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The cytotoxicity of garlic-related disulphides and thiosulfonates in WHCO1 oesophageal cancer cells is dependent on *S*-thiolation and not production of ROS

Muneerah Smith [a], Roger Hunter [b], Nashia Stellenboom [b], Daniel A. Kusza [b], M. Iqbal Parker [a,c], Ahmed N.H. Hammouda [b], Graham Jackson [b], Catherine H. Kaschula [b,*]

[a] Department of Integrative Biomedical Sciences, Division of Medical Biochemistry, University of Cape Town, Anzio Road, Observatory,7925 Cape Town, South Africa
[b] Department of Chemistry, University of Cape Town, Rondebosch, 7701 Cape Town, South Africa
[c] International Centre for Genetic Engineering and Biotechnology, Anzio Road, Observatory, 7925 Cape Town, South Africa

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ABSTRACT

Background: Garlic has been used for centuries in folk medicine for its health promoting and cancer preventative properties. The bioactive principles in crushed garlic are allyl sulphur compounds which are proposed to chemically react through (i) protein *S*-thiolation and (ii) production of ROS.
Methods: A collection of R-propyl disulphide and R-thiosulfonate compounds were synthesised to probe the importance of thiolysis and ROS generation in the cytotoxicity of garlic-related compounds in WHCO1 oesophageal cancer cells.
Results: A significant correlation ($R^2 = 0.78$, Fcrit (7,1) $\alpha = 0.005$) was found between the cytotoxicity IC_{50} and the leaving group pK_a of the R-propyl disulphides and thiosulfonates, supporting a mechanism that relies on the thermodynamics of a mixed disulphide exchange reaction. Disulphide (**1**) and thiosulfonate (**11**) were further evaluated mechanistically and found to induce G_2/M cell-cycle arrest and apoptosis, inhibit cell proliferation, and generate ROS. When the ROS produced by **1** and **11** were quenched with Trolox, ascorbic acid or *N*-acetyl cysteine (NAC), only NAC was found to counter the cytotoxicity of both compounds. However, NAC was found to chemically react with **11** through mixed disulphide formation, providing an explanation for this apparent inhibitory result.
Conclusion: Cellular *S*-thiolation by garlic related disulphides appears to be the cause of cytotoxicity in WHCO1 cells. Generation of ROS appears to only play a secondary role.
General significance: Our findings do not support ROS production causing the cytotoxicity of garlic-related disulphides in WHCO1 cells. Importantly, it was found that the popular ROS inhibitor NAC interferes with the assay.

1. Introduction

Exogenous factors are known to contribute to the development of many cancers, with nutritional intervention playing a major role in its prevention. Garlic (*Allium sativum*) is a medicinal plant used throughout the ages for its beneficial health effects, which include protection against cancer. Garlic consumption has been the subject of numerous clinical trials designed to establish a link to reduced cancer risk. Critical reviews of the epidemiological literature [1–5] are quite contradictory depending on the tumour examined, the specific garlic preparation and the analysis used, although there appears to be a consensus that while the available evidence supports this link, further research is needed. The complexity of the problem is compounded by the fact that crude garlic extracts contain numerous pharmacologically active compounds with varying activities and stability. The main compound in whole garlic is alliin which is converted into the pungent allicin by the enzyme alliinase when the clove is crushed (see Fig. 1). Allicin is unstable in concentrated form and readily self-reacts via *S*-thioallylation, or decomposes via an αH Cope-type elimination to give a plethora of second-generation allyl sulphur products [6], with some of the major degradation products displayed in Fig. 1. These compounds share a sulphide or polysulphide functional group flanked by allyl side groups such as allyl disulphide (DAS), diallyl disulphide (DADS), diallyl trisulphide (DATS), diallyl tetrasulphide (DAS4) and *S*-allylmercaptocysteine (SAMC). Owing to the reactivity of allicin and related garlic allyl sulphides in *S*-thioallylation, following ingestion, these compounds spontaneously react with free intracellular thiols such as GSH, or susceptible cysteine residues of redox-sensitive proteins to form mixed disulphides and allyl mercaptan as the leaving group [7].

Allicin and related garlic allyl sulphur compounds, have been shown to exert cytotoxic effects in cancer cells by inhibiting their cell growth through G_2/M cell cycle arrest, and inducing apoptosis both in vitro and in vivo [8,9]. In addition, these compounds appear to exhibit low

* Corresponding author.

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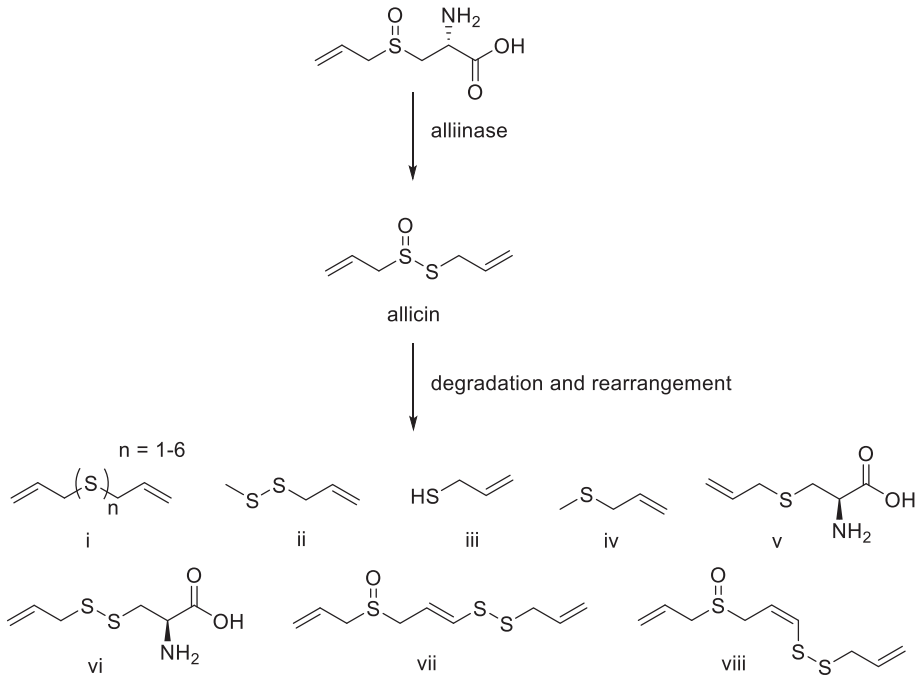


Fig. 1. The predominant secondary products of allicin degradation in crushed garlic. Upon crushing of the garlic clove the enzyme alliinase comes into contact with its substrate alliin and an elimination reaction ensues to generate allicin. Allicin degrades and rearranges to form secondary garlic products which include (i) the polysulfanes: diallyl disulphide (DADS) and diallyl trisulphide (DATS), (ii) allyl methyl disulphide (AMDS), (iii) allyl allylmercaptan, (iv) allyl methyl sulphide (AMS), (v) *S*-allyl cysteine (SAC), (vi) *S*-allylmercaptocysteine (SAMC), (vii) *E*-ajoene, and (viii) *Z*-ajoene.

cytotoxicity in animals [10], as well as a level of selectivity for tumour over normal cells in culture [11–13]. The underlying chemical biology behind their cytotoxicity in cancer cells is still unclear, although protein *S*-thiolation and the production of ROS have both been proposed to play a role [14]. There are a collection of studies which propose that the specific target of DADS [15], DATS [16], DAS4 [17], *Z*-ajoene [13] and SAMC [18] is β-tubulin, which has been shown by proteomics to be *S*-thioallylated by DATS in vitro [16]. This modification is postulated to inhibit the ability of β-tubulin to form microtubules during mitosis, an important step in cell division. Other protein targets that are *S*-thiolated by garlic allyl sulphides include human glutathione reductase [19] and sulphur transferases [20]. Although a handful of proteins have been identified as the specific targets of garlic allyl sulphur compounds, we have recently found that ajoene accumulates in the endoplasmic reticulum of MDA-MB-231 breast cancer cells where it *S*-thiolates a multitude of protein targets [21]. Bulk *S*-thiolation of ER proteins was found to interfere with the proper folding of newly synthesised proteins, and to activate the unfolded protein response [21]. If this mechanism is central to the cytotoxicity of garlic allyl sulphides in cancer cells then the ability of the compound to drive protein thiolysis in the forward direction should be a determinant in its activity.

In addition to protein *S*-thiolation, there is a growing body of evidence supporting the generation of ROS by garlic allyl sulphides in cancer cells. Within cells cytosolic glutathione is predominantly in the reduced state (GSH), in which a GSH:GSSG ratio in excess of 100:1 abounds [22]. Garlic allyl sulphides are reported to affect the GSH:GSSG ratio through spontaneous reaction with GSH to form *S*-allylmercaptoglutathione (GSS-allyl), with the expulsion of a thiol leaving group [23]. Some researchers believe that these metabolites are in fact the active principles in garlic. For example, allicin has been shown to readily react with cysteine to form *S*-allylmercaptocysteine (SAMC) [24], which may be the active metabolite of allicin in vivo, although SAMC itself is also a constituent of crushed garlic. The garlic compound 2-propenyl thiosulfate has been shown to spontaneously react with GSH at physiological pH to form GSS-allyl [20], and such a cross reaction would be expected to lower the GSH:GSSG ratio, thereby allowing a build-up of ROS to occur. Indeed, Schaferberg et al. [25] found that a fast uptake of ajoene accompanied an immediate reduction in the GSH:GSSG ratio in cultured BJA-B lymphoma cells. This was also reported following DAS4 treatment of U937 human histiocytic lymphoma cells [17]. In parallel though, ajoene has also been shown to act as an inhibitor of the SH metabolising enzyme glutathione reductase (GR), involved in the catalysis of GSSG to GSH, which could also play a role in decreasing the GSH:GSSG ratio [19]. Overall, the garlic compounds ajoene [26], DADS [27,28], DATS [29,30] and DAS4 [17] have all been shown to induce a time-dependent production of ROS in promyeloleukemic, glioblastoma, neuroblastoma, prostate and lymphoma cancer cell lines respectively.

In the current study, we aimed at probing whether thiolysis and ROS-production by a small library of garlic-related disulphides and thiosulfonates correlate with their cytotoxicity in WHCO1 oesophageal cancer cells. We utilised a mixed library of R-substituted propyl disulphides and R-substituted thiosulfonates with varying electronic effects feeding into the disulphide bond, and the cytotoxicity of these compounds in WHCO1 oesophageal cancer cells was evaluated. Using inhibitors of ROS, we then probed the relationship between ROS production and the cytotoxicity of disulphide and thiosulfonate compounds in the WHCO1 cells.

2. Materials and methods

2.1. Synthesis

Tetrahydrofuran was distilled under argon from lithium aluminium hydride and then sodium wire with benzophenone. Column chromatography was performed using silica-gel 60 (Merck 7734). Thin layer chromatography (TLC) was carried out on aluminium-backed Merck silica-gel 60 F_{254}. Compounds were visualised on TLC using a UV lamp. Infra-red (IR) absorptions were measured on a Perkin Elmer Spectrum one FT-IR Spectrometer and measured in chloroform. Nuclear Magnetic Resonance spectra were recorded on a Varian Unity 400 (100.6 MHz for ^{13}C) NMR instrument in chloroform-d. Chemical shifts (δ) were

recorded relative to residual chloroform ($\delta = 7.26$ ppm in ^{1}H NMR) and ($\delta = 77.16$ ppm in ^{13}C NMR). All chemical shifts are reported in ppm, and resonances are assigned according to IUPAC numbering, viz H-1 = H on C-1. High-resolution mass spectra were recorded on a Waters API Q-TOF Ultima machine at the Central Analytical Facility, University of Stellenbosch. Elemental analyses were performed using a Fisons EA 1108 CHNS elemental analyser. Reagents were purchased from Sigma-Aldrich or Merck.

2.1.1. Synthesis of thiosulfonates and disulphides

The disulphides (1–8) used in the study were synthesised from the two respective thiols using 1-chlorobenzotriazide (BtCl) as an oxidant [31], while the thiosulfonates (9–12) were synthesised from potassium *p*-toluenethiosulfonate and the respective R-substituted halide, RX (X = Cl or Br) in DMF at 60 °C for 3 h [32].

2.1.2. (S)-N-acetyl-S-((4-methoxybenzyl)thio)cysteine (13)

To a solution of **11** (150.0 mg, 0.486 mmol) in THF (5 mL) was added *N*-acetyl cysteine (40.0 mg, 0.243 mmol) dissolved in THF (0.5 mL) at 25 °C under nitrogen. The reaction was allowed to proceed for 16 h. The solvent was then removed under reduced pressure and the residue purified by silica gel chromatography using ethyl acetate/methanol to afford the mixed disulphide product **13** (53.6 mg, 0.188 mmol) in a 77% yield.

M.p. 112–114 °C; (found: C, 49.83; H, 5.37; N, 2.79; S, 18.03%. $C_{13}H_{17}NO_4S_2$ requires C, 49.51; H, 5.43; N, 4.44; S, 20.33%), found $[M+H]^+$ 316.0668, $C_{13}H_{17}NO_4S_2$ requires 316.0633; ν_{max}/cm^{-1} 3326, 1701, 1223, 1178, 1607, 1557, 1511, 543 (S–S); δ_H ($CDCl_3$, 300 MHz) 2.05 (3H, s, NAc), 2.88 (1H, dd, $J = 6.0, 14.1$ Hz, H-3), 2.94 (1H, dd, $J = 4.7, 14.1$ Hz, H-3), 3.78 (3H, s, OCH_3), 3.87 (2H, s, Bn), 4.75 (1H, m, H-2), 6.49 (1H, d, $J = 7.4$ Hz, NH), 6.74 (1H, s, OH), 6.85 (2H, d, $J = 8.8$ Hz, Ar), 7.22 (2H, d, $J = 8.8$ Hz, Ar); δ_C ($CDCl_3$, 101 MHz) 23.1, 39.5, 43.1, 52.3, 55.5, 114.3, 128.8, 130.7, 159.4, 171.5, and 173.2.

2.2. Cell lines and culture conditions

The oesophageal cancer cell-line WHCO1 was derived from a biopsy of primary oesophageal squamous cell carcinoma of South African origin [33]; the Het-1A cell line is an oesophageal epithelial cell-line derived from a 25-year-old black male, immortalised with the SV40 large T antigen (ATCC CRL-2692). Cells were all incubated at 37 °C under 5% CO_2 and cultured with antibiotics in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Gibco). For experiments, cells were plated at the specified cell density and allowed to recover overnight before treatment with compounds (dissolved in DMSO).

2.3. Cellular viability assay

Cytotoxicity of compounds was evaluated using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cellular viability assay (MTT assay). Briefly, WHCO1 or Het-1A cells were seeded at a density of 2.5 and 5.0×10^3 cells per well respectively (90 μL) in 96 well plates and allowed to recover overnight. Compounds in DMSO (0.1% v/v) were added to the cells (10 μL) at relevant concentrations (2-fold dilutions) and incubated for 24 or 48 h. Control cells received 0.1% DMSO or media alone. To confirm that the compound itself does not interfere with the assay, compound in the absence of cells was included as a control. Thereafter 5 mg/mL MTT (10 μL) was added to each well and incubated for 4 h at 37 °C, followed by addition of 100 μL of 10% SLS in 0.01 M HCl to solubilise the formazan crystals. The absorbance was read at 595 nm on a Multiscan FC plate reader (Thermo Scientific), and data was analysed using GraphPad Prism 4 software using sigmoidal dose–response variable slope curve fitting.

2.4. Cell proliferation assay

Cell proliferation was quantitated using the Cell Proliferation ELISA, BrdU kit (Roche) according to the manufacturer's instruction. Briefly, cells were plated and treated with compounds according to the cell viability protocol above. After 48 h, BrdU labelling solution (10 μL) was added for 4 h, followed by removal and addition of the fixative (100 μL) and treatment with the anti-BrdU-POD (100 μL) working solution for 90 min at RT. After removal of the anti-BrdU-POD, the cells were incubated with the peroxidase (POD) substrate and chemiluminescence was measured using a Veritas microplate luminometer (Promega).

2.5. Apoptosis quantification assay

Apoptosis was quantified using the Cell Death Detection ELISA kit (Roche) according to the manufacturer's instruction. Briefly, cells were plated and treated with compounds according to the cell viability protocol above. After the indicated incubation time, cell lysates were prepared and the cytosolic fraction (20 μL) was transferred to a streptavidin coated plate for analysis. Briefly, the freshly prepared immunoreagent (80 μL) containing anti-histone biotin and anti-DNA-POD was added to each well and the plate was incubated on a shaker at room temperature for 2 h. The solution was then removed by gentle tapping, washed (3×) and then incubated with ABTS (100 μL) for 15 min. The reaction was quenched by adding the ABTS stop solution (100 μL) after which the absorbance at 405 nm and 495 nm was read using a Multiscan FC plate reader (Thermo Scientific).

2.6. Cell-cycle analysis

WHCO1 cells were seeded for triplicate experiments (1×10^6 cells, 8 mL) in 100 mm cell culture dishes and allowed to recover overnight. To the cells were added DMSO solutions of each compound in media. Control cells received 0.1% DMSO alone. After the specified incubation time (0, 6, 12, or 24 h), cells were lifted with trypsin, re-suspended in 2 mL cold PBS, and fixed with cold 70% ethanol (8 mL). Cells were pelleted, washed with cold PBS, and re-suspended in PBS (100 μL) containing RNase A (50 μg/mL, Roche). Twenty minutes prior to analysis, propidium iodide (10 μg/mL) was added and incubated with the sample at 4 °C. Individual samples were run on a Becton Dickinson FACSCalibur flow cytometer using a 488 nm coherent laser and the acquisition software Cellquest Pro version 5.2.1. The cell population was identified and gated (R1) on a forward scatter (FSC) vs. side scatter (SSC) dot plot in acquisition mode. Fluorescent Channel 2 (FL2) at 575 nm was used for propidium Iodide detection. A dot plot of FL2A (area) vs FL2W (width) was used to identify single cells (R2) and to thus eliminate doublets. A histogram plot of FL2A was used to enumerate G_1/G_0, S-phase and G_2 populations and a threshold of 52 on the FSC channel was set to remove sample debris. Nile Red Fluorescent particles were used for instrument standardisation, stability and reproducibility. Data was analysed using MODFIT LT version 2.0, software.

2.7. Reactive oxygen species assay

Reactive oxygen species were measured using the cell-permeable fluorogenic probe 2′, 7′-dichlorodihydrofluorescin diacetate (DCFH-DA, Sigma). WHCO1 cells were seeded in quadruplicate in sterile white 96-well plates at 10×10^3 cells per well in 100 μL and allowed to recover overnight. The media were then removed and the cells were rinsed with PBS, followed by re-suspension in Krebs' Ringer Buffer (KRB, Gibco). If inhibitors were used, they were added and incubated with the cells for 30 min prior to adding the sulphur compound (10 μL); 7.5 mM *N*-acetyl-L-cysteine (NAC) (Sigma), 50 μM L-ascorbic acid (ascorbate) (Sigma) or 12.5 μM 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid (Trolox) (Calbiochem). DCFH-

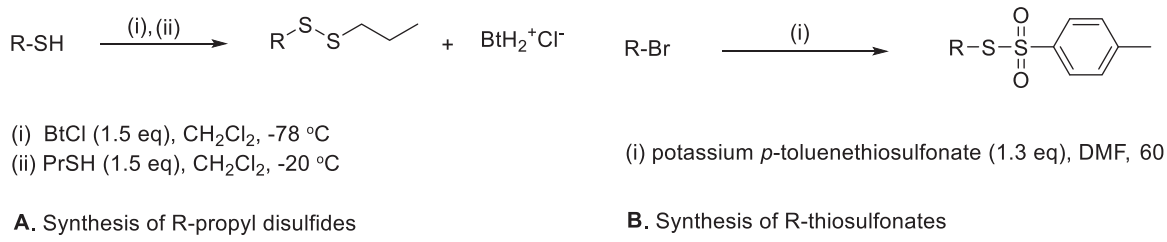


Fig. 2. Synthesis of R-propyl disulphides and R-thiosulfonates. (**A**) Disulphides were synthesised from the two respective thiols using 1-chlorobenzotriazide as an oxidant [31]. (**B**) Thiosulfonates were synthesised from potassium *p*-toluenethiosulfonate and the respective R-substituted halide [32].

DA (10 μM) was then added, followed by treatment of the cells with either **1** or **11** at the specified concentrations for the indicated time. Control cells were treated with 0.1% DMSO or media alone. To account for background DCFH-DA and/or compound fluorescence, wells without cells were treated with DCFH-DA or compound (**1** or **11**) alone and fluorescence was subtracted from the corresponding wells containing cells. Fluorescence was quantified on a Cary Eclipse fluorescence spectrophotometer (Varian), $E_{ex} = 480$ nm; $E_{em} = 530$ nm.

3. Results

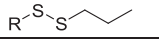
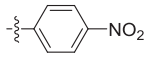
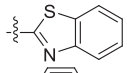
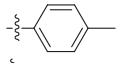
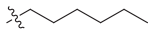
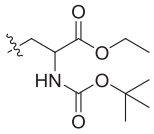
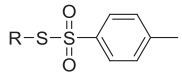
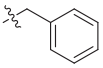
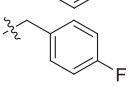
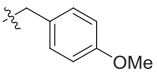
3.1. Relationship between leaving group pK_a and cytotoxicity of disulphides and thiosulfonates in WHCO1 cells

Previous studies in our laboratory have demonstrated that *S*-thiolation of *N*-Boc-cysteine by ajoene [32] is regioselective, with nucleophilic attack occurring selectively at the allyl-S rather than the vinyl-S of the ajoene disulphide during thiolysis exchange. Furthermore, we established that a dansyl group, strategically inserted on the allyl-S end of ajoene (to form a derivative called DP) [21], is transferred to numerous thiol groups of proteins following the addition of DP to MDA-MB-231 breast cancer cells. These findings were rationalised on the basis of the superior leaving ability of the vinylthio group, by virtue of delocalisation of incipient charge into the double bond, and this aspect was used as a crucial design element in the current library of disulphides chosen. Hence, for the disulphide library propyl-SSR, R was chosen as an anion stabilising group to favour regioselective attack involving propyl-S as the transferred group in the disulphide exchange and RS as the leaving group (see Fig. 3A). Specifically, R was varied as an aryl or heteroaryl group that could stabilise the thiolate leaving group in the exchange by resonance stabilisation, although some non-stabilising alkyl groups were also included as controls in which presumably regioselectivity was not secured. The disulphides were synthesised according to our previously published protocol [31] for unsymmetrical disulphide synthesis using 1-chlorobenzotriazole as the oxidant in the coupling of two different thiols (see Fig. 2A), and the compounds have been previously characterised.

All disulphides were tested for their ability to inhibit WHCO1 cancer-cell proliferation by an MTT cytotoxicity assay. This assay quantitates the enzymatic reduction of a yellow tetrazolium salt to purple formazan crystals by NAD(P)H-dependent cellular oxidoreductases. This reaction is only possible in metabolically active cells and therefore gives a quantitative measure of cell viability. The extent of inhibition is reported as a half maximal inhibitory concentration (IC_{50}) after a 48 h incubation period. All compounds were tested a minimum of three times, and the IC_{50} value is reported as an average thereof. Within this series of disulphides a wide variation in the cytotoxicity was observed, with values ranging from 10 μM to 50 mM, some 10,000 fold (see Table 1A).

The three most active compounds in the disulphide series were entries **1**–**3** with electron-withdrawing pyridyl, *p*-nitophenyl and benzothiazolyl groups respectively, with IC_{50}'s of 10–20 μM. Notably, these R groups all provide resonance stabilisation of the thiolate leaving group that is expected to drive the thiolysis exchange in the forward direction (Fig. 3A). Reducing the thiolate leaving ability in the form of entry **4** bearing a *p*-tolyl group (still resonance-stabilised, but lacking an electron-withdrawing group on the ring), resulted in a threefold reduction in activity ($IC_{50} = 61$ μM) in keeping with the relative electronic character and anion stabilising ability of the group. By comparison, disulphides **5**, **6**, **7** and **8** bearing different-length alkyl groups (**8** with an *N*-Boc cysteine Me ester group), all as poor leaving groups due to a lack of anion stabilisation, grouped together with an activity reduction around 1000-fold, pushing the activity into the millimolar range (8–50 mM). In keeping with this analysis, the leaving group stability, as reflected by the pK_a of its conjugate acid (shown in Table 2 and taken from literature data based on measurements carried out in water),

Table 1
Cytotoxicity of disulphide and thiosulfonate compounds against WHCO1 cancer cell proliferation. Disulphide (A) and thiosulfonate (B) compounds were quantitated for their anti-proliferative activity using the MTT assay. Extent of inhibition is reported as the IC_{50} defined as the concentration found to inhibit 50% WHCO1 cell growth after 48 h. Each IC_{50} value is an average of three independent determinations.

A		
No.	−R	$IC_{50} \pm SD$ (μM) WHCO1
1		9.9 ± 1.9
2		22 ± 3.0
3		23 ± 1.1
4		61 ± 6.3
5		$1.01 \pm 0.57 \times 10^4$
6		$4.93 \pm 1.24 \times 10^4$
7		$9.59 \pm 1.08 \times 10^3$
8		$8.51 \pm 7.87 \times 10^3$
B		
No.	−R	$IC_{50} \pm SD$ (μM) WHCO1
9		13 ± 2.3
10		16 ± 3.0
11		15 ± 1.9
12		13 ± 1.2

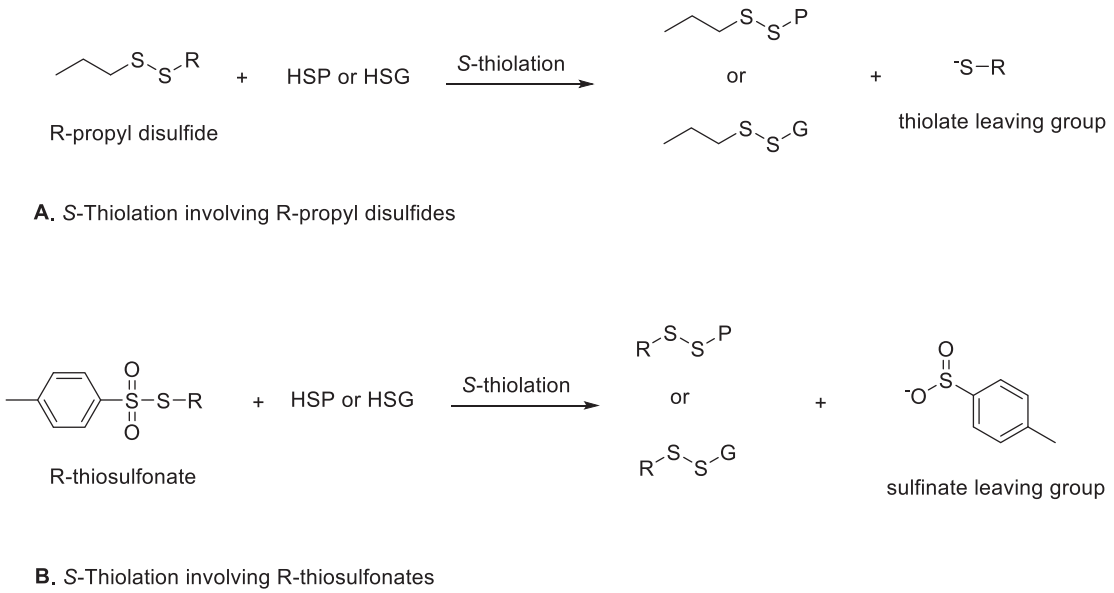


Fig. 3. Proposed thiolysis mechanism between reduced glutathione or protein thiol and a disulphide or thiosulfonate compound. (**A**) *S*-thiolation involving R-propyl disulphides with the expulsion of a thiolate leaving group; (**B**) *S*-thiolation involving R-thiosulfonates with expulsion of *p*-toluenesulfinic acid as a leaving group. GSH = reduced glutathione, PSH = protein thiol.

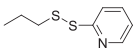
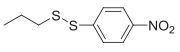
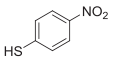
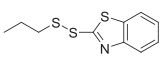
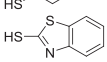
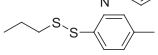
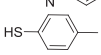
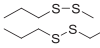
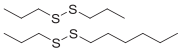
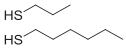
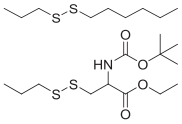
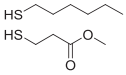
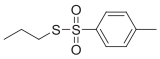
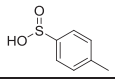
could be correlated with the observed cytotoxicity. This was done by converting the IC_{50}'s to Logs and plotting against the reported pK_a for the corresponding leaving group. The *p*-tolylsulfonyl leaving group of the thiosulfonates (see next section) also followed the observed trends and was included in the analysis. A significant correlation was found between the $LogIC_{50}$ and the pK_a of the leaving group with an $R^2 = 0.78$, Fcrit (7,1) $\alpha = 0.005$. (see Fig. 4).

In the thiosulfonate library the leaving group (LG) was kept constant as *p*-tolylsulfonyl, while the transferring group (T) was now varied from propyl (entry **12**) to a benzyl group (entry **9**), which in two cases was substituted with a *p*-substituent as electron-withdrawing (F) in entry **10** and electron-releasing (OMe) in entry **11**. Similar IC_{50}'s were found for all of the four thiosulfonates at around 15 μM (see Table 1B). These results indicate that the thiosulfonate moiety is solely responsible for the activity, in which the level of cytotoxicity once again closely aligns with the group's stability (leaving ability) as indicated through pK_a (2.8 for *p*-tolysulfinic acid — see Fig. 3B). The presence of two electron-withdrawing oxygens on the sulphur improves the activity from the *p*-tolylthio (disulphide) case in entry **4** ($IC_{50} = 61$ μM) about fourfold to be on a par with the most potent derivatives in the disulphide series (entries **1** and **2**) bearing strongly electron-withdrawing groups in the ring. Once again, these results further validate our hypothesis that thiolate stability (leaving ability) closely aligns with cytotoxicity, and in which thiolysis exchange is operating as the driver in the cytotoxic behaviour.

3.2. Cytotoxicity of 1 and 11 in WHCO1 cells

Evidence from the literature on the cytotoxicity of garlic allyl sulphides in cancer cells shows that although ajoene, allicin, DATS, DADS and DAS have different sulphur-containing functional groups, they share common mechanistic features. These include: inhibition of cell growth, G_2/M cell cycle arrest, increased levels of ROS and induction of apoptosis via the intrinsic pathway [34–36]. In this current report, our structure–activity data for both the disulphides and thiosulfonates support a common mechanism in which thiolysis exchange is a key

Table 2
Structures of disulphides and thiosulfonates and their respective leaving groups in a thiolysis exchange reaction.

No.	Disulphide	$LogIC_{50}$	Leaving group (LG)	LG pK_a
1		1.00		−1.07 [48,49]
2		1.30		5.1 [49,50]
3		1.36		7.09 [51]
4		1.79		6.82 [52]
5		4.01		10.0 [53]
6		4.69		12.2 [49,53]
7		3.93		10.2 [49,53]
8		3.98		10.4 [54]
12		1.11		2.8 [55]

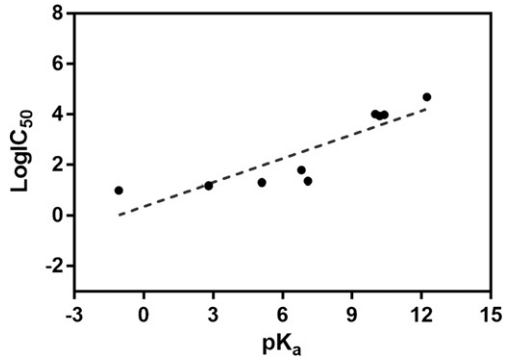


Fig. 4. Plot of pK_a vs $LogIC_{50}$. Correlation between the pK_a of the leaving group and the Log of the cytotoxicity IC_{50}. Compounds included in the plot are propyl derivatives **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, and **12**. Equation: $LogIC_{50} = 0.3168\ pK_a + 0.3365$. $R^2 = 0.78$, Fcrit (7,1) $\alpha = 0.005$.

reaction. Therefore, one disulphide and one thiosulfonate were selected for more detailed mechanistic evaluation. Compound **1** was selected from the disulphide group based on it displaying the lowest IC_{50}. As all the thiosulfonates displayed very similar IC_{50}s, **11** were chosen arbitrarily for further analysis.

We first set out to confirm that **1** and **11** do indeed inhibit cell proliferation, as the MTT assay measures viability, which is only an indirect measure of proliferation. For this purpose the BrdU cell-proliferation assay was used, which directly measures cell proliferation through quantification of incorporated tagged uracil into newly synthesised DNA. Compounds **1** and **11** were tested at $2 \times IC_{50}$ concentration for 48 h, with control cells treated with media alone or 0.1% DMSO in media alone. In agreement with the MTT results, complete inhibition of cell proliferation was observed for both compounds **1** ($p < 0.001$) and **11** ($p < 0.001$) (see Fig. 5A).

In order to assess whether **1** and **11** induce apoptosis in WHCO1 cancer cells, cells were treated with IC_{50} concentrations of each compound for 24 h. Morphology analysis of the treated cells was found to support apoptosis, with cells appearing condensed and rounded up following treatment, a characteristic feature of apoptosis. These cells were then harvested, and apoptosis was quantitated using an ELISA-based assay that detects histone-associated DNA fragments in the cytoplasm after treatment. Histone-associated DNA fragments were detected photometrically using the ABTS substrate. From Fig. 5B it is statistically evident that both **1** ($p < 0.005$) and **11** ($p < 0.001$) induce apoptosis in WHCO1 cancer cells.

In order to assess whether **1** and **11** display any selectivity for tumour over normal cells, the anti-proliferative IC_{50} was determined at the 24 h timepoint for both WHCO1 and Het-1A cells using the MTT assay. The WHCO1 cell line is derived from a biopsy of oesophageal squamous cell carcinoma, whereas the Het-1A cell line is a non-cancer cell line of oesophageal epithelial origin, immortalised with a SV40 large T antigen. As shown in Fig. 5D, the 24 h IC_{50} of **1** and **11** in the Het-1A cells was found to be 206 and 65 μM, which reveals a favourable 10- and 2.5-fold selectivity for tumour over normal cells respectively.

Garlic allyl sulphur compounds are reported to inhibit cancer cell growth by inducing G_2/M cell cycle arrest. We therefore tested whether compounds **1** and **11** affect cell cycle progression in WHCO1 cells. Cells were treated with $2 \times IC_{50,\ 24\ h}$ concentration of **1** or **11** for 24 h; control cells were treated with 0.1 % DMSO in media alone. Cells were then harvested and propidium iodide was used to stain the DNA and analysed by FACS. For WHCO1 cells treated with either **1** or **11**, a time-dependent increase in the population of cells in the G_2/M phase was observed, with a concurrent decrease in the S and G_1 populations (see Fig. 5C). An increase in the amount of debris formed after 24 h was also observed in both cases supportive of apoptosis induction at 24 h. Therefore, although analogues **1** and **11** are structurally different to natural garlic compounds, they contain an exchangeable disulphide or thiosulfonate pharmacophore that is responsible for the cytotoxic effects in WHCO1 cells. The latter include the low micromolar inhibition of proliferation and induction of apoptosis, as well as G_2/M cell cycle arrest.

3.3. Inter-relationship between ROS production and the cytotoxicity of 1 and 11 in WHCO1 cells

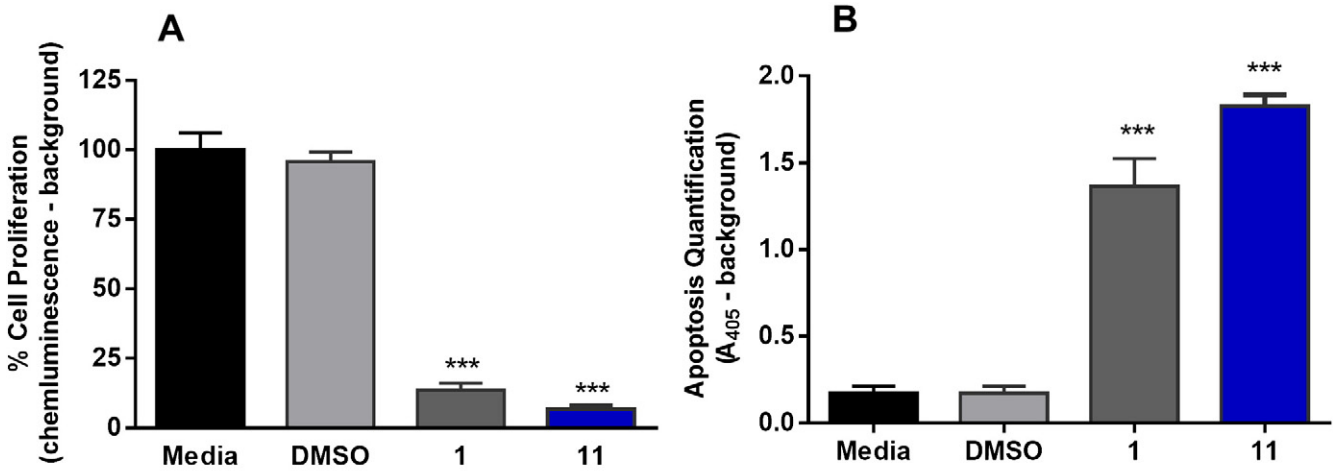
Garlic allyl sulphides are reported to induce ROS in cancer cells, which may play a role in their cytotoxicity. To investigate whether **1** and **11** produce ROS in WHCO1 cells, the 2,7-dichlorodihydroflourescin diacetate (DCFH-DA) reagent was used. DCFH-DA is able to permeate cells and only fluoresces upon ester cleavage and oxidation to dichlorofluorescein. The DCFH-DA reagent is oxidised by a broad spectrum of ROS and NOS which include hydrogen peroxide, hydroxyl, peroxy radicals, nitric oxide and peroxynitrite radicals making it a popular reagent for quantifying oxidative stress in cells. [37,38]. WHCO1 cells were treated with **1** or **11** at 1/2 $IC_{50,\ 24\ h}$, $IC_{50,\ 24\ h}$ or $2 \times IC_{50,\ 24\ h}$ concentrations, and subsequent fluorescence was recorded up to 6 h. As can be seen in Fig. 6A WHCO1 cells treated with **11** resulted in a time- and concentration-dependent increase in the production of ROS compared to the control. The levels of ROS in the untreated sample also increased with time due to cell proliferation and endogenous ROS being produced. By comparison, compound **1** failed to produce significant levels of ROS beyond the control up to 6 h at both 1/2 $IC_{50,\ 24\ h}$ and $IC_{50,\ 24\ h}$ concentrations, although low (yet significant) levels were detected at the $2 \times IC_{50,\ 24\ h}$ concentration at 6 h (Fig. 6A).

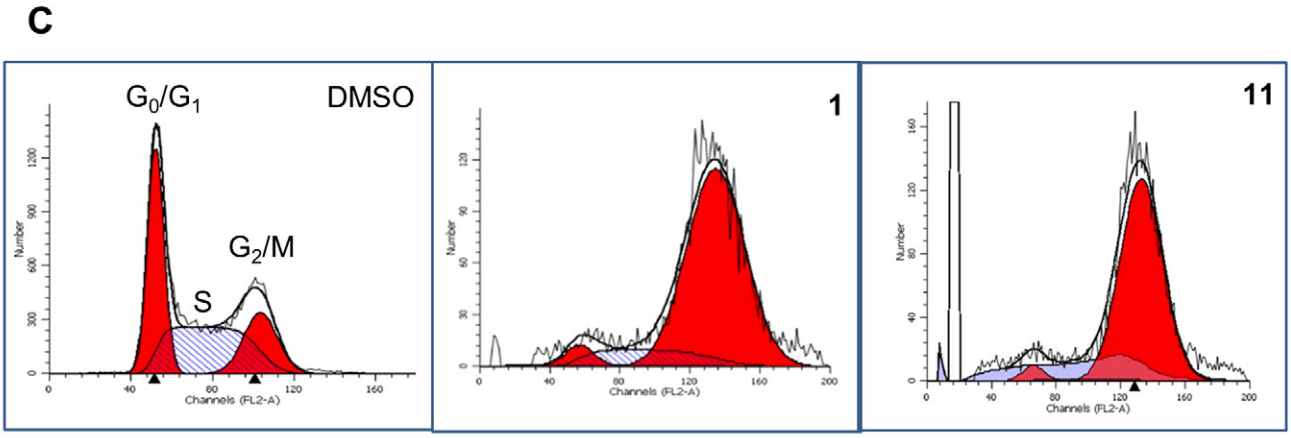
To probe the influence of ROS production on the cytotoxicity of **1** and **11** in WHCO1 cells, we evaluated cytotoxicity in the presence of ROS inhibitors. Three ROS inhibitors were chosen for this experiment, namely: *N*-acetyl cysteine (NAC), L-ascorbic acid (ascorbate or vitamin C) and Trolox (a soluble form of vitamin E). Vitamin E is the primary lipid soluble, small molecule antioxidant in cells, while vitamin C is the corresponding water soluble antioxidant that protects lipids and lipid structures against peroxidation [39]. NAC on the other hand is a synthetic antioxidant that stimulates GSH synthesis, and its free radical scavenging property arises either directly via the redox potential of thiols, or via increasing glutathione levels in the cells [40]. The results for compounds **1** and **11** will be discussed separately and then compared.

First, we first established that the chosen ROS inhibitors were capable of scavenging ROS. Here, pre-incubation of the cells with any of the three inhibitors resulted in a reduction of ROS to levels below that of endogenous (Fig. 6B(i)), although ascorbate was less effective than the other two. Treatment of the cells with thiosulfonate **11** (3 h, $IC_{50,\ 24\ h}$ concentration) in the absence of any inhibitor resulted in a 1.5 fold increase in the levels of ROS compared to the control (Fig. 6B(i), lane 2) which was completely suppressed following pre-incubation with any of the ROS inhibitors.

To determine how ROS production relates to the cytotoxicity of **11**, cells were pre-treated with the ROS scavengers NAC, ascorbate or Trolox and then treated with **11** for 24 h, followed by cytotoxicity quantification by the MTT assay. It is immediately evident from Fig. 6B(ii) that those cells pre-treated with inhibitors ascorbate (lane 6) and Trolox (lane 8) were still susceptible to the cytotoxic effects of **11**. However, in cells pre-treated with NAC (lane 4), cytoprotection was observed.

The results for disulphide **1** were somewhat different. Firstly, unlike thiosulfonate **11**, compound **1** failed to increase ROS above the background, suggesting a crucial difference between **1** and **11** in their relationship to ROS production. As with **11**, though, the inhibitors alone or a combination of inhibitor with **1** also resulted in a reduction of ROS to levels below the background, again with ascorbate proving the less effective of the three (see Fig. 6C(i)). A striking difference though is in the cytotoxicity study, where a similar profile to **11** was generated, with only NAC able to restore cell viability (lane 4, Fig. 6C(ii)), while





D

No.	WHCO1 $IC_{50} \pm SD$ (μM)	Het1A $IC_{50} \pm SD$ (μM)	Fold change
1	22 ± 1.5	206 ± 15	10
11	26 ± 1.5	65 ± 11	2.5

Fig. 5. Cytotoxic effects of compounds **1** and **11** in WHCO1 cells. (**A**) Anti-proliferative activity of **1** and **11** in WHCO1 cells using the BrdU assay. Cells were treated with the compounds at twice $IC_{50,\ 48\ h}$ for 48 h. (**B**) Apoptosis-inducing activity of **1** and **11** in WHCO1 cells through quantification of histone-associated DNA fragments in the cytoplasm after treatment with $IC_{50,\ 24\ h}$ concentration for 24 h. (**C**) WHCO1 cells were treated with **1** or **11** for 24 h and cells were stained with propidium iodide and analysed by FACS. Compounds **1** and **11** were found to induce complete G_2/M cell cycle arrest in WHCO1 cells when treated at twice $IC_{50,\ 24\ h}$ concentration for 24 h compared to untreated DMSO controls. (**D**) Fold selectivity of compounds **1** and **11** for WHCO1 cells over the normal epithelial control (Het1A) measured by the MTT cell proliferation assay for 24 h. All experiments were performed independently in triplicate and values are reported as the average ± standard deviation.

pre-treatment with ascorbate (lane 6) or Trolox (lane 8) failed to reverse the cytotoxicity of **1**. This important contrast indicates that NAC and **1** together restore cell viability via a mechanism other than scavenging ROS. Upon closer inspection of the chemical structure of NAC, we decided to test whether **11** and NAC may self-react through thiolysis (Fig. 7).

3.4. Evidence for S-thiolation between 11 and NAC

In a model reaction, **11** and NAC were incubated in THF at room temperature overnight. A product formed that was less polar on TLC than NAC and it was isolated by silica gel chromatography as an oil in 77% yield. It was characterised by ^{1}H and ^{13}C NMR spectroscopy and HRMS to be *N*-acetyl cysteine methoxybenzyl disulphide, demonstrating the feasibility of this exchange. Therefore, the reversal of **11**'s cytotoxicity by NAC, in view of the results with the other ROS inhibitors cannot be due to a quenching of ROS but is likely due to the removal of **11** from the system via direct reaction with NAC.

4. Discussion

There is much evidence to support a link between garlic consumption and reduced cancer risk, especially cancers of the gastrointestinal tract [2–4]. This link is attributed to the allyl sulphur compounds that

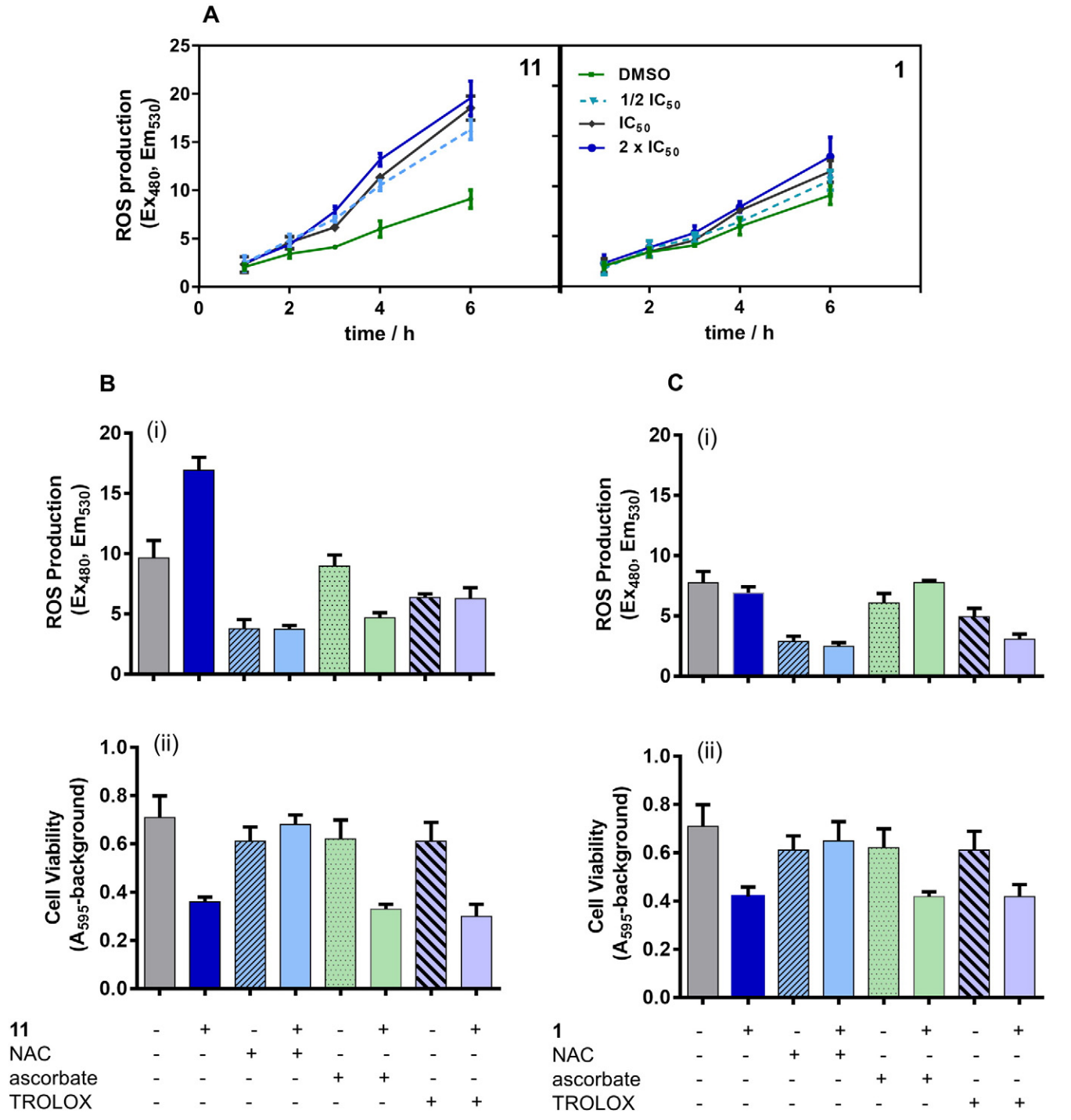


Fig. 6. ROS production by compounds **1** and **11** in WHCO1 cells. (**A**) Timecourse for ROS production by compounds **1** and **11** in WHCO1 cells treated at 1/2 $IC_{50,\,24\,h}$, $IC_{50,\,24\,h}$ and 2 × $IC_{50,\,24\,h}$ concentrations or DMSO alone (control) measured from 1–6 h using the DCFH-DA assay. (**B**) Effect of ROS inhibitors NAC (10 mM), ascorbate (50 μM) and Trolox (12.5 μM) by **11**on (i) production of ROS after 3 h measured by DCFH-DA assay and (ii) cytotoxicity after 24 h measured by MTT assay. (**C**) Effect of ROS inhibitors NAC (10 mM), ascorbate (50 μM) and Trolox (12.5 μM) by **1**on (i) production of ROS after 3 h measured by DCFH-DA assay and (ii) cytotoxicity after 24 h measured by MTT assay. All experiments were performed independently in triplicate and values are reported as the average ± standard deviation.

are found in crushed cloves sharing a sulphide/disulphide/polysulphide or thiosulfinate functional group as a common structural motif. In turn, this has prompted the notion that the underlying chemical biology behind the anti-cancer effects of these compounds involves a combination of glutathione and protein *S*-thiolation via the disulphide pharmacophore, and generation of reactive oxygen species, which together gives rise to a cascade of cytotoxic events leading to growth inhibition, G_2/M cell cycle arrest and induction of apoptosis.

We have recently found that ajoene targets the endoplasmic reticulum in MDA-MB-231 breast cancer cells where it *S*-thiolates a multitude of protein targets [21]. There is some evidence pointing towards the extent of *S*-thiolation being linked to the cytotoxicity of the garlic allyl sulphur compounds. For example, ajoene is expected to be a superior thiolating agent to diallyl disulphide due to the presence of the vinyl group which would be expected to increase the electrophilicity of the disulphide as well as provide a relatively more stable leaving

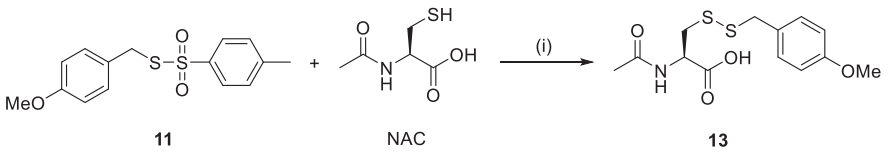


Fig. 7. Reaction between *N*-acetyl cysteine and thiosulfonate **11** to generate the mixed disulphide **13**.

group, thus promoting the forward thiolysis reaction. This may be reflected in its enhanced cytotoxicity in cancer cells (IC_{50}–20 μM) [13,32] relative to that reported for DADS (IC_{50}–60 μM) [16,41]. The present study thus sought to build on this idea by testing a small library of disulphides and thiosulfonates with varying leaving group activities. Indeed, the results of the cytotoxicity experiments as measured by the MTT assay revealed a significant correlation ($R^2 = 0.78$, $p = 0.002$) between the cytotoxicity IC_{50} in WHCO1 cells and the reported pK_a of the leaving group, a thermodynamic driving factor in mixed disulphide formation. In addition, a very wide rift (1000–10,000 fold) was observed between disulphides having a resonance-stabilising group attached to the thiolate leaving group and those containing non-stabilising (alkyl) groups. These results support the importance of *S*-thiolation in the cytotoxic mechanism of disulphides in WHCO1 cells. In our previous study [21], we found that ajoene accumulates in the ER of cancer cells by a trapping mechanism involving protein *S*-thiolation. Disulphides entering the cytosol are expected to become depleted by forming a mixed disulphide with glutathione (GSH), whereas those entering the lumen of the ER form mixed disulphides with free cysteine thiols of newly synthesised proteins (PSH). The cytosolic targeting to GSH vs ER targeting to protein sulfhydryl groups may be driven by the higher concentrations of GSH that are present in the cytosol over the ER [42]. Garlic related disulphides interfere with cellular processes by acting as GSSG mimics, and interfering with the redox state of the cell and with protein folding [21]. However, based on the superior leaving group ability of garlic disulphides and related analogues in thiolysis exchange, these mimics are expected to be superior thiolating agents compared to GSSG. Our findings that cytotoxicity correlates to *S*-thiolation, as measured by the thermodynamics of the leaving group in thiolysis exchange, supports this hypothesis. In addition to protein *S*-thiolation, there is much literature evidence supporting ROS generation by garlic allyl sulphides as an important factor in their cytotoxicity. More specifically, ajoene [25,26], DADS [27,28], DATS [29,30] and DAS4 [16] have all been shown to induce a time-dependent production of ROS in a variety of cancer cell lines. Therefore, to shed some light on this in context, mechanistic aspects of the cytotoxicity of thiosulfonate **11** and disulphide **1** were investigated. In agreement with reports for other related garlic allyl sulphur compounds, both were found to be strong inducers of G_2/M cell-cycle arrest and apoptosis. By comparison and of great significance were the contrasting results for **1** and **11** in ROS production (Fig. 6B and C(i)). In spite of almost identical cytotoxicity IC_{50}'s, thiosulfonate **11** produced high levels of ROS after only 3 h and at concentrations below the IC_{50}. Compound **1** however produced very low levels of ROS which were only significant at twice the IC_{50}. Since these two compounds have similar IC_{50}'s due to a similar degree of thiolysis, the contrasting ROS results may imply that the different leaving groups have a bearing on ROS generation.

Both allicin [43,44] and 2-propenylthiosulfinate [20] have been shown to spontaneously react with GSH in vitro. Therefore, it is expected that disulphides and thiosulfonates entering the cytosol will undergo spontaneous thiolysis exchange with GSH. This reaction would lower the levels of intracellular GSH and lead to increased ROS. However, the striking difference in final ROS levels for these two agents, in which those for **11** are significantly higher than those for **1** (Fig. 6), deserves comment. The assumption that these differences are not kinetic ones due to substrate rate differences in the disulphide exchange (assume complete conversion for each), implies that the observed ROS level attenuation is due to the leaving groups produced in the exchange, i.e. a 2-pyridylthiolate (or its neutral thione) for **1** and a thiosulfinate (or its conjugate acid) for **11**. In turn, the unlikelihood of these species being able to substitute for GSH in any enzyme-catalysed ROS detoxification process suggests that any ROS attenuation involving them is a chemically-based process. Here, the reactivity of thiosulfinate ion in solution may be a factor, but a clear and satisfactory explanation of the said results at this stage is not forthcoming, and will require further experimentation for clarification.

Importantly, our results demonstrate that ROS generation is a secondary effect as far as cytotoxicity is concerned for both **1** and **11**, since pre-treatment with ROS inhibitors Trolox and ascorbic acid did not negate the cytotoxic effects. Of importance was the significant correlation observed between the leaving group pK_a and the cytotoxicity of both classes of compound in the absence of any ROS considerations. By comparison, pre-treating in the individual case with the ROS inhibitor NAC did result in shutting down the cytotoxicity of both **11** and **1**, even though **1** failed to produce any significant increase in ROS. Inspection of the chemical structure of NAC suggests that NAC may chemically react with disulphides. This possibility was investigated by performing a chemical reaction between NAC and the disulphide **11** in THF overnight, which indeed resulted in a reaction product between inhibitor and compound to form a mixed disulphide. We therefore explain the apparent reversal of the cytotoxicity by **11** through the ability of NAC to reduce and inactivate the disulphide test compound.

A number of downstream events have been linked to ROS production by garlic allyl sulphur compounds that include activation of the cJun NH_2 terminal kinase cJun signalling cascade [27,29,45], hyperphosphorylation of Cdc25C [30] and activation of the mitochondrial membrane caspase cascade [46]. However quite a few of these studies have drawn their conclusions based on inhibitor studies using NAC [26,30,46,47]. Other ROS cytotoxicity studies, however, have also observed positive effects which do not make use of NAC. For example Filomeni et al. observed that overexpression of the antioxidant enzyme Cu–Zn SOD offered some protection against lipid peroxidation, protein carbonylation, G_2/M cell cycle arrest and apoptosis in SH-SY5Y neuroblastoma cells and that use of the spin trap DMPO offered some protection against G_2/M cell cycle arrest and apoptosis [27]. In another study, addition of the SOD and catalase mimetic, EUK134, was found to offer some protection against G_2/M cell cycle arrest and Cdc25C phosphorylation [29]. In a study by Das et al., ascorbate pre-treatment was found to reverse p38 MAPK phosphorylation and JNK1 activation by DAS and DADS, thereby highlighting the importance of ROS production in this signalling cascade in human glioblastoma T98G and U87MG cells. In a study by Kelkel et al., the authors failed to detect any ROS production by DAS4, and supportive of our results, found that NAC but not Trolox pre-treatment resulted in reversal of the cytotoxic effects by DAS4 in histiocytic lymphoma cells [17].

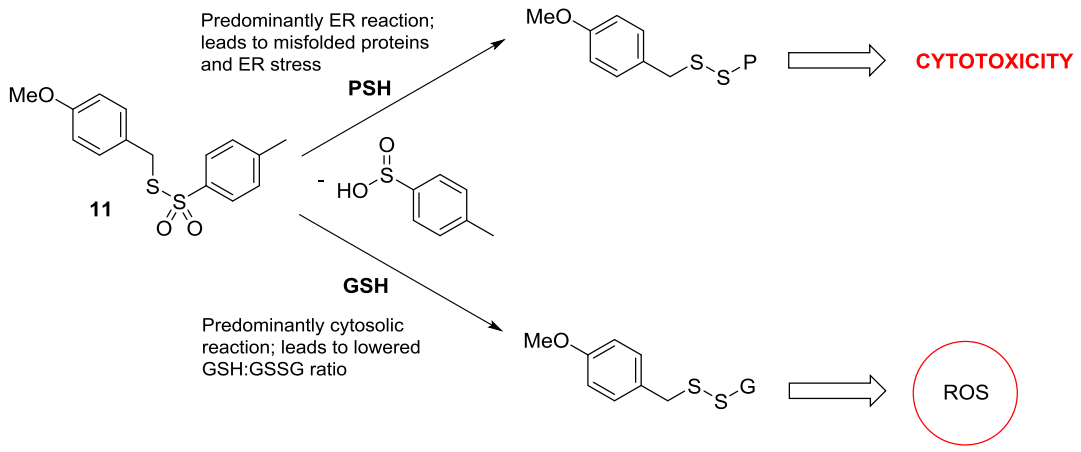


Fig. 8. Proposed cytotoxic mechanism of thiosulfonate **11** in WHCO1 cancer cells. Thiosulfonate (**11**) accumulates in the ER where it *S*-thiolates newly synthesised proteins and induces ER stress. This reaction is thermodynamically driven by the stability of the leaving group. Thiosulfonate **11** entering the cytosol *S*-thiolates glutathione leading to lowered GSH:GSSG levels; and subsequent increased levels of ROS.

In the current study the finding that NAC alone is able to nullify the cytotoxicity of thiosulfonate **11** and disulphide **1** strongly suggests a reaction between the inhibitor and the compound. If correct, this important finding suggests that results obtained from inhibitor studies between NAC and garlic-related disulphides should be interpreted with caution. Needless to say, there are inhibitor studies that utilise inhibitors other that NAC, which have found a positive correlation between ROS production and G_2/M cell cycle arrest and apoptosis in SH-SY5Y neuroblastoma cells [27], and G_2/M cell cycle arrest and Cdc25C phosphorylation in DU145 and PC3 prostate cancer cells [29].

We have recently found that ajoene targets the ER in MDA-MB-231 breast cancer cells [21]. Being lipophilic, we propose that ajoene and related disulfides may rapidly cross the membrane to enter the cytoplasm via passive diffusion [43]. The cytoplasm is highly reducing due to high levels of GSH which is expected to reduce the disulphides by thiolysis, thereby lowering the GSH levels and allowing ROS to increase (see Fig. 8). Thiolysis exchange in the current study on disulphides and thiosulfonates has been shown to be thermodynamically driven by the leaving group stability in a mixed disulphide reaction.

5. Conclusion

The current study has delineated the effects of thiolysis exchange and ROS production by disulphides and thiosulfonates in WHCO1 oesophageal cancer cells. The thermodynamics of thiolysis was found to correlate to cytotoxicity as measured by the pK_a of the leaving group in thiolysis exchange. This supports our previous findings on ajoene, which demonstrated ER targeting and an accumulation of misfolded proteins. We conclude that protein *S*-thiolation by these agents is the determining factor in their cytotoxicity, driving ER stress and the unfolded protein response. Our results also suggest that ROS is generated as a result of decreased GSH levels following thiolysis exchange and that the role of the leaving group on ROS attenuation needs further investigation. The current study has relevance to the design of effective cytotoxic agents against cancer cells.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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