Cancer incidence is rising and this global challenge is further exacerbated by tumour resistance to available medicines. A promising approach to meet the need for improved cancer treatment is drug repurposing. Here we highlight the potential for repurposing disulfiram (also known by the trade name Antabuse), an old alcohol-aversion drug that has been shown to be effective against diverse cancer types in preclinical studies. Our nationwide epidemiological study reveals that patients who continuously used disulfiram have a lower risk of death from cancer compared to those who stopped using the drug at their diagnosis. Moreover, we identify the ditiocarb–copper complex as the metabolite of disulfiram that is responsible for its anti-cancer effects, and provide methods to detect preferential accumulation of the complex in tumours and candidate biomarkers to analyse its effect on cells and tissues. Finally, our functional and biophysical analyses reveal the molecular target of disulfiram’s tumour-suppressing effects as NPL4, an adaptor of p97 (also known as VCP) segrease, which is essential for the turnover of proteins involved in multiple regulatory and stress–response pathways in cells.

Despite advances in the understanding of cancer biology, malignant diseases have a high global toll. Furthermore, the increasing average human life expectancy is predicted to have demographic consequences, including an increase in the incidence of cancer. The high cancer-associated morbidity and mortality highlight the need for innovative treatments. Given the high costs, failure rate and long testing periods of developing new drugs, using drugs that are approved for the treatment of diverse diseases as candidate anti-cancer therapeutics represents a faster and cheaper alternative, benefitting from available clinically suitable formulations and evidence of tolerability in patients. Among promising cancer-killing drugs is disulfiram (tetraethylthiuram disulfide, DSF), a drug that has been used for over six decades as a treatment for alcohol dependence, with well-established pharmacokinetics, safety and tolerance at the US Food and Drug Administration (FDA)-recommended dosage. In the body, DSF is metabolized to ditiocarb (diethylidithiocarbamate, DTC) and other metabolites, some of which inhibit liver aldehyde dehydrogenase. Because DSF showed anti-cancer activity in preclinical models and because adjuvant DTC was used to treat high-risk breast cancer in a clinical trial, DSF emerges as a candidate for drug repurposing in oncology. Additional advantages of DSF include a broad spectrum of malignancies sensitive to DSF, and its ability to also target the stem-like, tumour-initiating cells. Although the mechanism of DSF’s anti-cancer activity remains unclear and it has been suggested that the drug inhibits proteasome activity, it has been shown that DSF chelates bivalent metals and forms complexes with copper (Cu), which enhances its anti-tumour activity. In addition to the lack of a well-defined mechanism of action in cancer cells, the main obstacles for DSF repurposing have been: (i) uncertainty about the active metabolite(s) of DSF in vivo; (ii) the lack of assays to measure these active derivative(s) in tumours; (iii) missing biomarker(s) to monitor the impact of DSF in tumours and tissues; (iv) the lack of insights into the preferential toxicity towards cancer cells compared to normal tissues; and (v) the absence of a specific molecular target that could explain the potent anti-tumour activity of DSF. Here, we combine experimental approaches and epidemiology to address the important characteristics of DSF in relation to cancer, pursuing the goal of repurposing DSF for cancer therapy. We identify the active metabolite of DSF, and provide biological validation and mechanistic insights, including the discovery of a biologically attractive protein that has previously not been considered as the target for the anti-cancer activity of DSF.

**Epidemiological analyses of DSF and cancer**

The relative lack of cancer-related clinical trials with DSF prompted us to explore whether DSF use might reduce cancer mortality at a population level. Using the Danish nationwide demographic and health registries, we estimated hazard ratios of cancer-specific mortality associated with DSF use among patients with cancer for the first time during 2000–2013 (see Methods, Table 1 and Extended Data Fig. 1a). DSF users were categorized as (i) previous users, who were patients during 2000–2013 (see Methods, Table 1 and Extended Data Fig. 1a). DSF users were categorized as (i) previous users, who were patients during 2000–2013 (see Methods, Table 1 and Extended Data Fig. 1a).
used DSF. Notably, we also found reduced cancer-specific mortality for cancer overall (Table 1), as well as for cancers of the colon, prostate and breast among continuing users compared to previous DSF users (Extended Data Fig. 1a). Stratification by clinical stage (Table 1) revealed reduced cancer-specific mortality with continuing use of DSF even among patients with metastatic disease. Although it is not possible to draw conclusions about causality, these findings supported the hypothesis that DSF may exert anti-cancer effects among patients suffering from common cancers, prompting us to perform pre-clinical analyses.

**Anti-tumour activity of the DTC–copper complex**

Because DSF anti-cancer activity has been suggested to be copper-dependent, we compared groups of mice injected with human MDA-MB-231 breast cancer cells, fed with (i) normal diet; (ii) normal diet plus copper gluconate (CuGlu); (iii) normal diet plus DSF; or (iv) normal diet plus DSF and CuGlu (DSF/CuGlu) and tumour volume was measured over time (Fig. 1a and Extended Data Fig. 1b, c). Compared to matched controls, tumour volume in DSF- and DSF/CuGlu-treated groups at 32 days (at DSF doses equivalent to those used by alcoholics) was suppressed by 57% and 77%, respectively ($P = 0.0038$ in favour of the DSF/CuGlu treatment versus DSF alone). These results validate previous *in vitro* and *in vivo* studies, which indicated that DSF is an efficient anti-cancer agent and that copper potentiates its activity. As the reactive metabolite DTC forms complexes with metals, particularly copper, we argued that a DTC–copper complex (bis (diethyldithiocarbamate)–copper (CuET)) forms *in vivo* (Extended Data Fig. 1d), providing the anti-cancer metabolite. To test this hypothesis, we developed a high-resolution approach based on high-performance liquid chromatography–mass spectrometry to measure CuET in tissues, and readily detected CuET after a single oral dose of DSF (Extended Data Fig. 1e, f). Extracts from plasma, liver, brain and MDA-MB-231-xenografted tumours contained CuET in samples from mice treated for five days with DSF or DSF/CuGlu. The CuET levels in plasma and liver were slightly higher after DSF/CuGlu treatment compared to DSF alone. Notably, the CuET levels in the tumour specimens were almost an order of magnitude higher compared to corresponding levels in liver and brain tissues from the same animals (Fig. 1b), suggesting preferential accumulation of CuET in tumours. Importantly, we also confirmed formation of CuET in humans undergoing DSF treatment for alcoholism (Fig. 1c).

Next, we synthesized CuET and performed comparative cell culture and animal studies. Short-term (24-h) assays and long-term (colony-forming assay, CFA) assays consistently showed higher cytotoxicity of CuET than of the primary DSF metabolite DTC in various cancer cell lines (Fig. 1d and Extended Data Fig. 1g). The half-maximal lethal dose (LD$_{50}$) values of CuET in CFA experiments were $\leq 100$ nM in three out of three tested breast cancer cell lines and similar potency was observed among cell lines derived from human lung, colon and prostate tumours (Extended Data Fig. 2a). These data were corroborated by tetrazolium dye ((2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2h-tetrazolium–5-carboxanilide) (XTT))-based 48-h cytotoxicity tests on a wider panel of cell types (Extended Data Fig. 2b). Unexpectedly, only the most sensitive cell lines (for example, AMO-1, Capan1) showed markers of apoptosis, which included annexin V and activated caspases, whereas in most cell lines, for example, MDA-MB-231 and U2OS cells, CuET induced apoptosis-independent cell death (Extended Data Fig. 2c–f).

Direct therapeutic effects of CuET *in vivo* were then investigated using the MDA-MB-231 breast cancer (Fig. 1e) and AMO-1 myeloma (Fig. 1f) xenograft models treated intraperitoneally with a CuET–albumin formulation, with which the anti-tumour activity and good tolerability of this DSF metabolite was confirmed (Extended Data Fig. 1h, i).

**CuET inhibits p97-dependent protein degradation**

Next, we investigated the interaction between CuET and cellular protein degradation, one of the suggested explanations for anti-tumour effects of DSF. We confirmed that CuET induces phenotypic features shared with proteasome inhibitors, such as MG132 or bortezomib (BTZ), including accumulation of poly-ubiquitylated (poly-Ub) proteins (Fig. 2a and Extended Data Fig. 3a), rapid deubiquitylation of histone H2A (H2A)$^{19}$ (Extended Data Fig. 3b) and accumulation of ubiquitylated proteins in the cytoplasm $^{19}$ (Extended Data Fig. 3c). Furthermore, TNF (also known as TNFα)-induced degradation of IκBα (ref. 20) was blocked after 1-h treatment with CuET or BTZ (Fig. 2b). Finally, CuET inhibited degradation of Ub(G76V)–GFP (an ubiquitin–fusogenic degradation substrate)$^{21}$ in a dose-dependent manner (Fig. 2c). However, although these data confirmed a defect in protein degradation, CuET had no effect on the CT-like, C-like or T-like activity of the 20S proteasome$^{22}$ (Extended Data Fig. 3d, e). This was further corroborated by the lack of a stabilizing effect of CuET on p53 tumour suppressor protein in dicoumarol-treated cells, in which

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**Table 1 | Cancer-specific mortality associated with DSF use among Danish patients with cancer**

| Cancer type | Overall | Localized stage | Non-localized stage | Unknown stage |
|-------------|---------|----------------|--------------------|--------------|
|             | Number* | HR 95% CI | Number* | HR 95% CI | Number* | HR 95% CI | Number* | HR 95% CI |
| Any cancer† | 3,038 | 1.00 | 1,429 | 1.00 | 1,054 | 1.00 | 555 | 1.00 |
| Previous users | 1,177 | 0.66 | 0.58–0.76 | 0.000 | 355 | 0.71 | 0.59–0.87 | 0.001 |
| Continuing users | 236,950 | 0.68 | 0.64–0.73 | 0.000 | 73,933 | 0.80 | 0.73–0.88 | 0.000 |
| No prescriptions | 49,663 | 0.66 | 0.62–0.71 | 0.000 |

Hazard ratios (HR) and 95% confidence intervals (CI) comparing continuing and previous users of DSF, relative to the time of their cancer diagnosis. For DSF exposure categories, statistics and clinical stages, see Methods.

*Number of patients included.
†Except cancers of the liver and kidney.
CuET (1 μM) on CDC25A versus HIF-1α shows a differential effect of Bortezomib (BTZ) (1 μM), CuET (1 μM), and DBeQ (10 μM) on CDC25A versus HIF-1α in MG132-pretreated (4 h, 5 μM) cycloheximide (CHX, 1 h, 50 μg ml⁻¹)-exposed HeLa cells. BTZ (8 h, 1 μM) induces NRF1 120-kDa (top arrow) and 110-kDa (bottom arrow) forms; whereas CuET (8 h, 0.5 μM) only induced the non-cleaved 120-kDa form in NIH3T3 cells. FRAP quantification in U2OS Ub–GFP cells: slower mobility of accumulated cytoplasmic GFP–Ub after a 2-h pre-treatment with NMS873 (10 μM), CuET (1 μM) or BTZ (1 μM). a, b, d–g. Data are representative of two independent biological experiments. Data are linked means and individual values (c) and relative mean signal of the bleached region from 12 cells per treatment (g).

p53 turnover depends on the core 20S proteasome independently of ubiquitin23,24. In contrast to CuET, treatment with the 20S proteasome inhibitor BTZ stabilized p53 irrespective of dicoumarol (Extended Data Fig. 3f), indicating that 20S proteasome-dependent protein turnover remains operational with CuET treatment. Furthermore, CuET failed to inhibit 26S proteasome activity (Extended Data Fig. 3g), which was inferred from RPNI1-dependent deubiquitylation25. Collectively, these results suggest that CuET stabilizes ubiquitylated proteins by blocking a step upstream of the proteasome.

Next we considered p97-dependent processing of poly-Ub proteins, as this pathway operates upstream of the proteasome and its malfunction resembles phenotypes of proteasome inhibition26. Unlike BTZ or MG132, CuET induced only modest accumulation (a small subfraction) of HIF-1α (Fig. 2d), consistent with reported modest accumulation of HIF-1α after knockdown of p97 compared to cells with inhibited proteasomes27. Next, we pre-treated cells with MG132, followed by wash-off and 1-h cycloheximide (an inhibitor of translation) treatment combined with BTZ, CuET or DBeQ (a direct inhibitor of p97 ATPase activity)28. All tested inhibitors prevented degradation of CDC25A (a known p97 target)29, whereas degradation of the mostly p97-independent target, that is, most of HIF-1α22, was inhibited only by BTZ (Fig. 2e). Furthermore, consistent with cleavage of the 120-kDa species of the endoplasmic reticulum-tethered transcription factor NRF1 into an active 110-kDa form being a p97-dependent process30, appearance of the cleaved NRF1 form was inhibited by both CuET and NMS873 (another p97 ATPase inhibitor) (Fig. 2f and Extended Data Fig. 4a, b). These results suggest that the p97 pathway is compromised in cells treated with CuET.

Next, we asked whether CuET impairs the p97 segregase activity that extracts poly-Ub proteins from cellular structures, such as the endoplasmic reticulum, Golgi apparatus or chromatin for subsequent proteasomal degradation31. Using fluorescence recovery after photobleaching (FRAP) to investigate the mobility of accumulated poly-Ub proteins, we found that whereas GFP–ubiquitin in DMSO- or BTZ-treated cells diffused rapidly into bleached areas, this diffusion was slower after treatment with CuET or NMS873 (Fig. 2g and Extended Data Fig. 4c). This suggests that after treatment with CuET or NMS873 at least a subset of the accumulated poly-Ub proteins remains immobile, probably embedded into cellular structures. Consistently, upon detergent pre-extraction of mobile proteins, we observed greater immuno-fluorescence signals of extraction-resistant poly-Ub (K48) proteins (destined for proteasomal degradation) in NMS873- and CuET-treated cells compared to BTZ- or DMSO-treated controls (Extended Data Fig. 4d).

Western blot analysis of endoplasmic reticulum-rich microsomal fractions also revealed enrichment of poly-Ub proteins after CuET and NMS873 treatment (Extended Data Fig. 4e). Malfunction of p97 segregase is furthermore associated with a cellular unfolded protein response (UPR)32. We confirmed UPR in cells treated with CuET or NMS873 by detecting increased markers of UPR induction, including the spliced form of XBP1s, ATF4 and phosphorylated (p-) eIF2α33 (Extended Data Fig. 4f).

These studies are also of clinical relevance, because inhibition of p97 was suggested as an alternative treatment strategy for myeloma patients who had relapsed after therapy with BTZ (also known by the trade name Velcade)34 or carfilzomib (CFZ)35. Thus, we performed cytotoxicity tests with CuET on a panel of BTZ- or CFZ-adapted and non-adapted human cell lines or on cells derived from samples of patients with myeloma before therapy and with BTZ therapy. All pairs of adapted and non-adapted cells showed similar sensitivity to CuET treatment, in contrast to BTZ (Extended Data Fig. 5a–d). These results suggest that treatment with DSF (best combined with copper) or CuET might become a feasible therapeutic option for patients with relapsed, BTZ-resistant multiple myeloma.

**CuET binds and immobilizes NPL4**

To elucidate how CuET inhibits the p97 pathway, we first used an assay of p97 ATPase activity36. In contrast to treatment with NMS873, CuET had no effect on p97 ATPase activity (Extended Data Fig. 6a). Because NPL4 and UFD1 proteins are key components of the p97 segregase31, we examined whether CuET might target the pathway through these cofactors. Ectopic overexpression of NPL4–GFP, but not UFD1–GFP or p97–GFP, reduced CuET cytotoxicity, suggesting that NPL4 is a candidate target of CuET (Fig. 3a and Extended Data Fig. 6b). An analogous ‘rescue effect’ of ectopic NPL4–GFP was apparent from the reduction in accumulation of poly-Ub proteins caused by CuET (Extended Data Fig. 6c).

As shown by live-cell imaging, 2–3-h exposure to CuET induced prominent nuclear clustering of NPL4–GFP but not of UFD1–GFP or p97–GFP (Fig. 3b). Within 2–3 h, most of cellular NPL4–GFP became immobilized in nuclear clusters and also in cytoplasmic areas, as shown by FRAP (Fig. 3c). CuET-induced immobilization of endogenous NPL4 was confirmed by accumulation, which was detectable by western blot, in the detergent-insoluble fractions from various cell lines (Fig. 3d) and by immunofluorescence on pre-extracted cells (Extended Data Fig. 6d). Notably, the immobilization of NPL4 was also detectable in pre-extracted sections of cryopreserved tumours from mice treated with DSF or DSF and CuGlu, thus providing a potential biomarker of CuET activity towards the p97 pathway in vivo (Fig. 3e).

NPL4 is an attractive candidate for CuET binding, because this protein contains two zinc finger domains: a C-terminal NZF (NPL4–zinc finger) and a putative zinc finger–NPL4 zinc finger, which bind bivalent metals and metal complexes that might chemically resemble CuET37.

**Figure 2 | CuET inhibits p97 segregase-dependent protein degradation.**

a, CuET causes accumulation of poly-ubiquitylated proteins in MCF7 cells. b, TNF-induced IκBα degradation is compromised after 1-h treatment with CuET or BTZ. c, Dose-dependent inhibition of Ub(G76V)–GFP degradation by CuET. HeLa cells were treated for 3 h. n = 3 experiments. d, HIF-1α levels after 2-h treatments with MG132 (5 μM), CuET (1 μM), BTZ (1 μM) in HeLa cells. e, Differential effect of BTZ (1 μM), CuET (1 μM) and DBeQ (10 μM) on CDC25A versus HIF-1α in MG132-pretreated (4 h, 5 μM), cycloheximide (CHX, 1 h, 50 μg ml⁻¹)-exposed HeLa cells. f, BTZ (8 h, 1 μM) induces NRF1 120-kDa (top arrow) and 110-kDa (bottom arrow) forms; whereas CuET (8 h, 0.5 μM) only induced the non-cleaved 120-kDa form in NIH3T3 cells. g, FRAP quantification in U2OS Ub–GFP cells: slower mobility of accumulated cytoplasmic GFP–Ub after a 2-h pre-treatment with NMS873 (10 μM), CuET (1 μM) or BTZ (1 μM). a, b, d–g. Data are representative of two independent biological experiments. Data are linked means and individual values (c) and relative mean signal of the bleached region from 12 cells per treatment (g).
NPL4 aggregates trigger a heat-shock response

Although the nuclear NPL4 clusters occupied DAPI-unlabelled areas of chromatin (Extended Data Fig. 6d) co-localization with DAPI-excluded structures, such as nucleoli and nuclear speckles, were not found (Extended Data Fig. 7a). In late-G2 cells, NPL4 clusters were evidently excluded from the partially condensed chromatin (Extended Data Fig. 7b), suggesting that the NPL4 aggregates exclude chromatin rather than accumulating in specific nuclear areas. Both the nuclear clusters and the immobilized cytoplasmic NPL4 co-localized with poly-Ub proteins (confirmed by anti-Ub(K48) and FK2 antibodies), small ubiquitin-like modifiers (SUMOs) (only in nuclei) and with TDP43 (Extended Data Fig. 7c, e). Blockade of cellular ubiquitylation with a chemical inhibitor (MLN7243) of the El ubiquitin-activating enzyme failed to prevent either NPL4–GFP nuclear aggregation or cytoplasmic immobilization (Extended Data Fig. 7d), excluding the immobilization of NPL4 via recognition of ubiquitylated and SUMOylated substrates, but rather suggesting that immobilized NPL4 attracts ubiquitylated proteins or proteins that subsequently become ubiquitylated and/or SUMOylated. A key protein commonly associated with intracellular protein aggregates is HSP70, a chaperone implicated in aggregate processing42. Indeed, pre-extracted cells showed co-localization of HSP70 with both CuET-induced NPL4(WT)–GFP and spontaneous NPL4(MUT)–GFP aggregates (Fig. 4b, c). Both the CuET-induced NPL4(WT) aggregates and spontaneous NPL4(MUT) aggregates also co-localized with p97 (Extended Data Fig. 7f, g), as is particularly evident after pre-extraction. In the NPL4–GFP model, the amount of p97 immunoreactivity within...
that enhances the anti-tumour effects of DSF; it is unlikely that another DSF metabolite could represent the major anti-cancer agent as levels of non-CuET metabolites should be lowered by copper addition. We also present a method for CuET detection in tissues and plasma, as well as data suggesting that preferential accumulation of CuET in tumours may contribute to cancer cell toxicity, consistent with the high therapeutic tolerability of DSF, as documented even after years of daily administration at doses comparable to those we used in our mouse experiments. Considering the numerous studies on DSF and diverse opinions about the potential target of its anti-cancer effects, identification of NPL4, a key component of the p97–NPL4–UFD1 segregase complex, as the molecular target of CuET is surprising. The CuET–NPL4 interaction leads to rapid formation of protein aggregates and immobilization of this otherwise very mobile multifunctional protein complex, resulting in a severe phenotype, induction of HSR and eventually cell death. While additional potential targets of CuET cannot be excluded, the malfunction of the p97 pathway due to the NPL4–p97 aggregate formation explains the major cell phenotypes and the consequent cell death. Our work also reconciles the controversial studies, suggesting that the proteasome is the DSF target, by demonstrating that neither 20S nor 26S proteasome, but the processing of ubiquitylated proteins by the NPL4-dependent segregase, is targeted by CuET. Our results support the notion that the p97–NPL4 pathway is a promising therapeutic target in oncology. Indeed, reports on p97 overabundance correlating with progression and metastasis of carcinomas of the breast, colon and prostate are consistent with our present nationwide epidemiological analysis, which revealed an association between continued use of DSF and favourable prognosis, an intriguing finding that should be investigated further, particularly given the currently limited therapeutic options for patients with metastatic cancer. From a broader perspective, our study illustrates the potential of multifaceted drug-like small molecules identifies disulfiram as an inhibitor of prostate cancer cell growth. Clin. Cancer Res. 15, 6070–6078 (2009).

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### Supplementary Information

Supplementary Information is available in the online version of the paper.

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**Artificial ReSeArcH**

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METHODS

The experiments were not randomized. Epidemiological analyses and access to health registers. We conducted a population-based cohort study by combining Danish nationwide demographic and health registers. This study was approved by the Danish Data Protection Agency and Statistics Denmark’s Scientific Board. As the epidemiological study was based solely on register data and did not involve any contact with patients, no ethical approval was required from the Danish Scientific Ethical Committee. The cohort consisted of all Danes aged ≥85 years with a first-time diagnosis of cancer before January 2000 and December 2013. Because DSF (Antabuse) is a relative contra-indication among individuals with liver or kidney diseases, we excluded patients with cancers of the liver or kidney from the cohort. Cohort members were categorized according to use of DSF into two main groups: (i) those who filled at least one prescription of DSF within five years before the cancer diagnosis, but did not fill DSF prescription(s) during the first year after the diagnosis (previous users), that is, individuals suffering from alcoholism but taking DSF only before their cancer diagnosis; and (ii) those who used DSF before their cancer diagnosis and also filled one or more DSF prescriptions during the first year after the cancer diagnosis (continuing users), that is, individuals who underwent DSF therapy both before and after the cancer diagnosis. We also defined a category of patients with cancer who did not fill prescription(s) for DSF either before or after (≤1 year) the cancer diagnosis (never users). In the main analyses, we calculated hazard ratios and 95% confidence intervals estimating cancer-specific mortality among continuing DSF users compared to previous DSF users based on a Cox model regressing on both propensity scores and disulfiram use. By including propensity scores in the regression, we used demographic and comorbid conditions/diagnostic codes as well as prescription data for selected concomitant drugs, to balance baseline characteristics of previous and continuing users of DSF and to adjust estimated hazard ratios of cancer-specific mortality associated with DSF use. The patients with cancer were followed from one year after the diagnosis until death, migration or end of study (31 December 2014). The propensity scores thus estimate the probability of being treated with DSF in the exposure window 0–1 year after the cancer diagnoses conditional on the following other covariates in the calculation of propensity scores using logistic regression: gender, age at diagnosis, calendar time, highest achieved education and disposable income; medical histories of diabetes mellitus, chronic obstructive pulmonary disease, ischaemic heart disease, congestive heart failure, cerebrovascular disease, atrial fibrillation or atrial flutter, dementia and ulcer disease; and use of non-steroidal anti-inflammatory drugs (NSAIDs; including aspirin), non-aspirin antithrombotic agents (anticoagulants), statins, antihypertensive medication, other cardiovascular drugs, anti-diabetics and psychotropic drugs. In the Cox model, the propensity score is further included as a restricted cubic spline to model possible non-linearities, in addition to the categorical disulfiram use, which is the variable of interest. Analyses were run for cancer overall and for breast, prostate and colon cancer, separately. Furthermore, all analyses were stratified by stage (localized, non-localized or unknown). Statistical significance of DSF use was evaluated by likelihood ratio tests. We used the software R for statistical computing.

In vivo tumour experiments. The human breast cancer cell line MDA-MB-231 was injected (10⁶ cells transplanted subcutaneously) to grow tumours in athymic NUGNU female mice (AnLab Ltd) with a body weight of 23.6–26.9 g, aged 12 weeks. Inclusion criteria were: female, appropriate age and weight (15–30 g). Exclusion criteria were: tumour size must not exceed 20 mm (volume 4,000 mm³) in any direction in an adult mouse, the tumour mass should not proceed to the point where it significantly interferes with normal bodily functions, or causes pain or distress owing to its location, persistent self-induced trauma, rapid or progressive weight loss of more than 25%, for seven days. In none of the experiments were these approved ethical limits exceeded. After the tumours grew to 0.114–0.117 cm³ on average, mice were randomly divided into four groups, each of eight mice, and treated as follows: (i) normal diet; (ii) normal diet plus oral administration of 0.15 mg kg⁻¹ copper gluconate (CuGlu); (iii) normal diet plus oral administration of 50 mg kg⁻¹; DSF; (iv) normal diet plus oral administration of 0.15 mg kg⁻¹ CuGlu and 50 mg kg⁻¹ DSF. Administration of compounds was carried out as a blinded experiment (all information about the expected outputs and the nature of used compounds were kept from the animal technicians). CuGlu was administered each day in the morning (08:00) and DSF each day in the evening (19:00) to mimic a clinical trial combining DSF and CuGlu in treatment of tumours involving the liver (NCT00742911). After treatment began, mice were weighed and their tumours measured twice per week. At day 32, mice were euthanized, and the tumours were removed and frozen at −80 °C. The experiment was evaluated by comparing growth curves of tumours in the experimental groups with those in controls. The rates of tumour growth inhibition (TGI) were calculated by the formula TGI = (1 – V_treated/Control) Where V_treated is the mean tumour volumes in the treated group and V_control is the mean tumour volumes in the control group).

Mean tumour volume values at specific time intervals were statistically evaluated. To test directly the effect of CuET, we used MDA-MB-231 and AMO-1 models. MDA-MB-231 was injected (5 × 10⁵ cells were transplanted subcutaneously) to grow tumours in SCID mice (ENVIGO) aged 10 weeks (+2 weeks). AMO-1 xenografts were expanded in SCID mice. Each group consisted of 10 animals, each bearing two tumours. CuET was formulated in bovine serum albumin solution (1%) to a final concentration of 1 mg ml⁻¹. CuET was applied intraperitoneally with a schedule of five days on and two days off. All aspects of the animal study met the accepted criteria for the care and experimental use of laboratory animals, and protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine Charles University in Prague and Ethical Committee of Faculty of Medicine and Dentistry, Palacky University in Olomouc. For HPLC–MS and immunohistochemistry analysis, we used MDA-MB-231 xenografts treated with the same DSF and DSF plus copper gluconate regime as described for the anti-cancer activity assessment with the notable difference that mice were euthanized after five days of treatment.

HPLC–MS analysis of CuET. The HR-MRM analysis was performed on a HPLC-ESI-QTOF system consisting of HPLC chromatograph Thermo UltiMate 3000 with AB Sciex Triple TOF 5600+ mass spectrometer, using the DuoSpray ESI source operated at an ion source voltage of 5,500 V, ion source gas flow rates of 40 units, curtain gas flow rate of 30 units, declustering potential of 100 V and temperature 400 °C. Data were acquired in product ion mode with two parent masses (358.9 and 360.9) for analysis of CuET. Chromatographic separation was done by PTFE column, which was especially designed for analysis of strong metal chelators filled by C18 sorbent (IntelliMed, IM_301). Analysis was performed at room temperature and with a flow rate of 1,500 μl min⁻¹ with isocratic chromatography. Mobile phase consisted of HPLC grade acetone (Lachner) 99.9%, HPLC water (Merck Millipore) 0.1% and 0.03% HPLC formic acid (Sigma-Aldrich). Acquired mass spectra were evaluated in software PeakView 1.2. Extracted ion chromatograms of transitions 88.0 and 116.0 (common for both parent masses) with a 0.1 mass tolerance were Gaussian smoothed with width of two points. Peak area was then recorded and recalculated to ng ml⁻¹ according to the calibration curve.

Sample preparation for HPLC–MS analysis. Liquid nitrogen-frozen biological samples were cut into small pieces using a scalpel. Samples (30–100 mg) were immediately processed by homogenization in 100% acetone in a ratio of 1:10 sample: acetone (for plasma or serum the ratio was 1:4). Homogenization was done in a table-top homogenizer (Retsch MM301) placed in a cold room (4 °C) in 2 ml Eppendorf tubes with 2 glass balls (5 mm) for 1 min at 30 Hz. The tube was immediately centrifuged at 4 °C, 20,000 × g for 2 min. Supernatant was decanted into a new 1.5-ml Eppendorf tube and immediately centrifuged for 30 min using a small table-top centrifuge (BioSan FVL-2400N) placed inside a −80 °C freezer. Supernatant was quickly decanted into a glass HPLC vial and kept at −80 °C for no longer than 6 h. Just before the HPLC analysis, the vial was placed into a pre-cooled (4 °C) LC-sample rack and immediately analysed. To enable an approximate quantification of analysed CuET, a calibration curve was prepared. Various amounts of CuET were spiked in plasma, frozen in liquid nitrogen, and placed at −80 °C to mimic sample processing. Standards were then processed as the samples described above. To measure circulating CuET concentrations, mice were given a single oral dose of DSF (50 mg kg⁻¹) and euthanized at different time points. Serum was collected and frozen for analysis.

Blood collection from humans for HPLC–MS analysis of CuET. Blood samples were collected before and 3 h after oral application of DSF (Antabuse, 400 mg) dissolved in water. Phlebotomy needles were specific for metal analysis—Sarstedt Safety Kanule 21G × 1 ¼ inches, 85.116.600. Collection tubes were specific for metal analysis—Sarstedt, S-Monovette 7.5 ml LH, 01.1604.400. Immediately after blood collection samples were centrifuged in a pre-cooled centrifuge (4 °C at 1,300 rpm for 10 min). After centrifugation, tubes were placed on ice and the plasma fraction was aspirated into 1.5 ml Eppendorf tubes with approximately 500 μl per tube. The tubes with plasma were immediately frozen on dry ice and later stored in −80 °C. Blood samples were collected from volunteers who gave informed consent and were undergoing Antabuse therapy because of alcohol abuse. Participants were four males (aged 34, 38, 41, 60 years) and five females (aged 37, 56, 46, 59, 63 years). All individuals were freshly diagnosed for alcohol-use disorder and were scheduled for Antabuse therapy. Blood samples were collected before and after the first use of Antabuse. All relevant ethical regulations were followed for the study. The study, including the collection of blood samples, was approved by the Ethical Committee of Faculty of Medicine and Dentistry, Palacky University in Olomouc.

Cell lines. Cell lines were cultured in appropriate growth medium supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin; and maintained in a humidified, 5% CO₂ atmosphere at 37 °C. Cell lines cultured in DMEM medium were: HCT116 (ATCC), DU145 (ECACC), PC3 (ECACC), T47D (NCI60),
least 10,000 events were acquired per sample. Collected data were processed using BD FACSSuite (BD Biosciences) and exported into Microsoft Excel.

Initial culture medium and washing buffer were collected to include trypsinization. The next day, cells were treated as indicated. After 24 h, an XTT assay was performed according to the manufacturer's instructions (Applichem). XTT solution (500 μg/ml) 1 was added to the medium with geneticin was replaced every 2–3 days until 80% myeloma cells, based on IMWG criteria (BTZ-resistant) and an untreated patient with multiple myeloma (BTZ-sensitive). The purity of the cell samples was >80% myeloma cells, as assessed by morphology.

Viability assay of multiple myeloma cells. The CellTiter 96 MTS-assay (Promega) was used according to the manufacturer's instructions to determine the cell viability of BTZ (Janssen Cilag), CFZ and Cetuxin in cell lines and the absorbance of the formazan product was measured in 96-well microplates at 492 nm. The assay measures dehydrogenase enzyme activity found in metabolically active cells. For patient cells, the more sensitive luminescent CellTiterGlo assay (Promega) was used to determine cell viability, measured by ATP production of metabolically active cells. The primary myeloma cell samples were obtained after written informed consent obtained by Z. Czibotariu (Galen ethics committee—Eihkommission Ostschweiz), in accordance with ICH-GCP and local regulations. Malignant plasma cells were retrieved by PBMC isolation from a patient with multiple myeloma progressing under BTZ-containing therapy, based on IMWG criteria (BTZ-resistant) and an untreated patient with multiple myeloma (BTZ-sensitive). The purity of the cell samples was >80% myeloma cells, as assessed by morphology.

Immuno blotting and antibodies. Equal amounts of cell lysates were separated by SDS–PAGE on hard or precipitated tris–glycine gradient (4–20%) gels (Life Technologies), and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% bovine milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature, and then incubated overnight at 4°C or for 1 h at room temperature, with one of the following primary antibodies (all antibodies were used in the system under study (assay and species) according to the instructions of the manufacturer): anti-ubiquitin (1:1,000; Cell Signaling, 3933), anti-H2A, acidic patch (1:1,000; Merck Millipore, 07-146), anti-monoubiquitylated H2A (1:1,000; Merck Millipore, clone 6E6G), anti-lc-Bo (1:500; Santa Cruz Biotechnology, sc-371), anti-p53 (1:500; Santa Cruz Biotechnology, clone D5B10), anti-VCP (1:2,000; Abcam, ab11433), anti-VCP (1:1,000; Novus Bio, NB100-1557), anti-NPLOC4 (1:1,000; Novus Bio, NBPI-82166), anti-ubiquitin ly48-specific (1:1,000; Merck Millipore, clone Apu2), anti-β-actin (1:2,000; Santa Cruz Biotechnology, sc-1616; or 1:500; Santa Cruz Biotechnology, sc-87778), anti-GAPDH (1:1,000; GeneTex, clone ID4), anti-lamin B (1:1,000; Santa Cruz Biotechnology, sc-6217), anti-calnexin (1:500; Santa Cruz Biotechnology, sc-11397), anti-α-tubulin (1:500; Santa Cruz Biotechnology, sc-5286), anti-XBP1 (1:500; Santa Cruz Biotechnology, sc-7160), UFD1 (1:500; Abcam, ab155003), cleaved PARP1 (1:500; Cell Signaling, 5945), p-eIF2α (1:500; Cell Signaling, 3957), ATF4 (1:500; Merck Millipore, ABE387), HSP90 (1:500; Enzo, ADI-SPA-810), HSP70 (1:500; Enzo, ADI-SPA-830), HSF1 (1:500; Cell Signaling, 4336), p-HSP27 (1:1,000; Abcam, 159587), HSP27 (1:1,000; Abcam, 109576) followed by detection by secondary antibodies: goat anti-mouse IgG–HRP (GE Healthcare), goat anti-rabbit (GE Healthcare), donkey anti-goat IgG–HRP (Santa Cruz Biotechnology, sc-2001). Bound secondary antibodies were visualized by ELC detection reagent (Thermo Fisher Scientific) and images were recorded by imaging system equipped with CCD camera (ChemiDoc, Bio-Rad) operated by Image Laboratory software or developed on film (Amersham).

Immunofluorescence staining. Cells were grown in 24-well plates with a 0.170-mm pore size polycarbonate filter. In Forma 300 (BD Biosciences), a filter was added to the medium with geneticin was replaced every 2–3 days until 80% myeloma cells, based on IMWG criteria (BTZ-resistant) and an untreated patient with multiple myeloma (BTZ-sensitive). The purity of the cell samples was >80% myeloma cells, as assessed by morphology.

Microscopy, FRAP and image analysis. Samples were analysed using a Zeiss Axioscope Z.1 platform equipped with the Elyra PS.1 super-resolution module for structured illumination (SIM) and the LSM/780 module for CLSM. High resolution images were acquired in super-resolution mode using a Zeiss Plan Apo100×/1.46 oil objective (total magnification, 1,600 × ) with appropriate oil (Immersion 518F). SR-SIM setup involved five rotations and five phases for each image layer and up to seven photons per image. The CLSM setup for FRAP and life cells acquisition had a c-Apo 40×/1.2 W water immersion objective. Bleaching of regions of interest (ROIs) was performed using an Argon 488 nm laser. Lower resolution images of fixed samples were acquired using a Plan APO 63×/1.4 oil objective (total magnification 1,008 × ). FRAP and image acquisitions were performed using Zeiss Zen 11 software. For FRAP, internal Zen's 'Bleach'
and ‘Regions’ modules were used. Data from FRAP analysis involving multiple bleached ROIs were exported into Microsoft Excel and plotted. Basic processing of acquired images, such as contrast and brightness settings, was done in Adobe Photoshop on images exported as TIFFs. Quantitative microscopy-based cytometry of the immunofluorescence-stained samples was performed using an automatic inverted fluorescence microscope BX71 (Olympus) using ScanR Acquisition software (Olympus) and analysed with ScanR Analysis software (Olympus).

Cell fractionation for Triton-X100 insoluble pellets. Cells were treated as indicated, washed with PBS and lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM MgCl$_2$, 10% glycerol, 0.5% Triton X-100, protease inhibitor cocktail by Roche) for 10 min gently agitation at 4°C. Then, cells were scraped into Eppendorf tubes and kept for another 10 min on ice with intermittent vortexing. After that, the lysate was centrifuged at 20,000 rpm for 10 min at 4°C. The insoluble fraction and supernatant were separately re-suspended in 1× LSB buffer.

Isolation of microscopic fraction. After the desired treatment in cell culture, cells were washed with cold PBS and lysed (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail). Lysates were homogenized by Potter-Elvehjem PTFE homogenizer and kept on ice for 20 min. The homogenates were subjected to serial centrifugation steps (720g and 10,000g for 5 min each, and 100,000g for 1 h). Pellets and supernatants from the last ultracentrifugation step were resuspended in the 1× LSB buffer and used for western blot analysis.

Immunoperoxidase staining of pre-extracted tissue sections. Frozen sections (4–5μm thick) from xenograft-grown, cryopreserved tumour tissues were cut on a cryostat and placed on commercial adhesion slides (SuperFrost Plus, Menzel, Germany) and air-dried for 2 h at room temperature. The dried sections were carefully covered with the cold extraction buffer: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl$_2$, 5% glycerol, 1 mM DTT, 1% Triton X-100, 1% IGEPAF, protease inhibitor cocktail (Promega, Madison, WI), anti-cyclin B1 antibody (1:1000, sc-417, Santa Cruz Biotechnology) or control antibodies (1:500, NBP1-82166, Novus Biologicals) and Vectastain Elite kit as secondary reagents (Vector Laboratories, USA), followed by a nickel-sulfate-enhanced diaminobenzidine reaction without nuclear counterstaining, mounted and microscopically evaluated and representative images documented by an experienced oncopathologist.

Isothermal titration calorimetry (ITC). Experiments were performed at 25°C with a Nano ITC Low Volume (TA Instruments) and analysed by Nano Analyze Software v.2.3.6. During all measurements, injections of 2.5μL of ligand (16μM) were titrated into 250μL protein solution (2μM) with time intervals of 300 s, a stirring speed of 250 r.p.m. All ITC experiments were conducted with degassed buffered solutions titrated into 20μM HEPES buffer pH 7.5, in the presence of 1% DMSO. Purified GST–NPL4(WT) and GST–NPL4(MUT) proteins were used in ITC experiment. Drug affinity responsive target stability (DARTS). DARTS was performed according to a modified published protocol 26. Purified GST–NPL4(WT) and GST–NPL4(MUT) proteins were diluted by 100 mM phosphate buffer, pH 7.4 to final concentration of 0.03mg/mL. The proteins were treated with CuET (final concentration of 5μM; dissolved in DMSO) for 1 h and equal amounts of DMSO were added to the solutions, which served as control samples. Pronase (Sigma–Aldrich) was dissolved in TNC buffer (50 mM Tris–Cl, 50 mM NaCl, 10 mM CaCl$_2$, pH 7.5). The 0.025μg of pronase was added to 30μL of protein solution and incubated for 1 h at 37°C. Samples without pronase served as the non-digested controls. After 1 h of incubation at 37°C, inhibition of proteasome activity was measured by the release of hydrolysed free AMC as described above.

Ub(G76V)–GFP degradation. HeLa Ub(G76V)–GFP-OUD-Luc cells expressing Ub(G76V)–GFP were plated at a density of 10$^5$ cells per well in 96-well plates. The next day, cells were treated with 4μM MG132 for 3 h. After that, the medium was discarded and cells were washed twice with PBS and then incubated with the tested compound in the presence of 30μg/mL cycloheximide for another 3 h. The GFP signal was acquired using an ImageXpress automated microscope. For each well, four images were taken (corresponding to 200–250 cells). Cells were analysed every 30 min during 3 h of treatment. Normalized GFP signal intensity was calculated using the following formula: (test compound (background))/(basal GFP signal intensity × background) where ‘test compound’ is defined as the mean GFP signal intensity of Ub(G76V)–GFP expressing cells treated with the test compound. ‘Background’ is defined as the background GFP signal intensity of HeLa cells. ‘Basal GFP signal intensity’ is defined as mean GFP signal intensity of Ub(G76V)–GFP-expressing cells treated with DMSO. The degradation rate constant (k) was obtained from the slope of the linear range of plotting ln(normalized GFP signal intensity) versus time ranging from 90 to 180 min. The percentage of remaining k for each compound is calculated using the following formula (test compound/ DMSO) × 100.

p97 ATPase activity assay. P97 ATPase assay was performed as described previously 27. A total of 250 μM of p97 protein was diluted in assay buffer (50 mM Tris–HCl pH 7.4, 20 mM MgCl$_2$, 0.5 mM DTT). Compounds were added in DMSO (final concentration of DMSO was 5%). After 10 min of incubation, the reaction was started with ATP (100 μM final concentration) followed by a 1-h incubation at room temperature. The reaction was stopped by adding Bioimol green solution (Enzo) and free phosphate was measured according to the manufacturer’s instructions. Results are expressed as the percentage of activity of the control (a well containing only DMSO).

26S proteasome activity. The RP11 assay is described in ChemBioChem (AID5884931). In brief, a synthetic fluorescently labelled substrate, UbpeptOG, was used to measure RP11 activity. Fluorescence polarization assay was performed in a low-volume 384-well solid black plate in the presence of (i) 5μL of the compound, 1.10-pentanol and Culn in 3% DMSO or 3% DMSO control; (ii) 5μL of BioMol 26S proteasome; and (iii) 5μL of substrate (15 μM UbpeptOG). Fluorescence polarization is measured using a plate reader with excitation of 480 nm and emission of 520 nm filter set. The activity was normalized to DMSO control and fit to a dose–response curve.

Protein expression and purification. All proteins were expressed in E. coli BL21 (DE3) cells (Novagen). Ph-His (pET28a vector) and Ued1-His (pET28a vector) expression were induced by 1mM IPTG (Life Technologies) at an OD$_{600}$ of 0.6 for 10 h at 22°C. NPL4(WT) and NPL4(MUT) (pGEX-2TK) were induced by 0.4 mM IPTG at an OD$_{600}$ of 0.8 overnight at 16°C. For p97 and UFD1, the bacterial pellet was suspended in buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 2.5 mM MgCl$_2$, 20 mM imidazole, 5% glycerol) and lysed by sonication and centrifuged (14,000g for 20min). Proteins were purified by Ni-NTA chromatography (Qiagen) according to the manufacturer’s instructions. For p97, the protein was further purified by gel filtration (Superdex 200, GE Healthcare). For GST–NPL4(WT) and GST–NPL4(MUT), the bacterial pellet was suspended in phosphate buffer (PBS, 0.1% Triton X-100, 1% SDS, 50 mM NaCl) and lysed by sonication and centrifuged (14,000g for 10 min). Proteins were purified by glutathione sepharose 4B (Life Technologies) according to the manufacturer’s protocol. The proteins were further purified by gel filtration (Superdex 200, GE Healthcare).

Chemicals. CuET was prepared by direct synthesis from water solutions of diethylthiocarbamate sodium salt and copper(n) chloride as described previously 28. CuET for in vivo experiments was prepared equally with a slight modification. The reaction between diethylthiocarbamate sodium salt and copper(n) chloride was performed in a sterile 1% aqueous solution of bovine serum albumin. The resulting solution was used directly. The following chemicals were purchased from Sigma-Aldrich: bovine serum albumin, N-acetyl-L-cystein, N6-(2-aminopropyl)triamineethylenediamine disulfate, DTT (Sigma–Aldrich), sodium diethylthiocarbamate tributyrate (Sigma–Aldrich), copper(n)–gluconate (Sigma–Aldrich), BTZ (Velcide, Janssen-Cilag International N.V.), GM132 (Sigma–Aldrich), DBeq (Sigma–Aldrich), NMS873 (Abmole), cycloheximide (Sigma–Aldrich), dicoumarol (Sigma–Aldrich), 1,10-pentanethiol (Sigma) and MLN7243 (Active Biochem).

380/460 nm (TECAN, Infinite M200PRO). To measure proteasome activity in live cells, the cells were seeded in 24-well plate at a density of 0.2 × 10$^6$ cells per well. Cell lines were treated with CuET (1μM and 5μM), vehicle control or 1μM BTZ for 1h. After incubation, cells were twice washed with 0.5 mL of 1× ice-cold PBS and scraped into 100μL ice-cold lysis buffer and then centrifuged at 15,000 r.p.m. for 15 min at 4°C. Subsequently, the cell extract (10μg) was incubated with 20μL substrates to measure chymotrypsin-like, trypsin-like and caspase-like activities (Suc-LLVT-AMC, Ac-RLR-AMC and Z-LLE-AMC (Boston Biochem)) in 90μL of assay buffer (30 mM Tris–HCl, 0.035% sodium dodecylsulfate (pH 7.4)) in the presence CuET (1μM and 5μM) and BTZ (1μM) for the investigation of proteasome inhibition; BTZ as an equivalent volume of solvent (DMSO) was used as a control. After 2 h of incubation at 37°C, inhibition of proteasome activity was measured by the release of hydrolysed free AMC groups by fluorimeter at 520/50 nm filter set. The activity was normalized to DMSO control and fit to a dose–response curve.
Statistical analyses and reproducibility. For the epidemiological study, we calculated hazard ratios and 95% confidence intervals estimating cancer-specific mortality, based on a Cox model regressing of both propensity scores and disulfiram use, balancing baseline characteristics of previous and continuing users of DSF and adjusting estimated hazard ratios of cancer-specific mortality associated with DSF use51. The propensity score estimates were conditional on multiple covariates, based on using logistic regression (see ‘Epidemiological analyses and access to health registers’ for specifics of cohorts and covariates). In the Cox model, the propensity score is further included as a restricted cubic spline to model possible nonlinearities, in addition to the categorical disulfiram use as the variable of interest. Statistical significance of DSF use was evaluated by likelihood ratio tests, using the software R for statistical computing52.

For evaluation of the animal studies, STATISTICA software, v.12 (StatSoft) was used to estimate sample size. For a power of 80%, the level of significance set at 5%, 4 groups and RMSSE = 0.8, seven mice per group were estimated. For usage of non-parametrical statistical methods, the number of eight mice per group was finally planned. The differences between tumour volumes were statistically analysed by non-parametrical Kruskal–Wallis test, not requiring any assumptions of normality and homoscedascity. To test the effect of CuET treatment on survival of AMO-1-xenografted mice, a Kaplan–Meier graph and log-rank statistical test were used. For other experiments, the statistics, such as number of repetitions, centre value and error bars, are specified in figure legends.

Data availability. Most data generated or analysed during this study are included in the article and its Supplementary Information. Uncropped images of all gels and blots can be found in Supplementary Fig. 1. Source Data for all graphs are provided in the online version of the paper. Additional datasets generated during and/or analysed during the current study and relevant information are available from the corresponding authors upon reasonable request.

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Extended Data Figure 1 | Epidemiological and pre-clinical data of the anti-cancer effects of DSF. a, Summary of hazard ratios (HR) and 95% confidence intervals (CI) for cancer-specific mortality among Danish patients with cancer, comparing continuing and previous users of DSF for selected types of cancer (for statistical analysis and definitions of DSF exposure categories, see Methods). b, Photographs of subcutaneously growing human MDA-MB-231 tumours extracted from mice at day 32. c, Time-course diagram of mouse weight. n = 8 animals per group. d, Model of CuET formation during metabolic processing of orally administered DSF in the human body. e, Examples of mass-spectrometry spectra of CuET expressed as peaks of 4 MRM transitions in mouse serum after CuET spikes, compared to orally applied DSF (50 mg kg$^{-1}$). Data are representative of two independent experiments. f, Pharmacokinetic analysis of CuET levels in mouse serum after orally applied DSF (50 mg kg$^{-1}$), n = 2 animals per time point. g, Effect of DTC and CuET on MDA-MB-231 cells analysed by colony formation assay. n = 3 independent experiments. h, Time-course diagram of weight in CuET- and vehicle-treated mice. n = 10 animals per group. i, Extended time-course diagram of weight in CuET- and vehicle-treated mice. n = 10 animals per group. Data are mean ± s.d. (c, h, i) or linked means (g).
CuET is the major anti-cancer metabolite of DSF. a, CuET cytotoxicity measured by a colony-formation assay in human cell lines derived from breast, lung, colon and prostate carcinomas. Data are mean ± s.d. of three independent experiments (breast) or presented individually for two independent biological experiments for each cell line (lung, colon and prostate). b, IC50 values from two independent biological experiments documenting differential CuET-induced cytotoxicity across a panel of cancer and non-cancerous cell lines (48 h treatment). c, Analysis of annexin V signal in AMO-1 cells exposed to toxic doses of NMS873 (5 μM, 16 h) or CuET (100 nM, 16 h) and in U2OS cell exposed to toxic doses of NMS873 (10 μM, 16 h) or CuET (1 μM, 16 h). d, Analysis of caspase 3/7 activity in selected cell lines after apoptosis induction by NMS873 (AMO-1: 6 h, 5 μM; Capan1: 16 h, 10 μM; U2OS: 16 h, 10 μM; MDA-MB-231: 24 h, 10 μM) or CuET (AMO-1: 16 h, 100 nM; Capan1: 16 h, 250 nM; U2OS: 16 h, 1 μM; MDA-MB-231: 24 h, 1 μM). e, Absence of cleaved PARP1 after a toxic dose of CuET in U2OS cells, compared to etoposide treatment as a positive control. f, Analysis of cytochrome c (in red) release from mitochondria in U2OS cells during cell death induced by the positive control staurosporin (STS, 1 μM) compared to cell death induced by CuET (1 μM). Blue, DAPI. Scale bar, 10 μm.

c–f, Data are representative of two independent biological experiments.
Extended Data Figure 3 | CuET-induced proteasome inhibition-like response is not due to proteasome inhibition. a, Kinetics of poly-Ub protein accumulation in U2OS cells treated with CuET or the proteasome inhibitor BTZ. b, CuET treatment (1.5 h) induces rapid deubiquitylation of ubiquitylated histone H2A (uH2A) similarly to proteasome inhibitors BTZ or MG132 in U2OS cells. c, CuET treatment (1.5 h) induces rapid cytoplasmic accumulation of poly-ubiquitylated proteins (FK2 antibody staining) in U2OS cells, similar to BTZ and MG132 treatment. d, 20S proteasome activity is not inhibited by CuET as examined in live MDA-MB-231 cells (d) or in lysates from MDA-MB-231 cells (e). Data are mean ± s.d. of four independent experiments. f, CuET treatment (1 μM, 6 h) does not cause accumulation of p53 in the presence of dicoumarol (300 μM) in MCF7 cells. g, In vitro 26S proteasome function measured as RPN11 deubiquitylation activity, is not inhibited by CuET; 1,10-phenanthroline (1,10-OPT) served as a positive control. Data are representative of two (a–c, f) or three (g) independent experiments.

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Extended Data Figure 4 | CuET inhibits the p97 pathway and induces cellular UPR. a, MG132-treated cells (5 μM, 6 h) accumulate both forms of NRF1 (120-kDa and 110-kDa bands, top and bottom arrows, respectively), whereas CuET-treated cells (1 μM, 6 h) accumulate only the non-cleaved 120-kDa form. b, Inhibition of the NRF1 cleavage process (appearance of the lower band) by CuET and NMS873 (a p97 inhibitor; 5 μM) in mouse NIH3T3 cells co-treated with the proteasome inhibitor MG132 (5 μM for 6 h). c, Time-course example images from a FRAP experiment, for which the quantitative analysis is shown in Fig. 2g (U2OS cells, blue boxes mark areas before bleaching, arrows after bleaching). d, U2OS cells pre-extracted with Triton X-100 and stained for poly-Ub(K48). The antibody signal intensities for cells treated with DMSO, BTZ (1 μM), NMS873 (10 μM) and CuET (1 μM) are analysed by microscopy-based cytometry and plotted below. e, Western blot analysis of accumulated poly-Ub proteins in the ultracentrifugation-separated microsomal fraction from U2OS cells treated with mock, CuET (1 μM), NMS873 (10 μM) or BTZ (1 μM) for 3 h. f, UPR in U2OS and MDA-MB-231 cell lines induced by 6-h treatment with CuET (various concentrations) or positive controls (5 μM NMS873, 2 μg ml⁻¹ tunicamycin, 1 μM thapsigargin) is shown by increased levels of XBP1s, ATF4 and p-eIF2α. a–f, Data are representative of two independent experiments. All scale bars, 10 μm.
Extended Data Figure 5 | CuET kills BTZ-resistant cells. a, BTZ-adapted (BTZres), CFZ-adapted (CFZres) and non-adapted AMO-1 human myeloma cells are equally sensitive to treatment with CuET. b, BTZ-adapted, CFZ-adapted and non-adapted ARH77 human plasmocytoma cells are equally sensitive to treatment with CuET. c, BTZ-adapted and non-adapted RPMI8226 human myeloma cells are equally sensitive to treatment with CuET. d, Human myeloma cells derived from a patient with BTZ-resistant myeloma show CuET sensitivity comparable to myeloma cells derived from a patient with BTZ-sensitive myeloma. Data are means linked of three independent experiments (a–c) or data are from two independent experiments (d).
Extended Data Figure 6 | See next page for caption.
Extended Data Figure 6 | CuET targets NPL4, causing immobilization and nuclear clustering of NPL4. a, CuET (1 μM) does not inhibit ATPase activity of p97. NMS873 (5 μM) was used as a positive control. Data are mean ± s.d. from four independent experiments. b, Western blotting analysis showing levels of ectopic p97–GFP, NPL4–GFP and UFD1–GFP in stable U2OS-derived cell lines used for the CuET-treatment rescue and cluster formation experiments. c, Ectopic expression of NPL4–GFP alleviates CuET-induced (125 nM, 4 h) accumulation of poly-Ub proteins in U2OS cells. d, Distribution of NPL4 nuclear clusters relative to chromatin in cells treated with CuET (1 μM, 2 h). Scale bars, 2 μm. e, Schematic representation of site-directed mutagenesis within the amino acid sequence of the putative zinc finger domain of NPL4. f, ITC curve showing the lack of CuET binding to purified NPL4(MUT) protein. g, DARTS analysis of recombinant NPL4 proteins shows that differential pronase-mediated proteolysis after CuET addition is apparent for NPL4(WT) but not for NPL4(MUT); detected by either silver-stained SDS–PAGE (the most prominent differential bands are marked by red dots) or by blotting with an anti-NPL4 polyclonal antibody. h, Viability of cells expressing doxycycline-inducible NPL4(MUT)–GFP, treated with CuET for 48 h. Data are from three independent experiments, means are linked. i, Accumulation of K48-ubiquitinated proteins and activation of UPR in cells expressing the doxycycline-inducible NPL4(MUT)–GFP. b–d, f, g, i. Data are representative of two independent experiments.
Extended Data Figure 7 | Immobilized NPL4 forms insoluble protein aggregates. a, NPL4–GFP aggregates induced by CuET treatment (1 μM, 3 h) do not co-localize with nuclear speckles (stained by SC-35 antibody) or nucleoli (visible as a DAPI− nuclear signal). b, NPL4–GFP nuclear aggregates induced by CuET (1 μM, 3 h) are excluded from chromatin in early prometaphase U2OS cells. c, Co-localization of spontaneous NPL4(MUT)–GFP aggregates with SUMO2/3, poly-UB(K48) and TDP43 in pre-extracted U2OS cells. d, NPL4–GFP aggregates are formed independently of ubiquitylation, as shown in CuET-treated (1 μM, 3 h) cells pre-treated with a chemical UBA1 inhibitor (MLN7243, 10 μM, 1 h). The lack of cellular FK2 staining of ubiquitylated proteins validates the efficacy of the MLN7243 inhibitor. e, Co-localization of FK2 signal with the spontaneous NPL4(MUT)–GFP aggregates in pre-extracted U2OS cells. f, Analysis of p97 in CuET-induced (1 μM, 3 h) NPL4–GFP aggregates in pre-extracted U2OS cells. g, Analysis of p97 in spontaneous NPL4(MUT)–GFP aggregates in pre-extracted U2OS cells. a–g, Data are representative of two independent biological experiments. All scale bars, 10μm.
Extended Data Figure 8 | NPL4 aggregation immobilizes the p97 binding partner and induces a global cellular HSR. a, Immobilization of selected proteins in Triton X-100-resistant pellet fractions of CuET-treated (1 μM, 3 h) U2OS cells. b, Immobilization of selected proteins in Triton X-100-resistant pellet fractions from U2OS cells expressing doxycycline-inducible NPL4(MUT)–GFP (48 h after induction). c, CuET dose-dependent immobilization of p97 in Triton X-100 pre-extracted MDA-MB-231 cells (3 h). Scale bar, 10μm. d, Immunohistochemical staining showing non-extractable p97 in MDA-MB-231 xenografts from mice treated with DSF or DSF and CuGlu, compared to vehicle. Scale bar, 50μm. e, HSR after CuET (8 h treatment) is shown by various HSR markers detected by western blotting of U2OS cell extracts. f, HSR markers in U2OS cells expressing doxycycline-inducible NPL4(MUT)–GFP (24 h after induction). a–f, Data are representative of two independent biological experiments.
Experimental design

1. Sample size
   Describe how sample size was determined.
   STATISTICA software, ver. 12 (StatSoft Inc., USA) was used to estimate the sample size. For the power of 80%, the level of significance set at 5%, 4 groups and RMSSE = 0.8, 7 mice in each group was estimated. For usage of non-parametrical statistical methods, the number of 8 (and 10) mice in each group was finally planned.

2. Data exclusions
   Describe any data exclusions.
   No data were excluded.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All experiments were reproduced to reliably support conclusions stated in the manuscript.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Animals were randomly divided into experimental groups.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Administration of compounds was carried out as a blinded experiment (all information about the expected outputs and the nature of used compounds were kept from the animal-technicians).

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed

   - [x] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - [x] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - [x] A statement indicating how many times each experiment was replicated
   - [x] The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - [x] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - [x] The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   - [x] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - [x] Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

7. Software

Describe the software used to analyze the data in this study.

The data were analyzed using Microsoft Excel 2016, STATISTICA 12, Graphpad Prism 4, PeakView 1.2, Image Lab 4.1, Carl Zeiss Zen 2011 SP6 (black), Nano Analyze Software 2.3.6, Olympus ScanR Analysis 1.3.0.3.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials used are fully available from commercial sources with the exception of LAPC4 cell line, that we obtained from Zoran Culig, University of Innsbruck.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- anti-ubiquitin (Cell Signaling, cat. n.: 3933; lot 4), anti-H2A, acidic patch (Merck Millipore, cat. n.: 07-146; lot 2880748), anti-monoubiquityl-H2A (Merck Millipore, clone E6C5; lot 2239798), anti-IκBα (Santa Cruz Biotechnology, cat. n.: sc-371), anti-phospho(Ser32/36)-IκBα (Cell Signaling, clone 5A5), anti-p53 (1:500; Santa Cruz Biotechnology, clone DO-1; D0915), anti-HIF1α (BD Biosciences, cat. n.: 610958; lot 47858), anti-Cdc25A (Santa Cruz Biotechnology, clone DCS-120; our own clone commercially available by Santa Cruz), anti-NRF1 (Cell Signaling, clone DSB10; lot 1), anti-VCP (Abcam, cat. n.: ab11433; lot GR298429-3), anti-VCP (Novus Bio, cat. n.: NBP100-1557; lot A1), anti-NPLOC4 (Novus Bio, cat. n.: NBP1-82166; lot A96635), anti-ubiquitin lys48-specific (Merck Millipore, clone Apu2; lot 2724416), anti-β-actin (Santa Cruz Biotechnology, cat. n.: sc-1616; lot B2206), anti-β-actin (Santa Cruz Biotechnology, clone C4, cat. n.: sc-47778; lot C0916), anti-GAPDH (GeneTex, clone 1D4; lot 821603479), anti-Lamin B (Santa Cruz Biotechnology, M20, cat. n.: sc-6217; lot J2313), anti-calnexin (Santa Cruz Biotechnology, H70, cat. n.: sc-11397; lot C1214), anti-α-Tubulin (Santa Cruz Biotechnology, B7, cat. n.: sc-5286; lot C1313), anti-Xbp1 (Santa Cruz Biotechnology, M-186, cat. n.: sc-7160; lot A2314), CHOP (Cell Signaling, L63F7, cat. n.: 2895; lot 10), Ufd1 (Abcam, cat. n.: ab155003; lot GR119674-2), cleaved PARP1 (Cell Signaling, cat. n.: 9544; lot 4), p-eIF2a (Cell Signaling, cat. n.: 3597; lot 9), ATF4 (Merck Millipore, cat. n.: ABE387 lot 2736396), HSP90 (Enzo, cat. n.: ADI-SPA-810; lot 05051501), TOP-43 (Proteintech, cat. n.: 10782-2-AP; lot number not provided by manufacturer), HSP70 (Enzo, cat. n.: ADI-SPA-830; lot 05021648), HSF1 (Cell Signaling, cat. n.: 4356; lot 2, pHSP27 (Abcam, cat. n.: 155987; lot GR117377), HSP27 (Abcam, cat. n.: 109376; lot GR61497-8), FK2 antibody (Enzo, cat. n.: BML-PW8810), Sumo2/3 (Abcam, cat. n.: ab3742; lot GR8249-1), Cytochrome c Alexa Fluor 555 conjugated (BD Pharmeding, cat. n.: 558700).

Secondary antibodies: goat-anti mouse IgG-HRP (GE Healthcare), goat-anti rabbit (GE Healthcare), donkey-anti goat IgG-HRP (Santa Cruz Biotechnology, sc-2020), Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, 1:1000). Antibodies critical for novel conclusions were validated by elimination of signals upon KD experiments and/or by functional assays. All antibodies were used in the system under study (assay and species) according to the profile of manufacturer.
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. HCT116 (ATCC), DU145 (ECACC), PC3 (ECACC), T47D (NCI60), HS578T (NCI60), MCF7 (ECACC), MDA-MB-231 (ATCC), U-2-OS (ECACC), HeLa (ATCC), NIH-3T3 (ATCC), CAPAN-1 (ATCC), A253 (ATCC), FaDu (ATCC), h-TERT-RPE1 (ATCC), NCI-H358 (ATCC), NCI-H52 (ATCC), HCT-15 (ATCC), AMO-1 (ATCC), MM-15 (ATCC), ARH77 (ATCC), RPMI8226 (ATCC), OVCAR-3 (NCI60), CCRF-CEM (ATCC), K562 (ATCC), 786-O (NCI60), U87-MG (ATCC), SiHA (ATCC), A549 (ATCC), HT29 (ATCC), LAPC4 (kindly provided by prof. Zoran Culig, University of Innsbruck), RWPE-1 (ATCC)
   
   b. Describe the method of cell line authentication used. All cell lines authenticated by STR method.
   
   c. Report whether the cell lines were tested for mycoplasma contamination. All cell lines were tested for mycoplasma contamination.
   
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. None of the used cell lines is listed in ICLAC database.

11. Description of research animals
   In this study were used athymic nu/nu female mice (AnLab Ltd.) median age 13 weeks (+/- 1 week) and SCID female mice (ENVIGO, NL) median age 10 weeks (+/- 2 weeks).

12. Description of human research participants
   Human participants were 4 males (age of 34, 38, 41, 60 years) and 5 females (age of 37, 56, 46, 59, 63 years). All freshly diagnosed for alcohol use disorder and dedicated for Antabuse therapy. Blood samples were collected before and after first application of Antabuse.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation
For all flow cytometry data, confirm that:

- 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- 3. All plots are contour plots with outliers or pseudocolor plots.
- 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.

Cell cultures were treated as indicated and harvested by trypsinization. Initial culture medium and wash buffer were collected to include detached cells. Cells were centrifuged (250g, 5min) and resuspended in staining buffer (140 mM NaCl, 4 mM KCl, 0.75 mM MgCl2, 10 mM HEPES). Then cell number was determined and after centrifugation, cells were resuspended in appropriate amount of staining buffer to get concentration of 1 million cells per 900 microliters. For annexinV analysis, 1x10^5 cells was incubated in 100 microliters of staining buffer containing 2.5 mM CaCl2, Annexin V-APC (1:20, BD Biosciences) and 2.5 μg/ml 7-AAD (BD Biosciences) for 15 minutes on ice in the dark. For caspases 3/7 activity assay 1x10^5 cells was incubated in 100 microliters of staining buffer supplemented with 2% FBS, 0.5 μM CellEvent™ Caspase-3/7 Green Detection Reagent (ThermoFisher Scientific) for 45 minutes at room temperature in the dark. Subsequently, 0.5 μg/mL DAPI was added before analysis by flow cytometry. Samples were analyzed by flow cytometry using BD FACSVerse (BD Biosciences), at least 10,000 events were acquired per sample. Collected data were processed by BD FACSSuite (BD Biosciences).

6. Identify the instrument used for data collection.

BD FACSVerse (BD Biosciences) equipped with 405nm, 488nm and 640nm lasers, manufactured in October 2012.

7. Describe the software used to collect and analyze the flow cytometry data.

BD FACSSuite (BD Biosciences)

8. Describe the abundance of the relevant cell populations within post-sort fractions.

Cell sorting not employed

9. Describe the gating strategy used.

Using the FSC/SSC gating, debris was removed by gating on the main cell population. Positivity threshold for each cell line was defined on the basis of mock-treated (DMSO) sample. Identical positivity threshold was applied to all samples within cell line.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☑