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Exploring the Structural Requirements for Inhibition of the Ubiquitin E3 Ligase Breast Cancer Associated Protein 2 (BCA2) as a Treatment for Breast Cancer

Ghali Brahemi,[†] Fathima R. Kona,[‡] Annalisa Fiasella,[†] Daniela Buac,[‡] Jitka Soukupová,[†] Andrea Brancale,[†] Angelika M. Burger,*[,‡] and Andrew D. Westwell*[,†]

[†]*Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff, CF10 3NB, Wales, United Kingdom, and* [‡]*Barbara Ann Karmanos Cancer Institute, Department of Pharmacology, Wayne State University, Detroit, Michigan*

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The zinc-ejecting aldehyde dehydrogenase (ALDH) inhibitory drug disulfiram (DSF) was found to be a breast cancer-associated protein 2 (BCA2) inhibitor with potent antitumor activity. We herein describe our work in the synthesis and evaluation of new series of zinc-affinic molecules to explore the structural requirements for selective BCA2-inhibitory antitumor activity. An N(C═S)S—S motif was found to be required, based on selective activity in BCA2-expressing breast cancer cell lines and against recombinant BCA2 protein. Notably, the DSF analogs (**3a** and **3c**) and dithio(peroxo)thioate compounds (**5d** and **5f**) were found to have potent activity (submicromolar IC_{50}) in BCA2 positive MCF-7 and T47D cells but were inactive ($IC_{50} > 10\,\mu M$) in BCA2 negative MDA-MB-231 breast cancer cells and the normal breast epithelial cell line MCF10A. Testing in the isogenic BCA2 +ve MDA-MB-231/ER cell line restored antitumor activity for compounds that were inactive in the BCA2 −ve MDA-MB-231 cell line. In contrast, structurally related dithiocarbamates and benzisothiazolones (lacking the disulfide bond) were all inactive. Compounds **5d** and **5f** were additionally found to lack ALDH-inhibitory activity, suggestive of selective E3 ligase-inhibitory activity and worthy of further development.

Introduction

The balance between the production of new cellular proteins and their targeted degradation is part of the normal choreographed life cycle of the cell, and numerous previous studies have elucidated the role of the ubiquitin-proteosome system in the highly regulated degradation of >80% of cellular proteins.[1] Polyubiquitination (tagging by the small protein ubiquitin) of target protein substrates is carried out by three classes of enzymes, of which the diverse and abundant ubiquitin E3 ligase family catalyze the final mechanistic step of ubiquitin transfer to specific lysyl residues of target proteins prior to proteosomal degradation.[2] Ubiquitin E3 ligase biology presents a number of therapeutic targets, since deregulated E3 ligase activity is known to be a feature of proliferative diseases such as cancer.[3] An example of a well-studied E3 ligase whose deregulation has been exploited for potential therapeutic benefit is murine double minute 2 protein (Mdm2[a]), which targets the tumor suppressor protein p53 for destruction.[4] Inhibition of the protein−protein interaction between Mdm2 and p53 for therapeutic gain is illustrated by the development of the Nutlin class of antitumor agents currently being studied in clinical trials for cancer.[5] Other components of the ubiquitin-proteosome system have provided additional targets for cancer therapy, leading to the development of the 26S proteosome inhibitor bortezomib as an approved agent for the treatment of multiple myeloma.[6]

BCA2 is an E3 ubiquitin ligase that was isolated from an invasive breast cancer cell line, and has been shown to be highly expressed in 56% (530/945) of invasive breast cancers, but not in most normal tissues examined.[7] Down-regulation of BCA2 has been shown to inhibit breast cancer cell growth and invasiveness.[7] BCA2 is expressed in the cytoplasm and nucleus, and nuclear BCA2 correlates with positive estrogen receptor status ($p < 0.004$). The classification of BCA2 as a Really Interesting New Gene (RING)-finger protein[8] with ubiquitin E3 ligase activity implicates the presence of a double Zn^{2+}-binding motif arranged in a cross-brace structure (Figure 1), termed RING-finger, as being essential for ubiquitin E3 ligase catalytic activity. The critical role of the Zn^{2+}-containing RING domain of BCA2 has been demonstrated by point mutation of key zinc-binding cysteine residues leading to the complete loss of enzyme activity.[7,9] BCA2 (also known as Rabring7) has been found to complex with a cytoplasmic binding partner Rab7, a small GTPase involved in cellular endocytosis and trafficking of oncogenic receptor tyrosine kinases (such as epidermal growth factor receptor (EGF-R)) for destruction in the lysosome.[10,11] Hence, targeting BCA2 within breast cancer cells may allow Rab7 to fulfill its function in tyrosine kinase receptor degradation, preventing the receptor recycling and sustained mitogenic signaling known to contribute to the development of resistance to tyrosine kinase-inhibitory therapeutics.[10] Taken together, the data outlined

*Authors to whom correspondence should be addressed: Chemistry; E-mail, WestwellA@cf.ac.uk. Phone: +44 2920 875800. Biology; E-mail, burgera@karmanos.org. Phone: +1 313 576 8302.

[a] Abbreviations: ABC, ATP binding cassette; ALDH, aldehyde dehydrogenase; BAAA, bodipy-aminoacetaldehyde; BCA2, breast cancer associated protein 2; CHX, cycloheximide; DEAB, diethylaminobenzaldehyde; DSF, disulfiram; EGF-R, epidermal growth factor receptor; Mdm2, murine double minute 2 protein; MTT, methyltetrazolium; PIFA, phenyliodonium di(trifluoroacetate); RING, Really Interesting New Gene.

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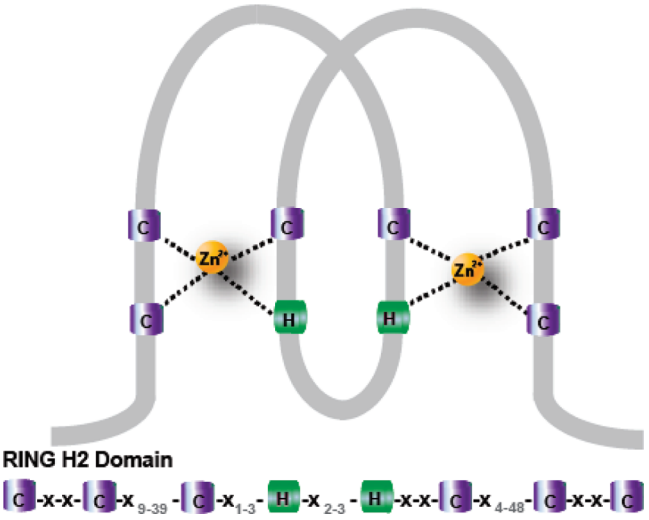


Figure 1. Cross brace structure of the RING-H2 domain as found in the ubiquitin E3 ligase BCA2. C, cysteine (purple); H, histidine (green); Zn^{2+}, zinc ions (yellow); gray, number of other amino acids between the cysteine and histidine motifs.

above suggest that BCA2 could prove an important therapeutic target within the E3 ubiquitin ligase class for the future treatment of breast cancer.

Given the crucial role of Zn^{2+} ions in the catalytic RING domain of BCA2, a series of "zinc-ejecting" compounds from the National Cancer Institute database have been screened for their ability to inhibit BCA2 activity in BCA2-expressing breast cancer cell lines such as MCF-7. These studies have led to the identification of DSF (NSC25953),[10,12,13] a registered drug for the treatment of alcoholism by virtue of additional ALDH1 activity, as a potent BCA2-inhibitory antitumor agent. In this paper, we describe the synthesis and antitumor evaluation of four series of novel "zinc-affinic" agents, in order to optimize selective activity against recombinant BCA2 and BCA2-expressing breast cancer cell lines.[14] From this antitumor data, we derived SAR insight into BCA2-inhibitory pharmacophore requirements, leading to the identification of potent and selective BCA2-inhibitory antitumor agents without accompanying ALDH-inhibitory activity.

Chemistry. Rationale for Choice of Candidate Compound Series. Four series of molecules having the potential ability to remove Zn^{2+} ions from the RING domain of BCA2 were synthesized, based on structures where the core group is known for zinc-binding activity (Figure 2). Initial studies focused on agents structurally related to the lead compound DSF (bis(diethylaminothiocarbonyl)disulfide; tetraethylthiuram disulfide), and derived from different secondary amine starting materials, in order to explore the structure−activity relationships (SAR) around DSF with respect to BCA2-inhibitory antitumor activity.

The ability of compounds such as DSF (and its pyrrolidine and piperidine analogs) to form chelation complexes with Zn^{2+} has previously been reported.[15,16] An alternative mechanism for removal of Zn^{2+} ions from the RING domain of BCA2 is the process of zinc-ejection, where modification of active-site cysteine residues causes ejection of Zn^{2+} from the active site. In the case of DSF and close analogues, the zinc-ejection process appears to be favored for interactions with zinc-binding proteins. For example, the inhibition of the histone demethylase JMJD2A by zinc-ejection, following treatment with a range of potential Zn^{2+} ejectors including DSF, has recently been described.[17]

A further "symmetrical" disulfide structurally related to the DSF-like compounds—4,4′-dithiodimorpholine (4,4′-dimorpholinedisulfide)—was synthesized in order to explore the structural requirement of a dithioester function for BCA2-inhibitory antitumor activity. 4,4′-Dithiodimorpholine has previously been studied as a potential lead compound for the treatment of neoplastic lesions of the cervix by targeting zinc-binding domains of the E6 oncoprotein of human papillomavirus.[18]

The carbamo(dithioperoxo)thioate series was designed as a series of "unsymmetrical" disulfides containing a cleavable S−S bond and the N(C=S)S—S group characteristic to DSF. This series presented the opportunity to vary the R and R^1 groups (Figure 2) to explore SAR requirements for BCA2-inhibitory antitumor activity. It is noteworthy that carbamo(dithioperoxo)thioates (mixed disulfides derived from DSF) have previously been reported as inhibitors of human mitochondrial[19] and sheep liver[20] ALDH, most likely via zinc-ejection. The registered drug status of DSF itself is based on its inhibitory activity against ALDH.

In order to further explore the structural requirements for BCA2-mediated antitumor activity, we synthesized a diverse series of dithiocarbamates. The dithiocarbamates bear close structural similarities with DSF analogs, but lack the S−S single bond, making this series an ideal candidate for exploring the importance of the disulfide bond in mediating antitumor activity.

Benzisothiazol-3-one derivatives have been reported to have a zinc-ejection effect, and have been tested for antiretroviral activity and their ability to eject Zn^{2+} from the HIV nucleocapsid zinc-finger protein NCP7.[21] In addition, we were interested in the benzisothiazolone structure as a known zinc-ejector lacking a labile S−S bond.

Synthesis of DSF Analogs (Bis(dialkylthiocarbamoyl)-disulfides; 3a−c) and 4,4′-Dithiodimorpholine (4). DSF (**1**) is commercially available (Sigma-Aldrich). Other DSF analogue thiuram disulfides (**3a−c**) were synthesized in low to moderate yields via a one-pot reaction (General Method A, Experimental Section, Scheme 1), by mixing the appropriate secondary amine (**2a−c**) with carbon disulfide under basic conditions (aqueous sodium hydroxide). Without further purification, the (presumed) intermediate dithiocarbamic acid was oxidized to the required thiuram disulfide in low to moderate yields as previously described using either aqueous iodine/potassium iodide[22] (compounds **3a** and **3b**) or hydrogen peroxide[23] in acetic acid (compound **3c**). More recently, an efficient synthesis of thiuram disulfides has been reported using carbon tetrabromide to promote reaction of amines with carbon disulfide and subsequent disulfide bond formation.[24] Alternative methods for the chemical oxidation of thiols to disulfides are also available, for example, the use of bromine on hydrated silica.[25] The structurally related dithiodimorpholine (**4**) was synthesized as previously described,[18] via treatment of morpholine with sulfur monochloride under basic conditions (Scheme 1).

Synthesis of Carbamo(dithioperoxo)thioates. A series of carbamo(dithioperoxo)thioates (**5a−j**) that are structurally related to DSF analogues were synthesized in order to extend our understanding of SAR within the class of BCA2-inhibitory zinc-ejectors and to further explore whether an S−S single bond is necessary for BCA2-inhibitory antitumor activity. The one-pot synthesis of carbamo(dithioperoxo)thioates is essentially

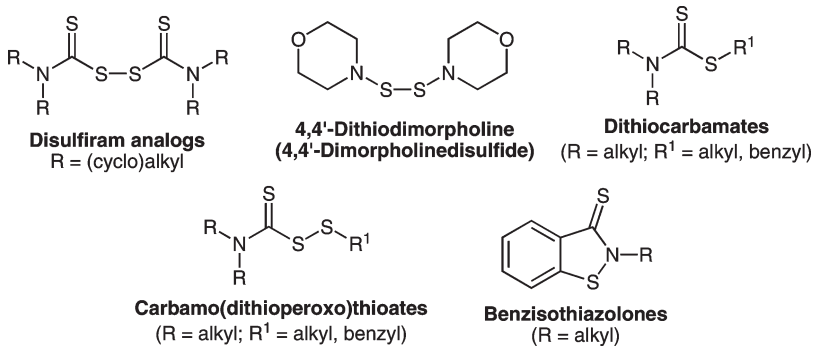
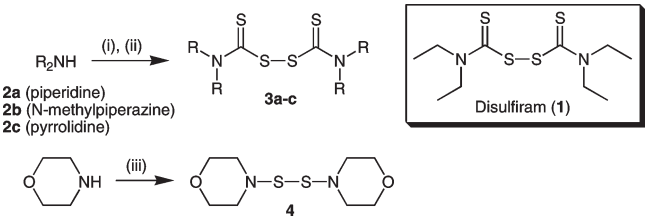


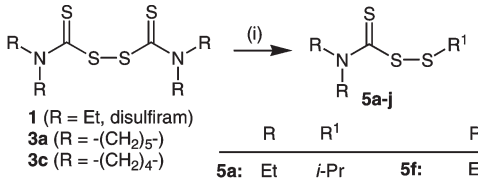
Figure 2. Potential zinc-binding BCA2 inhibitors.

Scheme 1[a]



[a] Reagents: (i) CS_2, NaOH (aq); (ii) I_2, KI (aq) or H_2O_2, AcOH; (iii) S_2Cl_2, NaOH, hexane/H_2O, 0 °C.

Scheme 2[a]



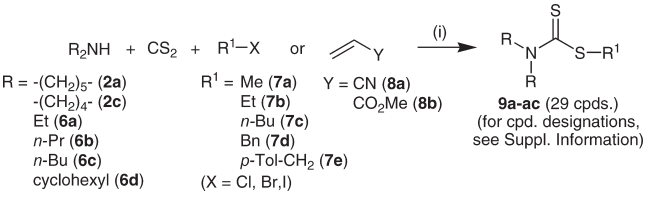
	R	R^1		R	R^1
5a:	Et	*i*-Pr	**5f:**	Et	CH_2CH_2OH
5b:	Et	*n*-Bu	**5g:**	Et	4-$C_6H_4CO_2H$
5c:	Et	*i*-Bu	**5h:**	Piperidinyl	*n*-Bu
5d:	Et	*n*-Hex	**5i:**	Piperidinyl	Cyclohexyl
5e:	Et	Cyclohexyl	**5j:**	Pyrrolidinyl	Cyclohexyl

[a] Reagents: (i) R^1SH, EtOH, reflux, 16 h.

straightforward, and is based on previous described methods (General Method B, Experimental Section, Scheme 2).[26,27] Briefly, stirring commercially available DSF (or synthetic analogues **3a** or **3c**) with the appropriate thiol in ethanol under refluxing conditions (16 h) yielded the required carbamo(dithioperoxo)thioate product (**5a**−**j**, Scheme 2) in low to moderate yield following purification by column chromatography using mixtures of ethyl acetate and hexane as eluent.

Synthesis of Dithiocarbamates. Dithiocarbamates (**9a**−**ac**) were readily synthesized in a one-pot reaction according to the method of Azizi et al.[28,29] (General Method C, Experimental Section; Scheme 3). The appropriate secondary amine (**2a**, **2c**, **6a**−**d**) was mixed with carbon disulfide and the required alkyl halide (**7a**−**e**) or Michael acceptor (**8a**−**b**) in equimolar ratios in water (5−18 h). In some instances, extraction of the aqueous phase using ethyl acetate gave rise to the pure product directly after solvent evaporation, whereas on other occasions, it was necessary to purify products by column chromatography using mixtures of ethyl acetate and hexane as eluent. Spectroscopic and analytical data of dithiocarbamate products **9a**−**ac** are recorded in Supporting Information.

Scheme 3[a]



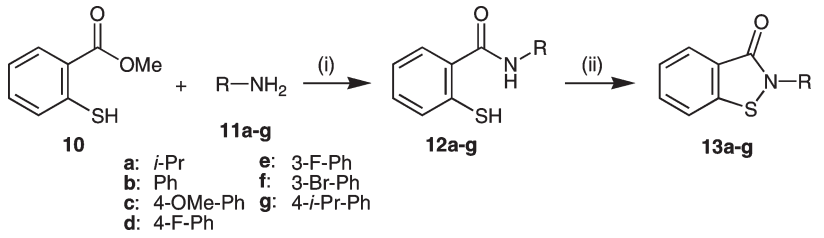
[a] Reagents: (i) H_2O, 5−18 h.

Synthesis of Benzisothiazolones. The general procedure for the synthesis of benzisothiazolones was adopted from the previously reported method of Correa et al. (General Method D, Experimental Section; Scheme 4).[30] The first step of the synthesis involves formation of the amide (**12a**−**g**) by reacting methyl thiosalicylate (**10**) with different primary amines (**11a**−**g**), followed by oxidative ring closure using phenyliodonium di(trifluoroacetate) (PIFA) to give the required benzisothiazolones (**13a**−**g**) in low to moderate overall yield.

Biological Results

Screening of Zinc Affinic Compounds in $BCA2^+$ and $BCA2^{low/-}$ Cells. A total of 50 compounds representing the four different classes of sulfur-based structures described above were evaluated for antiproliferative activity by methyltetrazolium (MTT) assay in the BCA2-positive breast cancer cell lines MCF-7, T47D, MDA-MB-231/ER, the BCA2-low/negative ($^{low/-}$) breast cancer cell line MDA-MB-231 and the normal breast epithelial cell line MCF10A (Figure 3A,B). MDA-MB-231/ER is an isogenic subclone of MDA-MB-231 generated by transfection with ERα. ERα expression activates BCA2 expression via transcriptional regulatory mechanisms.[31] MCF10A was used as a surrogate for assessing normal tissue toxicity of the zinc ejection agents. A compound was considered active if the IC_{50} was $< 10\ \mu M$. We selected $10\ \mu M$ as a cutoff point, because our lead compound DSF has an IC_{50} in BCA2 positive cells between 0.1 and 0.3 μM. Potential selectivity for BCA2 inhibition by a candidate compound was considered if growth inhibition was observed in BCA2 positive cells, but not MDA-MB-231 or MCF10A cells. None of the 29 tested dithiocarbamates (**9a**−**9ac**) or the seven benzisothiazolones (**13a**−**g**) showed activity in any of the breast cancer cell lines (data not shown). All of the ten dithioperoxothioates (**5a**−**j**) were active with IC_{50}'s ranging from 0.15 to 2.75 μM in $BCA2^+$ cells, and 0.9 to $> 10\ \mu M$ in MDA-MB-231 or MCF10A cells (Table 1, Figure 3B). However, only **5d** and

Scheme 4[a]



[a] Reagents: (i) $AlMe_3$, CH_2Cl_2, 0 °C to room temp.; (ii) $PhI(OCOCF_3)_2$, CF_3CO_2H, CH_2Cl_2.

Table 1. Activity of DSF Analogues and Carbamo(Dithioperoxo)Thioates against Human Breast Cancer and Normal Cell Lines[a]

	mean $IC_{50} \pm$ SD (μM)[b] in cell lines[c]				
compound	MCF-7	MDA-MB-231	MDA-MB-231/ER	T47D	MCF10A
1 (DSF)	0.1 ± 0.01	>10	0.32 ± 0.14	0.17 ± 0.03	10 ± 0.2
3a	0.03 ± 0.001	>10	0.59 ± 0.12	0.27 ± 0.02	>10
3b	0.35 ± 0.05	3.0 ± 0.4	0.29 ± 0.1	0.33 ± 0.02	>10
3c	0.38 ± 0.03	>10	0.25 ± 0.1	0.28 ± 0.02	>10
4	>10	>10	>10	>10	>10
5a	0.39 ± 0.28	0.96 ± 0.03	0.25 ± 0.1	0.23 ± 0.01	>10
5b	0.4 ± 0.3	7.4 ± 2.6	0.3 ± 0.13	0.15 ± 0.02	>10
5c	0.45 ± 0.25	6.0 ± 2.8	2.1 ± 0.43	0.20 ± 0.02	>10
5d	0.43 ± 0.1	>10	0.35 ± 0.28	0.23 ± 0.02	>10
5e	0.3 ± 0.28	0.96 ± 0.01	0.28 ± 0.17	0.18 ± 0.02	>10
5f	0.5 ± 0.24	>10	2.75 ± 0.17	0.30 ± 0.03	>10
5g	0.5 ± 0.2	6.3 ± 2.6	0.55 ± 0.12	0.15 ± 0.01	>10
5h	0.65 ± 1.2	8 ± 1.6	0.39 ± 0.17	0.35 ± 0.03	>10
5i	0.6 ± 0.1	6.7 ± 2.5	2.3 ± 0.22	0.25 ± 0.03	>10
5j	1.4 ± 1.8	4.7 ± 3.9	0.25 ± 0.08	1.35 ± 0.02	>10

[a] Determined by MTT assay (72 h drug exposure); see ref 42 for details. [b] Compounds tested in triplicate, data expressed as mean values of three independent experiments. [c] Cancer cell line origin: MCF-7 (breast; BCA2 +ve; ER +ve), MDA-MB-231 (breast; BCA2 low/-ve; ER -ve), MDA-MB-231/ER (isogenic stable ER transfected clone of MDA-MB-231, BCA2+ve, ER +ve), T47D (breast, BCA2 +ve, ER +ve). MCF10A, normal breast epithelial cell line.

5f were active in $BCA2^+$, but inactive in $BCA2^{low/-}$ cells; **5d** was more potent than **5f** (Table 1). Similarly, DSF analogues (**3a**−**c**) were very active in $BCA2^+$ cells and inactive in BCA2 $^{low/-}$ cells with the exception of **3b** (Table 1). 4,4′-Dithiomorpholine (**4**) was inactive in both $BCA2^+$ and $BCA2^{low/-}$ cells. The most potent DSF derivative was **3a** with an IC_{50} in MCF-7 of 0.03 μM (Table 1, Figure 3A). On the basis of their activity profile compounds **3a**, **3c**, **5d**, and **5f** were considered for further biological testing in comparison to DSF. Representative growth curves showing the antiproliferative activity of **3a** and **5d** in the BCA2-positive and BCA2-negative cell lines are depicted in Figure 3A,B.

To test our hypothesis that the DSF analogues and carbamo(dithioperoxo)thioates act through a zinc ejection mechanism, MCF-7 cells were treated with compounds **1**, **3a**, and **5d** in the presence of zinc(II) chloride or with zinc chloride alone (Figure 3C). When zinc chloride was added at a range of concentrations between 600 nM and 75 μM to MCF-7 cells exposed to the respective IC_{50}'s of **1**, **3a**, and **5d** (Table 1), their growth inhibitory activity was completely abolished (Figure 3C). Zinc chloride alone had no effect on the growth of MCF-7 cells. These results are consistent with our working hypothesis that the active DSF and carbamo-(dithioperoxo)thioate analogues are causing inhibition of BCA2-mediated cell growth via a reversible zinc ejection mechanism.

Evaluation of Lead Compounds for Inhibition of BCA2 E3 Ligase Activity/Autoubiquitination. Lead compounds from the cell-based screening procedure were tested for their potential to inhibit the E3 ligase activity of BCA2. A hallmark of RING-finger ubiquitin E3 ligases is the capability of autoubiquitination, hence the ubiquitination efficacy of such ligases can be studied in absence of a functional substrate.[7,9] Recombinant wild-type human BCA2 produced in *E. coli* was used together with ATP, a rabbit E1 and the recombinant human E2 UbcH5b. Compounds active toward breast cancer cell lines expressing BCA2 (MCF-7, T47D, MDA-MB-231/ER), but inactive in $BCA2^{low/-}$ MDA-MB-231 breast cancer and normal MCF10A cells (Figure 4A), plus DSF were added to the ubiquitination reaction at concentrations of 5 and 50 μM (Figure 4B). Because this assay employs highly purified, recombinant BCA2, E1 and E2 enzymes and drugs are only incubated with the enzyme mixture for 30 min; therefore, drug concentrations higher than those inhibiting cell growth to 50% or 100% in a 5 day assay were used. Autoubiquitination of BCA2 was detected with antiubiquitin antibodies. In the event of the autoubiquitination, a high molecular weight polyubiquitin signal is seen (Figure 4B, 5 μM reactions) and low molecular weight ubiquitin bands are weak or absent. In contrast, when autoubiquitination is inhibited, ubiquitin is not attached to the E3 ligase and shows strong bands, whereas polyubiquitin signals are absent (Figure 4B, 50 μM reactions). All putative BCA2 inhibitory compounds were able to inhibit the autoubiquitination of recombinant BCA2 at a concentration of 50 μM, but not 5 μM (Figure 4B). Identical experiments performed using the recombinant RING-E3 ligase Mdm2 did not show inhibition of Mdm2 autoubiquitination (data not shown). These data suggest that our cell-based screening system was predictive of direct BCA2 enzyme inhibition.

Effects of Zinc Ejecting Compounds 3a and 5d on BCA2 Protein Stability in Cells. To confirm that inhibition of the

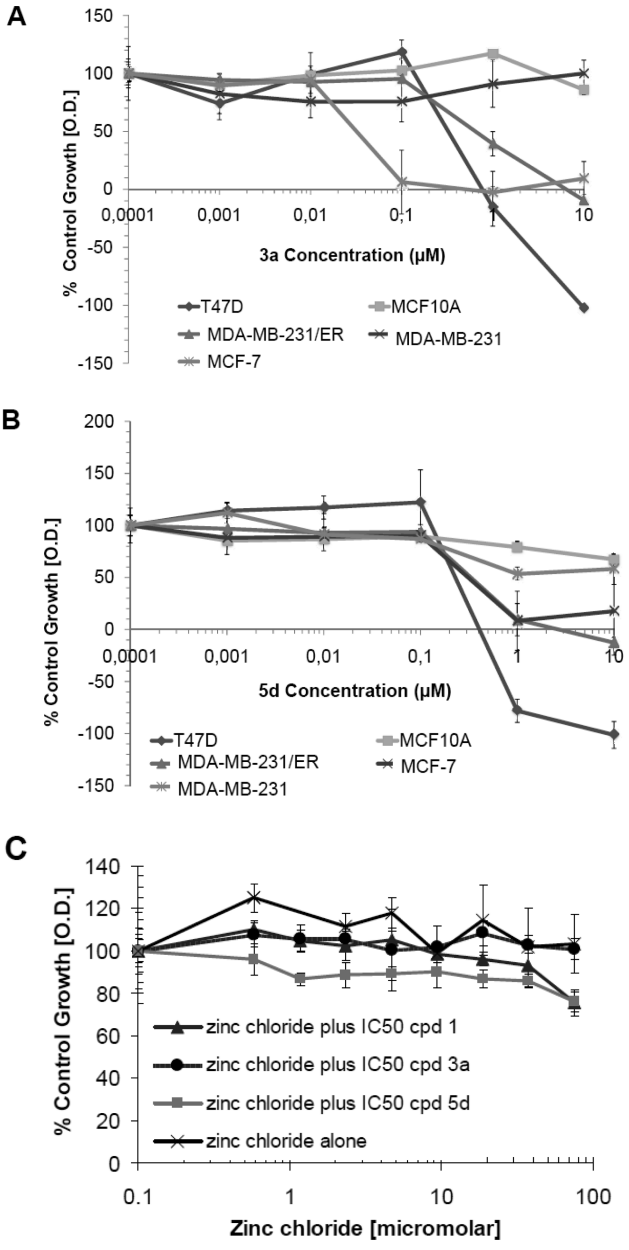


Figure 3. (A) Representative growth curves for compound **3a** in BCA2-positive and BCA2-negative cell lines from Table 1. (B) Representative growth curves for compound **5d** in BCA2-positive and BCA2-negative cell lines from Table 1. (C) Effects of compounds **1**, **3a**, and **5d** in the presence of zinc chloride. To assess whether the addition of Zn^{2+} can rescue the growth inhibitory effects of zinc ejecting compounds in BCA2-positive MCF-7 cells, various concentrations of $ZnCl_2$ were added together with IC_{50} concentrations of **1**, **3a**, or **5d** in MCF-7 cells. The value set as 100% is vehicle control treated MCF-7 cells without $ZnCl_2$ and drug. $ZnCl_2$ did prevent growth inhibition by compounds **1**, **3a**, and **5d** at all concentrations tested.

BCA2 catalytic E3 ligase activity by zinc ejecting compounds seen in the autoubiquitination experiments is of relevance under physiological conditions, we performed cellular assays with the $BCA2^{+}$ MCF-7 breast cancer cell line (Figure 5). We selected the most potent compound from each of the DSF analogue (**3a**) and dithioperoxothioate series (**5d**). MCF-7 cells, when exposed for 16 h (approximate half-life of $BCA2$[7]) to both compounds at their IC_{100} concentrations, showed a marked reduction of BCA2 protein levels (Figure 5A,B, lanes 2). The addition of the protein synthesis inhibitor cycloheximide (CHX) to **3a** or **5d** showed steady-state levels (Figure 5A) or further reduction of BCA2 (Figure 5B). Importantly, when the proteasome inhibitor N-(benzyloxycarbonyl)-leucinylleucinylleucinal (MG-132)[32] was added to the zinc ejector and CHX combination or the zinc ejector treatment alone (Figure 5A,B, lanes 4−5), BCA2 was stabilized and accumulated even in absence of protein synthesis (Figure 5A,B, lanes 4). These studies show that treatment with **3a** or **5d** leads to down-regulation of BCA2 protein, and that this process is dependent on the ubiquitin-proteasome pathway. If proteasomal degradation of ubiquitinated BCA2 protein is inhibited by **14** (MG-132), the E3 ligase accumulates (Figure 5).

Testing for ALDH Inhibition. To study whether novel BCA2 inhibitory disulfide analogues **3a** and **3c**, or the dithioperoxothioates **5d** and **5f**, retained the ability to inhibit ALDH1 like DSF, we performed an ALDH1 inhibition test. To detect ALDH1 positive cancer cells, a flow cytometry based method was recently developed using the leukemia cell line K562, known to have high levels of ALDH1.[33] Thus, we analyzed the ability of DSF, **3a**, **3c**, **5d**, and **5f** to inhibit the ALDH1-positive cell fraction in K562 cells compared to a known inhibitor of ALDH activity, diethylaminobenzaldehyde (DEAB) (Table 2). While DSF and **3a** inhibited ALDH1 to 57% and 79% respectively, **3c**, **5d**, and **5f** showed little inhibition of this enzyme. DSF analogue **3c** inhibited only a negligible fraction of ALDH1 (3.4%) and is therefore the most selective BCA2 E3 ligase inhibitor among the series of disulfides tested in this study. Dithio(peroxo)thioate compounds **5d** and **5f** also exhibited low levels of ALDH inhibition (9.8% and 8.6% relative to control, respectively).

Discussion

During this study, we aimed to identify potent and selective BCA2-inhibitory antitumor agents devoid of the ALDH-inhibitory activity characteristic to DSF. Considerable progress has been made toward these goals. In terms of the identification of potent and selective activity in BCA2-expressing breast cancer cell lines, the thiuram disulfide compound **3a** was found to be the most active (IC_{50} = 30 nM) in the MCF-7 cell line, with no activity (IC_{50} > 10 μM) in the BCA2 negative MDA-MB-231 breast cancer cell line and MCF10A normal breast epithelial cells. The activity of **3a** in MCF-7 cells was found to exceed that of DSF itself (IC_{50} = 100 nM). The similar activity profile of the DSF analogue **3a** to DSF is perhaps not surprising given its structural similarity; however, it is notable that the other closely related DSF analogues were substantially less potent and/or less selective (**3b**) compared to DSF and **3a**. The lack of activity for dithiodimorpholine (**4**) is suggestive of the need for a dithioester function for BCA2 inhibitory activity.

Among the series of dithio(peroxo)thioates (**5a**−**j**) structurally related to DSF, two compounds (**5d** and **5f**) stand out as having selective activity in the submicromolar IC_{50} range against the BCA2-expressing breast cancer cell lines compared to BCA2-negative/low MDA-MB-231 and normal MCF10A cells. Surprisingly, however, other dithioperoxothioate compounds more closely related in structure to DSF (such as **5a** and **5e**) did not display selective activity against BCA2-expressing cancer cell lines. The selectively active compounds **5d** and **5f** reinforce our hypothesis that an N(C=S)S—S group is required for selective activity in the BCA2-expressing breast

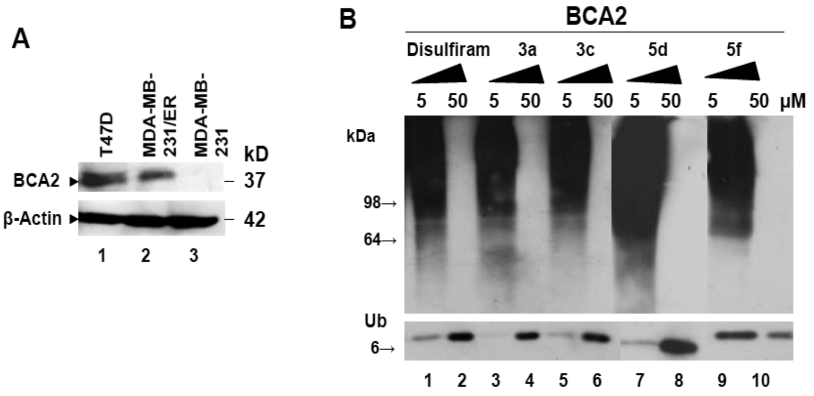


Figure 4. (A) Western blot showing expression of endogenous BCA2 in T47D and the isogenic MDA-MB-231 parental and MDA-MB-231/ER breast cancer cell lines. Anti-BCA2 antibodies were developed by us and used as previously described.[7] β-actin was used as equal loading control. (B) Western blot analysis of BCA2 autoubiquitination in the presence of disulfide analogues at 5 and 50 μM of drug dissolved in dimethylsulfoxide. The membrane was probed with antiubiquitin antibodies. Shown are the ubiquitin signal (8 kD) and high molecular weight polyubiquitinylated BCA2 (> 98 kD). Polyubiquitinated proteins are typified by a high molecular weight smear.

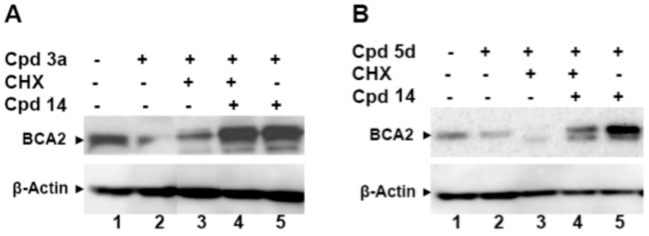


Figure 5. Western blots of endogenous BCA2 protein expression in MCF-7 breast cancer cells treated for 16 h with the BCA2 ligase inhibitors **3a** (**A**) and **5d** (**B**) in the presence and absence of the protein synthesis inhibitor CHX and the proteasome inhibitor **14**. (A) MCF-7 cells treated with vehicle only (DMSO, lane 1); compound (cpd) **3a** at its 100% growth inhibitory concentration (0.25 μM, lane 2); **3a** and CHX (lane 3); **3a**, CHX and **14** (lane 4); and **3a** plus **14** (lane 5). β-actin was used as equal loading control. (B) MCF-7 cells treated with vehicle only (DMSO, lane 1); compound (cpd) **5d** at its 100% growth inhibitory concentration (1 μM, lane 2); **5d** and CHX (lane 3); **5d**, CHX and **14** (lane 4); and **5d** plus **14** (lane 5). β-actin was used as equal loading control.

cancer cell lines, and the cellular data were further validated by the ability of compounds **3a**, **3c**, **5d**, and **5f**, plus DSF, to abolish the autoubiquitination activity of recombinant BCA2 at 50 μM (Figure 4). Moreover, for the most potent compounds of each class, **3a** and **5d**, we could show that inhibition of isolated enzyme activity translates into down-regulation of endogenous BCA2 at physiologically relevant drug concentrations (IC_{100}) that is dependent on its degradation through the ubiquitin-proteasome system (Figure 5). The importance of reversible zinc ejecting properties of **3a** and **5d** similar to that of DSF (compound **1**) for their mode of action, was demonstrated by the zinc chloride rescue studies shown in Figure 3C. When Zn^{2+} ions were added to **1**, **3a**, or **5d** concentrations that inhibit the growth of the BCA2 positive cell line MCF-7 to 50% under physiological Zn^{2+} levels, their growth inhibitory activity was abolished even at the lowest concentration tested.

At the outset of our studies, we were interested in the discovery of selective BCA2-inhibitory antitumor agents that lacked the ALDH-inhibitory activity that is the basis for the registered drug status of DSF in the treatment of alcoholism. Although DSF is a safe drug, the ingestion of alcohol causes a "hangover" effect leading to symptoms such as flushing of the skin, accelerated heart rate, shortness of breath, nausea, vomiting, and circulatory collapse. Interestingly, studies on the role of ALDH1 as a marker of normal and malignant mammary stem cells associated with a poor clinical outcome[34] suggest that retention of ALDH-inhibitory activity alongside BCA2 inhibition may not necessarily be an adverse property for the development of new anticancer agents.

Table 2. ALDH Inhibition in K562 Cells for Lead Compounds

compound (15 μM)	$ALDH^+$ K562 cells (%)	ALDH inhibition (%)
BAAA (positive control)	40.39 ± 2.21	0
DEAB (negative control)	0.53 ± 0.11	98.68
1 (disulfiram)	17.26 ± 2.21	57.26
3a	8.3 ± 1.7	79.45
3c	39.01 ± 0.8	3.4
5d	36.47 ± 3.82	9.8
5f	36.9 ± 3.15	8.6

By virtue of its zinc-[12] and copper-[35]binding properties, DSF has been shown to exhibit a number of interesting antitumor properties. The antiproliferative and pro-apoptotic effects of DSF have been attributed (in various cancer model systems) to include inhibition of proteasome activity,[35,36] nuclear factor kB,[37] ATP binding cassette (ABC) drug transporter protein activity,[38,39] and angiogenesis.[40] Pharmacological profiling of DSF using human tumor cell lines and tumor cells from patients has provided further evidence of antitumor potential.[41]

The literature around the antitumor activity of DSF is strongly indicative of a diversity of molecular targets and drug properties, mediated largely through the binding of metal ions (zinc and copper) and the interaction of DSF with key cysteine residues in target proteins.[42] Given that the literature on interactions of thiuram disulfides and dithioperoxothioates with zinc-dependent proteins is dominated by zinc-ejection effects,[17,42] we hypothesize that inhibition of BCA2 is likely to be related to reversible zinc-ejection from the enzyme active site via modification of key cysteine residues. This hypothesis is supported by our zinc chloride rescue data shown in Figure 3C. Despite its rather indiscriminate biochemical and pharmacological profile, DSF is notable for being very well tolerated following long-term treatment at relatively high doses (routinely 300−500 mg per day), and for its high oral bioavailability (> 80% bioavailability, with approximately 20% of drug remaining in the body for

1−2 weeks postingestion).[42] It is likely that the most active thiuram disulfides (**3a** and **3c**) and dithio(peroxo)thioates (**5d** and **5f**) synthesized in this study will also exhibit a diverse spectrum of antitumor activity, given their structural similarity to DSF. Further studies are ongoing to further define the antitumor potential of the lead compounds synthesized here, and their associated stability and toxicology.

Conclusions

The synthesis and evaluation of a variety of experimental agents based on the lead compound DSF has uncovered new antitumor agents with potent and selective antiproliferative activity against the E3 ubiquitin ligase BCA2. Potent and selective activity was observed in BCA2-expressing breast cancer cell lines (e.g., compound **3a**, **5d**), and biochemical and cellular studies with recombinant and endogenous BCA2, respectively, confirmed the ability of active compounds to inhibit the RING E3 ligase. In three cases (**3c, 5d** and **5f**) new BCA2-inhibitory molecules were found that lacked the ALDH-inhibitory activity characteristic to DSF and analogues.

Experimental Section

Chemistry. Melting points were measured on a Griffin apparatus and are uncorrected. Mass spectra were recorded on a Bruker MicroTOF LC instrument or at the EPSRC National Mass Spectrometry Centre (Swansea, U.K.). NMR spectra were recorded on a Bruker AVANCE 500 MHz instrument; coupling constants (J values) are in Hz. Merck silica gel 60 (40−60 μM) was used for column chromatography. All commercially available starting materials were used without further purification. Following purification, all new compounds were determined to possess ≥95% purity as determined by melting point/NMR/mass spectrometry and comparison with published data (for previously reported compounds), or a combination of spectroscopic analysis and combustion analysis (% CHN tested in duplicate) for new compounds. All compounds described were synthesized in the laboratory except the initial lead compound DSF, which is commercially available (Sigma-Aldrich, T1132, >97%).

General Method (A) for the Synthesis of Thiuram Disulfides (3a−b).[22] A solution of sodium hydroxide (160 mg, 4.0 mmol) in water (4 mL) was added to a mixture of secondary amine (4.0 mmol) in water (5 mL). After stirring at room temperature for 20 min, carbon disulfide (0.24 mL, 4.0 mmol) was added, and the mixture was stirred for a further 90 min. A solution of iodine in potassium iodide was prepared by adding potassium iodide (3.19 g, 19.2 mmol) and water (8 mL) to iodine (1.02 g, 4.0 mmol). The I_2/KI(aq) solution was then added dropwise to the reaction mixture, whereupon the solution initially turned yellow followed by formation of a precipitate. After stirring for a further 3 h, the precipitate was collected by filtration in vacuo and washed with excess water and 1 M aqueous sodium thiosulfate. The crude product was purified by recrystallization from petroleum ether and toluene to give the required thiuram disulfide (**3a−b**) as a yellow solid in low to moderate yield.

Bis(piperidinylthiocarbonyl)disulfide (3a). From piperidine. Yield = 28%. Mp 124 °C (lit.[24] 124−126 °C). ^{1}H NMR ($CDCl_3$) δ 4.25 (8H, m, NCH_2), 1.78 (12H, m). ^{13}C NMR ($CDCl_3$) δ 192.88 (C=S), 55.92 (NCH_2), 45.68, 24.41. m/z (EI+) 320 (M^+), 147.

Synthesis of Bis(pyrrolidinylthiocarbonyl)disulfide (3c).[23] A solution of pyrrolidine (0.49 mL, 6.0 mmol) in THF (1 mL) was added to a solution of sodium hydroxide (240 mg, 6.0 mmol) in water (2 mL) with stirring at 0 °C. Carbon disulfide (0.36 mL, 6.0 mmol) was then added dropwise and the mixture stirred at 0 °C for 30 min. Crushed ice (4.5 g) and acetic acid (0.9 mL) were then added, leading to the formation of a white precipitate. After a further 30 min, hydrogen peroxide (0.37 mL of 27.5% solution, 3.0 mmol) was added dropwise while maintaining the reaction temperature at 0 °C. n-Hexane (2.5 mL) was then added leading to the formation of precipitate after 30 min that was collected by filtration and washed with hexane and 2% aqueous acetic acid. The crude product was recrystallized (toluene/hexane) to give the product as a white solid in 39% yield. Mp 135−137 °C (lit.[43] 137−139 °C). ^{1}H NMR ($CDCl_3$) δ 3.98 (8H, m, NCH_2), 2.17 (4H, m), 2.01 (4H, m). ^{13}C NMR ($CDCl_3$) δ 189.31 (C=S), 57.12 (NCH_2), 51.10 (NCH_2), 26.75, 24.42. m/z (EI+) 292 (M^+), 146.

Synthesis of Dithiodimorpholine (4).[18] A solution of sulfur monochloride (3.20 mmol) in hexane (10 mL) was slowly added to a rapidly stirred two-phase mixture of morpholine (3.20 mmol), water (20 mL), hexane (10 mL), and sodium hydroxide (4.22 mmol) at 0 °C. The reaction mixture was then stirred at room temperature for a further 1 h; then, the separated aqueous layer was washed with hexane (20 mL) and the combined organic layers washed with 1 N HCl (aq) (10 mL), then water (10 mL). The organic layers were dried (Na_2SO_4), followed by concentration in vacuo (<40 °C) and recrystallization from n-hexane to give the product as a pale yellow solid in 65% yield. Mp 117−118 °C (lit.[18] 118−120 °C). ^{1}H NMR ($CDCl_3$) δ 3.74 (8H, m, OCH_2), 2.81 (8H, m, NCH_2). ^{13}C NMR ($CDCl_3$) δ 67.44 (OCH_2), 55.92 (NCH_2). m/z (CI+) 237 ($M^+ + 1$).

General Method (B) for the Synthesis of Carbamo(dithioperoxo)-thioates (5a−j). An equimolar mixture of the appropriate thiol (9.0 mmol) and thiuram disulfide (9.0 mmol) in ethanol (20 mL) were heated under reflux for 16 h. Following concentration of the reaction mixture in vacuo, the pure product was obtained in low yield after purification by column chromatography using hexane/ethyl acetate as eluant.

Hexyl Diethylcarbamo(dithioperoxo)thioate (5d).[19] From DSF and n-hexanethiol. Yellow oil, 9% yield. ^{1}H NMR (DMSO-d_6) δ 4.02 (2H, q, J = 6.8 Hz, NCH_2), 3.83 (2H, q, J = 6.8 Hz, NCH_2), 2.78 (2H, t, J = 6.7 Hz, SCH_2), 1.59 (2H, m), 1.40 (2H, m), 1.11−1.35 (10H, m), 0.92 (3H, t, J = 6.5 Hz, $CH_3CH_2CH_2$). ^{13}C NMR (DMSO-d_6) δ 51.08 (NCH_2), 46.78 ($\overline{N}CH_2$), 37.97 (SCH_2), 30.82 (CH_2), 27.83 (CH_2), 27.47 (CH_2), 21.97 (CH_2), 13.80 (CH_2), 12.93 (CH_3), 11.15 (CH_3). m/z (CI+) 266.1 ($M^+ + 1$).

2-Hydroxyethyl Diethylcarbamo(dithioperoxo)thioate (5f).[19] From DSF and β-mercaptoethanol. Thick green oil, 12% yield. ^{1}H NMR (DMSO-d_6) δ 4.84 (1H, t, J = 6.0 Hz, OH), 3.99 (2H, q, J = 6.8 Hz, NCH_2), 3.83 (2H, q, J = 6.8 Hz, NCH_2), 3.60 (2H, q, J = 6.4 Hz, CH_2OH), 2.89 (2H, t, J = 6.4 Hz, SCH_2), 1.25 (6H, m, 2 × CH_3). ^{13}C NMR (DMSO-d_6) δ 59.15 (CH_2OH), 51.18 (NCH_2), 46.82 (NCH_2), 40.70 (SCH_2), 12.95 (CH_3), 11.17 (CH_3). m/z (CI+) 226.0 ($M^+ + 1$).

General Method (C) for the Synthesis of Dithiocarbamates (9a−ac).[28,29] A mixture of secondary amine (10 mmol), carbon disulfide (10 mmol) and alkyl halide/Michael acceptor in water (50 mL) were stirred at room temperature for 16 h. The crude product was extracted using ethyl acetate (3 × 50 mL), dried ($MgSO_4$), and concentrated in vacuo. Purification by column chromatography using ethyl acetate/hexane as eluant gave the crude product (**9a−9ac**) in moderate yield. See Supporting Information for details of spectroscopic data.

General Method (D) for the Synthesis of Benzisothiazolones (13a−g).[30] A solution of $AlMe_3$ (11.52 mmol, 2.0 M in toluene) was added dropwise to a cooled (0 °C) suspension of the amine (11.52 mmol) in dichloromethane (30 mL). When the addition was complete, the reaction mixture was allowed to warm to room temperature and stirring was continued for 30 min. Methylthiosalicylate (0.7 mL, 5.76 mmol) was added and the mixture was heated under reflux (16 h), then allowed to cool and slowly quenched with 5% aqueous HCl (20 mL). The resulting precipitate was removed by vacuum filtration, and the filtrate transferred to a separating funnel, where the organic phase was collected. The aqueous phase was extracted using dichloromethane (3 × 15 mL), and the organic extracts were combined

and washed with a saturated solution of $NaHCO_3$ (15 mL) and brine (15 mL), followed by drying (Na_2SO_4) and concentration in vacuo. Column chromatography (ethyl acetate/hexane) gave the intermediate amides (**12a**−**g**), which were used in the oxidative ring closure step without further purification. A solution of phenyliodonium di(trifluoroacetate) (PIFA) (249 mg, 0.58 mmol) in CH_2Cl_2 (9 mL) was added at 0 °C to a solution of the intermediate benzamide (0.39 mmol) and TFA (0.09 mL, 1.16 mmol) in CH_2Cl_2 (6 mL). The reaction mixture was stirred for 1 h; then, the solvent was removed in vacuo to yield a residue that was purified using a column chromatography (ethyl acetate/hexane). See Supporting Information for details of spectroscopic data.

Biology. Cell Culture. The human breast cancer cell lines T47D, MDA-MB-231, and the human chronic myelogenous leukemia cell line K562 were obtained from American Type Culture Collection (Manassas, VA). The breast cancer cell line MCF-7 and the normal mammary epithelial cell line MCF10A were obtained from the Karmanos Cancer Institute (formerly known as Michigan Cancer Foundation) Cell Repository. MDA-MB-231/ER was generated by transfecting an estrogen receptor alpha (ERα) pcDNA3 construct into parental MDA-MB-231 following the Lipofectamine method as described by the manufacturer (Invitrogen, Carlsbad). Stable clones were derived by G418 selection. ERα expression was confirmed by immunostaining.[7] All cancer cell lines were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone from Fisher Scientific, Pittsburgh, PA). MDA-MB-231/ER was cultured in the presence of 1 mg/mL G418 (Invitrogen, Carlsbad, CA). MCF10A cells were grown in mammary epithelial growth medium (MEGM Bullet Kit, Cambrex Bio Science, East Rutherford, NJ). Cells were passaged routinely and kept at 37 °C and 5% CO_2. Exponentially growing cells were used in all experiments.

MTT Assay. The MTT proliferation assay was performed to assess at which concentration these compounds would produce 50% growth inhibition (IC_{50}) in tumor cells in order to identify leads that could be developed as anticancer agents. The doses employed ranged from 0.0001 to 10 μM. Compounds requiring IC_{50} concentrations > 10 μM were considered inactive. The MTT assay was essentially performed as previously described by us in detail.[44] For compounds found to be active (IC_{50} < 10 μM), three independent experiments were performed and IC_{50} values determined as mean ± standard deviation (SD). Growth curves were generated and IC_{50} and IC_{100} concentrations (100% growth inhibition) were delineated from the growth curves as reported.[44] The MTT assay was further used to assess whether the addition of $ZnCl_2$ can rescue the growth inhibitory activity of compounds **1**, **3a**, and **5d** in BCA2-positive MCF-7 cells. In brief, BCA2-positive MCF-7 cells were treated with vehicle control (growth set as 100%) and the IC_{50} concentrations of **1**, **3a**, and **5d** in the presence of a range of zinc chloride concentrations between 600 nM and 75 μM for 5 days followed by MTT assay as described above.

Western Blotting. Western blots were done for assaying the expression of endogenous BCA2 in the breast cancer cell lines MCF-7 and MDA-MB-231, MDA-MB-231/ER, and T47D. Exponentially growing cells were collected to generate cell lysates, and Western blotting was performed as described by us previously.[7]

Effects of Zinc Ejecting Compounds on Recombinant BCA2 Autoubiquitination. Drug stocks (10 mM) were prepared in DMSO and diluted to 100 μM in 1× ubiquitination assay reaction buffer. Ubiquitination assays were essentially performed as described previously by using recombinant wild-type His-BCA2 in the presence and absence of E2 conjugating enzyme UbcH5b extract, and with or without specific zinc ejecting compounds.[7] In brief, 10 ng recombinant His-BCA2 was used with diluted drugs to obtain a final concentration of 5 or 50 μM zinc ejector in 30 μL reaction volume, and the mixtures were incubated at room temperature for 30 min before performing the ubiquitination assay. The protein−drug solutions were then mixed with 3 μL of 20 mM ATP, 1 μL ubiquitin (1 μg, Sigma, St. Louis, MO), 1 μL E1 (20 ng, rabbit E1, Calbiochem, San Diego, CA), and 1 μL E2 (20 ng, recombinant human UbcH5b, Boston Biochem, Boston MA) in 50 mM Tris HCl pH 8.0 plus 3 μL 10× reaction buffer (500 mM Tris HCl pH 8.0, 20 mM DTT, 50 mM $MgCl_2$) and 21 μL H_2O to obtain a final reaction volume of 30 μL. Each mixture was incubated at 30 °C for 1 h, then 10 μL 4× SDS-gel loading buffer was added and the mixture was boiled for 3 min, followed by separation of reactions on a 4−20% gradient SDS-PAGE gel (Novex from Invitrogen) and immunoblotting.

Effects of Zinc Ejecting Compounds on Endogenous BCA2 Protein Stability. Experiments were essentially performed as in described by us previously.[7] In brief, 1×10^6 MCF-7 cells were seeded into 6-well plates and grown overnight. Compounds **3a** (IC_{100} = 0.25 μM) and **5d** (IC_{100} = 1 μM) were then added at the 100% growth inhibitory concentrations for 16 h alone or in combination with the protein synthesis inhibitor CHX (Sigma, 100 μM) and/or the proteasome inhibitor **14** (Calbiochem, 10 μM). Control cells were treated with vehicle (DMSO) for 16 h. Whole cell lysates were analyzed for BCA2 protein expression by Western blotting.

Determination of ALDH1 Inhibition. K562 cells (human immortalized myelogenous leukemia line) are known to express very high levels of ALDH1. Thus, these cells were used to analyze effects of our lead BCA2 inhibitory compounds and DSF for the inhibition of ALDH1. The ALDH assay kit ALDEFLUOR (StemCell Technologies, Vancouver, BC) was used according to the manufacturers instructions. Briefly, K562 cells were suspended in ALDEFLUOR assay buffer at concentration of 1×10^6 cells/mL. To prepare a substrate for ALDH1, bodipy-aminoacetaldehyde diethyl acetal (BAAA-DA) was activated to bodipy-aminoacetaldehyde (BAAA) in 1 N HCl. Cells were incubated with BAAA in the presence or absence of DEAB, an inhibitor for ALDH1 for 45 min at 37 °C. DEAB was added at concentrations of 15 μM from a 1.5 mM stock prepared in ethanol. Thus, we compared the effects of DEAB on ALDH1 activity to those of our lead compounds at the same concentration and by preparing dilutions in ethanol. Cells were washed once with ALDEFLUOR assay buffer, and then cellular fluorescence based on bodipy-aminoacetate converted BAAA by ALDH1 was measured by BD LSR I through channel 1 (FL1, BD Biosciences, San Jose, CA). $ALDH1^+$ cells were analyzed by a FL1 (horizontal) versus side scatter (SSC) dot plots by comparing fluorescence obtained from cells incubated with DEAB. The percentage of $ALDH1^+$ cells in the presence and absence of DEAB and compounds was determined and the percentage of ALDH1 inhibition calculated.

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Supporting Information Available: Spectroscopic data for thiuram disulfide (**3b**), carbamo(dithioperoxo)thioate (**5a**, **5b**, **5c**, **5e**, **5g**, **5h**, **5i**, **5j**), dithiocarbamate (**9a-9ac**) and benzisothiazolone (**13a**−**g**) compounds.This material is available free of charge via the Internet at http://pubs.acs.org.

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