for drug repurposing in oncological therapy [50,27]; in fact the idea of delivering CuDDC complex in a single formulation has been recently proposed to have substantial advantages over disulfiram itself [36]. Indeed, even with the regular dosing of disulfiram (250–500 mg) used in the treatment of alcoholism, the plasma concentrations of disulfiram and its metabolites are in low (single-digit) μM concentration range [14,20]. These concentrations are certainly in the same concentration range where the effects of disulfiram and CuDDC were noted in the current study.

Taken together, the results presented in the current report are consistent with the conclusions that disulfiram, via its metabolite CuDDC acts as an inhibitor of CBS and a scavenger of H_2S, which, in turn, potently suppresses H_2S levels in various cell types. We hypothesize that this effect may contribute to some of the previously demonstrated pharmacological actions of disulfiram and CuDDC in various oncological and non-oncological models *in vitro* and *in vivo*. Hence, (in a cell-type-specific fashion), disulfiram and/or CuDDC may be considered as potential agents for the experimental therapy of various pathophysiological conditions associated with H_2S overproduction.

5. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

6. Ethics approval and consent to participate

All experiments were performed according to relevant guidelines and regulations. The current study does not involve human materials or patients.

7. Consent for publication

There is no contains about any individual person's data in any form and there is no need to obtain consent for publication.

Funding

This work was supported by a grant from the LeJeune Foundation to C.S.

CRediT authorship contribution statement

Karim Zuhra: Conceptualization, Methodology, Writing - original draft. **Theodora Panagaki:** Methodology. **Elisa B. Randi:** Methodology. **Fiona Augsburger:** Methodology. **Marc Blondel:** Conceptualization, Writing - review & editing. **Gaelle Friocourt:** Writing - review & editing. **Yann Herault:** Conceptualization, Writing - review & editing. **Csaba Szabo:** Conceptualization, Resources, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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