Anti-alcohol abuse drug disulfiram inhibits human PH

of its active tetrameric form through a specific cy

Quentin Spillier[1,2], Didier Vertommen[3], Séverine Ravez[4], Roma

Themans[5], Cyril Corbet[2], Olivier Feron[2], Johan Wouters[5], Rapha

[1]Medicinal Chemistry Research Group (CMFA), Louvain Drug Research Inst

Catholique de Louvain, B-1200 Brussels, Belgium.

[2]Pole of Pharmacology and Therapeutics (FATH), Institut de Recherche Exp

(IREC), Université Catholique de Louvain, B-1200 Brussels, Belgium.

[3]de Duve Institute, Université catholique de Louvain, B-1200 Brussels, Belgi

[4]UMR-S1172 - JPArc - Centre de Recherche Jean-Pierre AUBERT Neuroscien

Lille, Inserm, CHU Lille, F-59000 Lille, France

[5]Department of Chemistry, NAmur MEdicine & Drug Innovation Center (N

Université de Namur, 61 rue de Bruxelles, B-5000 Namur, Belgium.

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Abstract

Due to rising costs and the difficulty to identify new targets, drug
viable strategy for the development of new anti-cancer treatments
disulfiram (DSF), an anti-alcohol drug, to treat cancer was reported
very recently that one anticancer mechanism-of-action was highlig
the inhibition of the p97 segregase adaptor NPL4, which is esse
proteins involved in multiple regulatory and stress-response intrace
recently DSF was also reported as one of the first phosphoglycerate
inhibitors, a tetrameric enzyme catalyzing the initial step of the ser
is highly expressed in numerous cancer types. Here, we investig
relationships (SAR) of PHGDH inhibition by disulfiram analogues
of action of DSF on PHGDH via enzymatic and cell-based evaluatio
mutagenesis experiments.

Introduction

Disulfiram (bis(diethylthiocarbamoyl) disulfide = DSF), commercia
used since 1948 (FDA-approved in 1951) as an alcohol-aversive
alcohol dependence.[1] Its mechanism of action probably involves
sensitivity to ethanol by inhibition of the enzyme acetaldehyde dehy

Starting from the 2000s, numerous studies have reported anti-tum
and its repurposing in the therapy of cancer is foreseen. This woul
drug, avoiding expensive development phases before its commerci
well-controlled ADME profile[7] and a fairly broad efficiency on v
clinical models.[8]

Different mechanisms accounting for the anticancer activity of I
group of Cassidy showed for instance in 2003 that DSF was able
kappa B (NF-κB), a protein implicated in immune response, hence
of cancer cells to 5-fluorouracil (5-FU).[9] Other data evidenced tha
apoptotic cell death of breast cancer cell lines by inhibition of the
However it is only very recently that a clear anticancer mechani
when Skrott *et al.* demonstrated that an *in vivo* metabolite of DSF c

Finally, in 2016,[11] the Cantley lab demonstrated that DSF was phosphoglycerate dehydrogenase (PHGDH), the first and limiting s synthetic pathway (SSP),[12] thus suggesting that DSF itself could dis *via* an alternative mechanism-of-action. In fact, in 2011, the gr demonstrated that PHGDH silencing leads to a significant decreas several PHGDH-overexpressing cells.[13] However, tumorigenesis s need to be detailed.

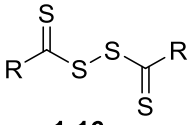
Recent results in our lab led to the identification of new PHGDH i screening campaign[14] and, similarly to the Cantley lab, DSF was 0.59 µM. Given the importance of PHGDH in cancer metabolism a repurposing DSF for cancer therapy, we set out to examine struc (SAR) in a series of DSF analogs and to elucidate its mechanism-of-

Results and Discussion

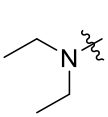
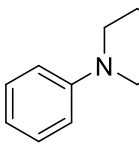
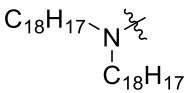
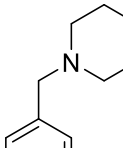
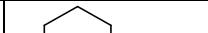
As a first step to detail our understanding of the binding of preliminary SAR were investigated around the bis(dithiocarbamate) a library of 20 DSF analogues developed earlier[15] by our team PHGDH using an isolated enzymes inhibition assay.[14] Compared

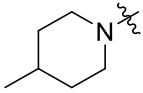
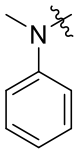
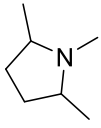
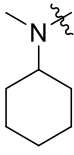
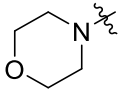
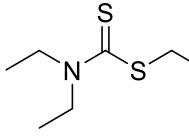
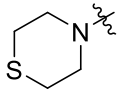
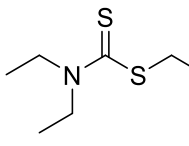
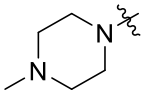
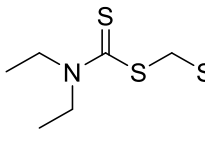
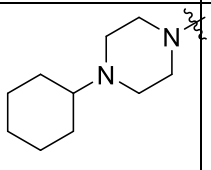
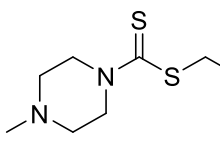
PHGDH active site (allosteric binding) or possibly (iii) covalent i
question we set out to detail the mechanism-of-action of DSF on PH

Table 1: PHGDH inhibition (IC_{50}) of DSF analogues **1-20**



1-16

Cmpd	R	PHGDH inhibition (IC_{50}, µM)[a]	Cmpd	R
1 (DSF)		0.59 [0.37-0.95]	**11**	
2		> 1 mM	**12**	
				

5		0.58 [0.44-0.77]	**15**	
6		0.42 [0.36-0.48]	**16**	
7		0.21 [0.18-0.25]	**17**	
8		0.34 [0.23-0.51]	**18**	
9		0.17 [0.14-0.21]	**19**	
10		0.57 [0.46-0.69]	**20**	

[a]All experiments to determine IC_{50} values were performed in trip

Then, both a rapid dilution and an incubation assays were per
possible formation of a covalent adduct between PHGDH and DS
other targets.[16]

As reported on **Figure 1A**, PHGDH inhibition increases, along
inhibition (100% residual activity) in the absence of DSF, to 100
incubation with DSF. These results suggest that DSF acts as a ti
PHGDH. Moreover, after a rapid dilution of the enzyme/inhibit
activity was not restored indicating that DSF shows most probably
mechanism (**Figure 1B**).

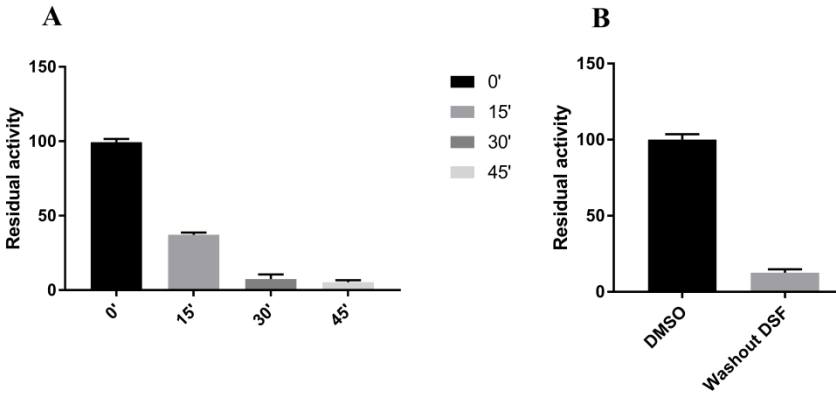


Figure 1: Characterization of PHGDH inhibition by DSF. Residu

Because previous studies showed that DSF anti-cancer activity
Skrott et al. found that the DSF metabolite diethyldithiocarbama
(CuET) is responsible for the anti-cancer activity through inhibiting
NPL4, we verified whether PHGDH inhibition could result from th
complex. To this end, we synthesized the diethyldithiocarbamat
CuEt and analyzed wtPHGDH inhibition. As a result, an IC_{50} in the
16-fold weaker compared to DSF itself, was obtained (**Figure 2**). T
although CuEt is known to be responsible, at least in part, for the a
PHGDH inhibition is not driven by the formation of this DSF metab

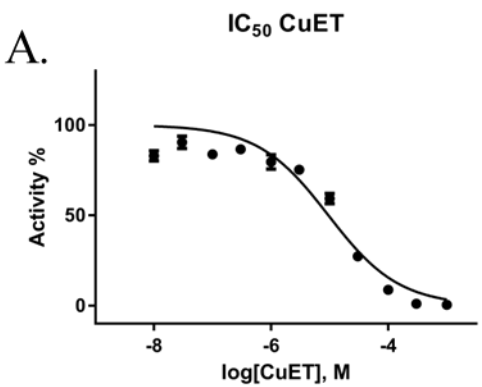
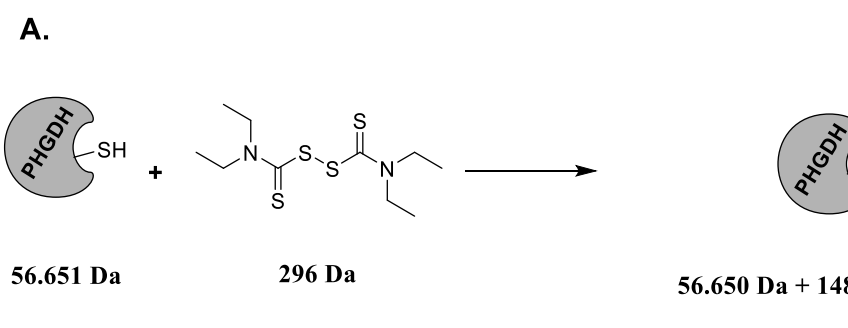


Figure 2: **A.** Dose-response curve of CuET on WT PHGDH **B.** PH
CuET [a]. All experiments to determine IC_{50} values were perform
compound dilution.Under bracket: 95 % confidence interval. Dat

to free sulfhydryl group of cysteine, providing a 462 Da size shift cysteine oxidized thus resulting in a change in migration (**Figure 3**) what we expect with DSF although with a mass shift of 147 D oxidized. As observed from **Figure 4**, when comparing PHGDH al after incubation with DSF (line C) no clear shift in mass between probably reflecting a very small shift in mass by the reaction of D residue. On the contrary, a clear and large shift in mass can be seen alone (line A) with PHGDH after incubation with AMS (line B), i several cysteine residues by AMS. Finally, when PHGDH is first then with AMS a broader mass profile is observed, suggesting, in pa AMS and DSF leading to several distinct masses (line D). Altogethe hypothesis that PHGDH is oxidized by DSF through cysteine(s) mod



groups of free (reduced) cysteine residues forming a mixed disulfi
are given for only one reactive cysteine.

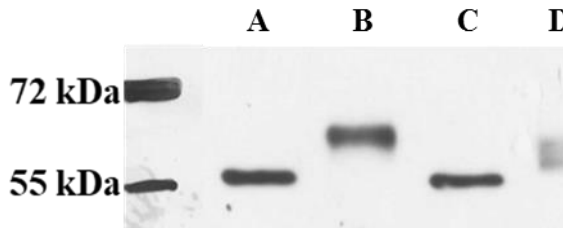


Figure 4: Western-blot of the different conditions. All samples
and/or AMS during 1h at room temperature before running on a 12
gel. **A**. PHGDH. **B**. PHGDH with 2mM AMS. **C**. PHGDH with
with 100µM DSF and 2mM AMS. Original uncropped Wester
Supplementary Information File Figure S3.

To unambiguously confirm this hypothesis and possibly ident
involved in this interaction, mass spectrometry experiments v
PHGDH was incubated for 30min with various concentrations of

relative abundances before and after DSF treatment based on the
peptides sequences. From these 12 cysteine residues, 9 are not oxi
and 3 are subject to oxidation (Cys111, 116, 281), although at
Supporting Information Table S1). Among these 3 cysteine resid
to a lesser extent Cys111, are found to be oxidized by DSF at the IC
µM (**Figure 5**). Interestingly, these data are in agreement with rece
Marletta[18] which demonstrated that PHGDH could also be inh
nitrosation of the same Cys116 residue. This particular cysteine th
role in PHGDH activity and moreover could constitute a novel in
inhibition.

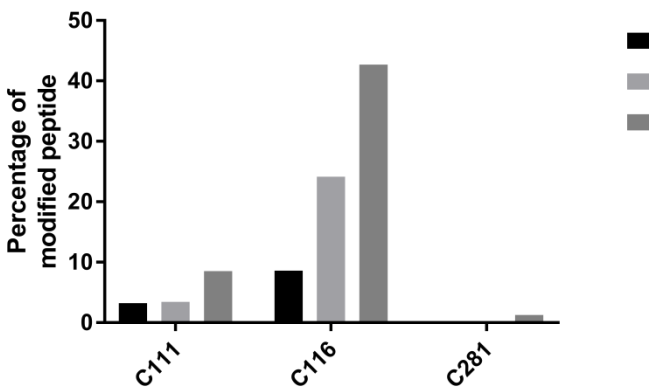


Figure 5: Percentage of the three oxidized cysteine residues (C111,

serine residue (PHGDH C116S). Interestingly, this mutant is kn
activity comparable to the wild type enzyme.[18] We actually showe
C116S mutant is only weakly inhibited by DSF, with a 20-fold
potency, in comparison to the wild type enzyme (**Table 2**). Altho
clear indication that C116 oxidation is critical for PHGDH inhibi
oxidation of other cysteine's such as C111 and C281 might be invo
albeit to a lesser extent as demonstrated recently in the works of Ma

Table 2: WT and C116S PHGDH inhibition (IC_{50}) of DSF

PHGDH	**PHGDH inhi**
Wild Type	0.59
C116S mutant	10.23

[a]All experiments to determine IC_{50} values were performed in trip
dilution, and all IC_{50} values were averaged when determined in
experiments. Under bracket: 95 % confidence interval.

With a view to understand the interaction of DSF with PHGDH on

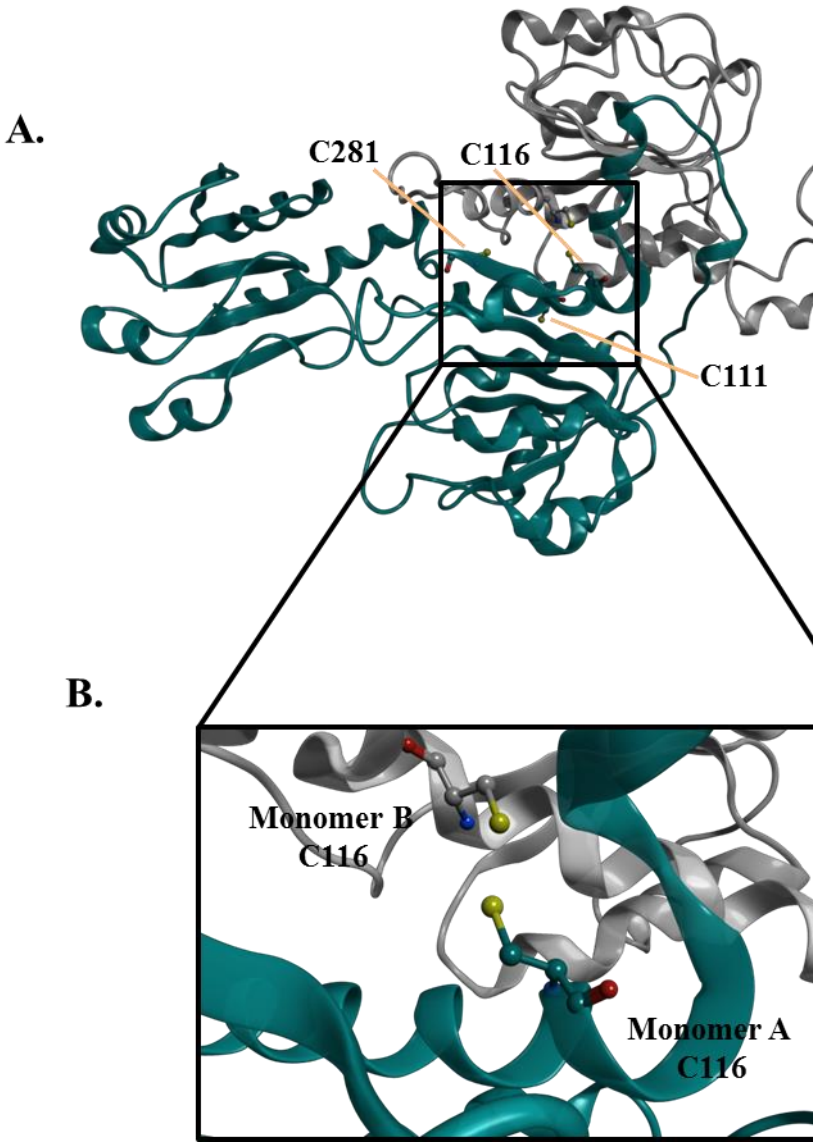


Figure 6: **A**. Overview of the PHGDH cysteine residues (111, 116 a

the Cys116 residue by DSF would similarly lead to PHGDH inhibit
oligomeric state.

To confirm this hypothesis, a cross-linking experiment, using bis-s
(BS3) as cross-linker, was finally undertaken with PHGDH alone o
with increasing concentrations of DSF. As clearly observed from **F**
alone is in a tetrameric form as previously reported,[11] PHGDH inh
concentration-dependent shift from the tetrameric to the dimeric, a
monomeric, form of PHGDH, thus corroborating our hypothesis
induce the formation of disulfide bridges through the formation of
intermediate as exemplified on **Figure 3A**,[19] the results obtained he
inhibit PHGDH by disruption of the active tetramer either into a
from the formation of a disulfide bridge between two Cys116
monomers, or to a lesser extent to an inactive diethyl(dithi
monomer.

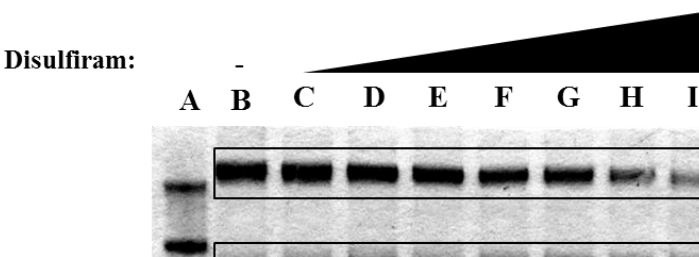


Figure 7: Cross-linking experiment of PHGDH with BS3 at variou
MW marker. **B**. 0 µM. **C**. 1 µM. **D**. 5 µM. **E**. 10 µM. **F**. 50 µM. **G**
500 µM.). PHGDH was incubated with DSF during 30' before c
used as control without DSF. Lane A (MW marker) was used to d
state of PHGDH. Original exposure of the uncropped gel is availa
Information File (Figure S4) .

Finally to detail the relationship between the ability of DSF to disr
its anti-cancer activity, we set out additional experiments aiming to
on two cancerous cell lines : UM-UC-3 human transitional cell can
express PHGDH (UM-UC-3-PHGDH+) and a variant of these
PHGDH (UM-UC-3-PHGDH-) (**Figure 8**)

When cells that do not express PHGDH (UM-UC-3-PHGDH-) are
cell proliferation inhibition (IC_{50}) of 3,64 µM is obtained, whereas
(UM-UC-3-PHGDH+) appear to be more susceptible to DSF treat
µM. Although the difference remains weak (~5-fold), proba
proliferative mechanisms are involved upon DSF treatment, tumor c
is higher for cells expressing PHGDH.

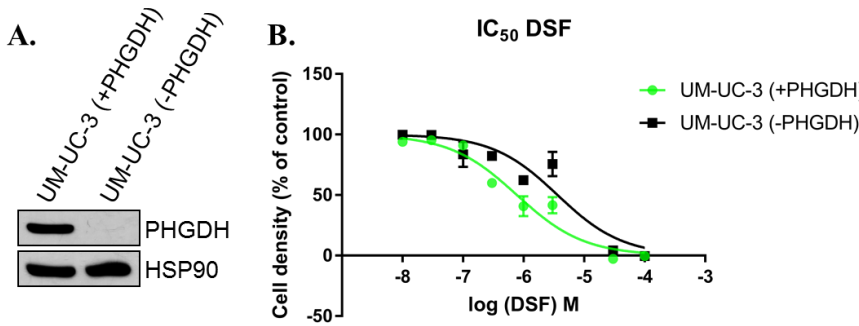


Figure 8: A. Representative immunoblot for PHGDH on UM-UC response curves of DSF. C. UM-UC-3-PHGDH- and UM-UC-3-P inhibition by DSF. All experiments to determine IC50 values were compound dilution, and all IC50 values were averaged on tw experiments. Under bracket: 95 % confidence interval.

Conclusion and Perspectives

In conclusion, in this paper, we have shown that DSF, a dehydrogenase inhibitor used as a treatment for chronic alcohol analogues are PHGDH inhibitors. Through biochemical and mass s we detailed the mechanism-of-inhibition of PHGDH by DSF involves the disruption of the active PHGDH tetramer into either an linked by a disulfide bridge involving Cys116 on adjacent monome

action involving PHGDH inhibition. Also, the maximum plasm
(Cmax) being reported to be 1.28 μM, that is around 2-fold highe
PHGDH in the cell-based assay (0.77μM), one can hypothesized tha
could be sufficient to provide anticancer activity *via* PHGDH inhibi

Methods

PHGDH Assay. Enzymatic assay was adapted from a previous
NADH fluorescence emission (Ex 340 nm/Em 460 nm) was follow
performed in PHGDH assay buffer (50 mM Tris, pH 8.5, and 1 m
enzyme concentrations were as follows: 3-PG, 240 μM; NAD^+, 12
PHGDH, 12 ng/μL; PSAT1, 20 ng/μL. The final concentration of D
was set to 5%.

Dilution Experiment. Dilution experiment was conducted followi
DSF (5 μM) or DMSO control was incubated with PHGDH for 45
DSF (5 μM) was included as a positive control for inhibition.

WT and C116S PHGDH Purification. pET28a human PHGDH a
PHGDH were transformed into BL21 *Escherichia coli*. A single
OD_{600} 0.6 in 1 L of Luria broth. Protein expression was induc

elution buffer (50 mM Tris, pH 8.5, 10 mM $MgCl_2$, 250 mM NaC
imidazole) and collected (1-mL fractions). Fraction protein con
Bradford assay. The most concentrated fractions were pooled and d
of dialysis buffer (50 mM Tris, pH 8.5, 10 mM $MgCl_2$, 250 mM N
2-mercaptoethanol). Protein purity was assessed via SDS/PAGE and

Mass spectrometry experimentsOrbitrap Lumos. Mass spectr
carried out on an Orbitrap Lumos, following a previously repor
modifications.[21] A local protein database containing the human PH
Uniprot O43175) was used to process the obtained MS/MS data.
ppm for precursor ions and 0.6 Da for fragment ions. Oxida
(+147.025 Da) and carbamidomethyl (+57.021 Da) were considered
on Cys.

Cross-linking experiments. PHGDH (3 μg) was incubated with D
50 μM, 100 μM, 250 μM, 500 μM) or vehicle control (DMSO) in
and 1 mM NAD^+ in 18 μL total volume for 30 min on ice. BS3 (S
dissolved in PBS was added to a final concentration of 5 mM and i
shaking at room temperature. The reaction was then quenched for 1
pH 7.5, to a final concentration of 28 mM. Cross-linked proteins

(DSMO) was added and cells were grown for 48 hours. Viability

Blue reagent (Life Technologies) according to manufacturer's instru

Chemicals. All reagents were purchased from chemical sup purification. Copper/DSF complex was obtained according procedure.[22]

Immunoblots. Western blot and immunoblot analysis were perfo procedure [23].

Author information

Corresponding Author: Pr. Raphaël Frédérick

*Phone: 32 (2)764 73 41. Fax: 32 (2)764 73 63. E-mail: raphael.fred

Author Contributions

Q.S., R.F., O.F. and J.W. designed the experiments, analyzed the manuscript. Q.S. performed all experiments except the cross-linkin analysis (done by D.V.). Q.S., S.R. and R.M. performed the scree developed the enzymatic assay. C.C developed the UM-UC-3 (PH Q.T. prepared the figures. R.F. supervised all aspects of the project.

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