Antiproliferative effect of natural tetrasulfides in human breast cancer cells is mediated through the inhibition of the cell division cycle 25 phosphatases

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Abstract. For many years, *in vitro* and *in vivo* studies have reported that organosulfur compounds (OSCs), naturally found in *Allium* vegetables, are able to suppress the proliferation of various tumor cells. In spite of recent advances, the specific molecular mechanisms involved in OSC activity are still unclear. Considering the antiproliferative effects observed in cancer cells, we postulated that OSCs might target the cell division cycle (Cdc) 25 phosphatases which are crucial enzymes of the cell cycle. Our findings suggest phosphatases Cdc25 as possible targets of naturally occuring polysulfides contributing to their anticancer properties. We report on the inhibitory activity of tetrasulfides occurring naturally in garlic and onion towards the human Cdc25 phosphatases. Diallyl- and dipropyltetrasulfides have emerged as interesting irreversible inhibitors of the Cdc25 isoforms A and C *in vitro*. Furthermore, growth of both sensitive (MCF-7) and resistant (Vcr-R) human breast carcinoma cells was significantly decreased by these tetrasulfides. The observed antiproliferative effect appeared to be associated with a G_2-M cell cycle arrest.

Introduction

For many centuries, *Allium* vegetables, such as garlic and onion, have been used to prevent a large variety of health disorders. Several epidemiologic studies have supported the current inverse correlation between dietary intake of *Allium* vegetables and cancer risk (1-3). These anticarcinogenic properties were described to be due to biologically active sulfur compounds found in *Allium* vegetables. Amongst them, allicin plays a crucial role in garlic chemistry because its degradation results in the production of various organic sulfur compounds, such as the well-known diallylmonosulfide (DAS), -disulfide (DAS_2) and -trisulfide (DAS_3) (reviewed in ref. 4). These diallylsulfides have been shown to offer protection against cancer induced by chemical agents in animal models (5). In addition, sulfur compounds are able to suppress the proliferation of various types of cultured cancer cells by inducing apoptosis and/or cell cycle blockage. For example, recent studies revealed that these compounds inhibit the growth of human prostate and colon cancer cells in association with G_2-M phase cell cycle arrest (6,7). Interestingly, studies performed *in vitro* and *in vivo* showed that sulfides could selectively target the tumor cells without affecting significantly healthy cells (7,8). In spite of recent advances made in the understanding of anticancer effects of organic sulfur compounds, the specific molecular mechanisms involved in their activity are still not clearly elucidated.

The cell division cycle (Cdc) 25 dual-specificity phosphatases are important regulators of eukaryotic cell cycle progression. Three members of the Cdc25 family exist in humans, all of which feature an active site cysteine residue. They play a crucial role in the activation of cyclin-dependent kinases (Cdk) by dephosphorylating the phospho-Thr14 and phospho-Tyr15 residues (reviewed in ref. 9). Cdc25 A appears to be mainly implicated in the progression to the S phase while Cdc25 B and C are known to be required specifically for entry into mitosis. However, the three Cdc25 isoforms are able to control G_1-S and G_2-M transition by activating Cdk1 and Cdk2, respectively (10). Over-expression of Cdc25 A and B was reported in various types of human malignancies including breast cancer (9). Moreover, Cdc25 over-expression has been correlated with either poor prognosis or tumor aggressiveness (11). Taken together, these data suggest that Cdc25 enzymes are attractive targets in cancer therapy.

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Considering the antiproliferative effects induced by organic sulfur compounds in cancer cells, the ability of polysulfides to modify cysteine residues in proteins and enzymes and the crucial role of Cdc25 phosphatases in cell cycle progression, we postulated that these enzymes might be potential targets of such compounds. The direct inhibition of Cdc25 activity by polysulfides found in garlic and onion is reported herein for the first time. The most potent *in vitro* Cdc25 inhibitors, diallyltetrasulfide (DAS_4) and dipropyltetrasulfide (DPS_4), are able to prevent breast cancer cell proliferation through a cell cycle arrest in G_2-M.

Materials and methods

Synthesis of organosulfur compounds. Organosulfur compounds were obtained from the Jacob Group. Diallylsulfide and diallyldisulfide were purchased from Sigma (Bornem, Belgium) and distilled before use. Diallyltrisulfide was synthesized according to the method of Milligan *et al* (12) and purified by vacuum distillation; diallyltetrasulfide was synthesized following the method of Derbesy and Harpp (13) and purified by column chromatography. Identity and purity of the compounds were confirmed by GC-MS, ^{1}H-NMR, ^{13}C-NMR or GC-MS and HPLC, respectively. Pure oils were stored at -80˚C and dissolved in DMSO immediately before use and kept throughout the experiment. The propyl-analogues were synthesized and purified according to the procedures employed for the allyl-compounds, with one minor difference: propylbromide was used instead of propylchloride for the synthesis of dipropyltrisulfide while propyl mercaptane was used instead of allyl mercaptane for the synthesis of dipropyltetrasulfide. Analytical data obtained for the dipropylsulfides were in accordance with the literature.

Cell culture. The human mammary adenocarcinoma cell line MCF-7 (ECACC, UK) and its counterpart resistant to vincristine Vcr-R (14) were used in this study. The cells were cultured in RPMI-1640 medium (Eurobio, France) supplemented with 10% (v/v) fetal bovine serum and penicillin (100 UI/ml)/ streptomycin (100 μg/ml) at 37˚C in a 5% CO_2 humidified atmosphere.

Cytotoxicity assay. Cytotoxicity was assessed by the standard MTT test (15) in conditions previously described (16). Briefly, cells were seeded at 10,000 cells/well in 96-well plates and allowed to growth for 24 h. The medium was then changed and the cells were treated for 24, 48 and 72 h with solutions containing the sulfur compounds.

Production and purification of the GST-Cdc25 fusion proteins in E. coli. Glutathione S-transferase (GST)-Cdc25 recombinant enzymes Cdc25A, Cdc25B and Cdc25C were obtained in our laboratory using transformed *Escherichia coli* BL21 (DE3) encoding the gene fusion constructs GST-Cdc25s. Fusion proteins were expressed by induction with 0.5 mM IPTG (isopropyl ß-D-thiogalactopyranoside, Eurobio, France) for 5 h at 30˚C. Then, cell pellets were thawed in 30 ml of lysis buffer [50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF, 3% (v/v) protease inhibitor cocktail (Sigma Chemical, USA)] and lysed by sonication (5x30 sec). After cell lysis, lysate was cleared by a 25,000 x g centrifugation for 45 min and fusion proteins were purified by affinity chromatography using glutathione-agarose gel as described (17).

In vitro phosphatase assay. Prior to *in vitro* assays, enzymes were purified by a Microcon® centrifugal filter unit with cut-off of 30 kDa (Millipore) in order to clear out of buffer containing reducing agents. Phosphatase activity was determined using 3-O-methyl fluorescein phosphate (OMFP) as substrate. The assay was performed in 96-well plates in a final volume of 100 μl. The final concentrations were 315 ng/well for Cdc25 A, 500 ng/well for Cdc25 B and 414 ng/well for Cdc25 C. The concentrations of enzyme used in these assays allow to obtain comparable fluorescence values for each Cdc25 isoform. The enzymes were incubated with compounds for 1 h at 30˚C in reaction buffer [50 mM Tris-HCl (pH 8.2), 50 mM NaCl, 1 mM EDTA, 0.1% SAB]. The reaction was initiated by adding 500 μM of substrate. After 2 h at 30˚C, fluorescence emission from the dephosphorylated product, 3-O-methyl fluorescein (OMF), was measured with Cytofluor® (Biosystem; excitation filter: 485 nm and emission filter: 510 nm). The efficiency of this method was controlled using a known Cdc25 inhibitor, BN82002 (Sigma-Aldrich) (18). Data represent means of at least three independent experiments. Determination of the median inhibitory concentration IC_{50} was performed using generalized linear models, as described (19).

Reversibility assays were performed by pre-incubating large amounts of Cdc25 C phosphatase (10 μg) with inhibitory concentrations of DAS_4 or DPS_4 (10 μM) for various times (0-90 min). Then, the enzyme-inhibitor mixture was 100-fold diluted in reaction buffer [50 mM Tris-HCl (pH 8.2), 50 mM NaCl, 1 mM EDTA, 0.1% SAB]. This dilution leads to a 0.1 μM concentration of inhibitor, which is inactive towards Cdc25 C (not shown). Phosphatase activity was subsequently measured as described above in the presence of 414 ng/well of Cdc25 C and after adding 500 μM of substrate (OMFP). Results are representative of four independent experiments.

Cellular growth assay. Cells were plated at a density of $2x10^5$ per well in 6-well plates. After 24 h, cells were treated with DAS_4 or DPS_4 (25 and 50 μM) or solvent alone (DMSO, 0.5% v/v). Treatment with DAS (50 μM), a compound that does not inhibit the Cdc25 phosphatases, was used as negative control. At indicated time points, adherent cells were trypsinized and harvested together with the floating cells. Then, the total amount of cells was quantified by the trypan blue exclusion counting method over the following 4 days.

Cell cycle analysis. Cells ($1x10^6$) were seeded in 100-mm dishes and allowed to attach overnight. Cells were exposed to 50 μM DAS_4 or DPS_4. An experiment with solvent alone (DMSO, 0.5% v/v) was performed as negative control. After incubation at 37˚C for the desired time, floating and adherent cells were harvested, stained with propidium iodide solution (50 μg/ml) and analyzed for DNA content with FACSCalibur flow cytometer (Becton-Dickinson, USA).

Table I. Structures of *Allium* compounds and screening for Cdc25 inhibition.[a]

	Compound	Structure	Residual activity of phosphatase (% of control)		
			Cdc25 A	Cdc25 B	Cdc25 C
garlic	Allicin		26 ± 16	67 ± 8	7 ± 2
	Diallyl sulfide (DAS)		116 ± 4	94 ± 4	88 ± 8
	Diallyl disulfide (DAS_2)		76 ± 15	97 ± 6	50 ± 7
	Diallyl trisulfide (DAS_3)		70 ± 14	87 ± 6	24 ± 6
	Diallyl tetrasulfide (DAS_4)		8 ± 10	48 ± 6	0 ± 1
	Allyl mercaptan	SH	115 ± 15	94 ± 4	82 ± 8
onion	Propyl allicin		48 ± 11	67 ± 5	16 ± 4
	Dipropyl sulfide (DPS)		119 ± 17	99 ± 4	96 ± 4
	Dipropyl disulfide (DPS_2)		99 ± 4	96 ± 1	68 ± 3
	Dipropyl trisulfide (DPS_3)		85 ± 13	84 ± 2	20 ± 3
	Dipropyl tetrasulfide (DPS_4)		12 ± 8	58 ± 8	2 ± 2
	Propyl mercaptan	SH	100 ± 22	79 ± 4	50 ± 3
	1,9-decadiene		129 ± 18	111 ± 6	99 ± 14
	3-isothiocyanatopropene	N=C=S	116 ± 12	100 ± 9	72 ± 8

[a]Phosphatase activity was evaluated using the OMFP dephosphorylation assay. The three isoforms of human Cdc25 phosphatases were incubated with 100 μM of each compound for 1 h at 30˚C. Activity was expressed as % of residual activity of control in the absence of compound. Results are mean ± SD of three independent experiments.

Cell lysate preparation and immunoblotting. MCF-7 cells were plated at a density of $1x10^6$ in 100-mm dishes. After 24 h, the cells were treated with DAS_4 and DPS_4 for 6 and 12 h. Cells were then harvested and lysed in lysis buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 0.1% SDS, 10% glycerol, 0.5% deoxycholate supplemented with protease inhibitors cocktail (Sigma Chemical), 1 mM PMSF and 2 mM Na_3VO_4] for 30 min on ice. Then, the cells were sonicated (3 times for 10 sec, VibraCell, Fisher Bioblock Scientific, Illkirch, France) and centrifuged (12,000 x g, 15 min, 4˚C). Lysate proteins (50 μg) were separated on a 14% gel by SDS-PAGE and transferred on a nitrocellulose membrane. Immunoblotting was performed using anti-phospho-Cdk2 (1:1000) (Santa Cruz Biotechnology), anti-phospho-Cdk1 (1:1000) (Cell Signaling Technology). Primary antibodies were detected with horseradish peroxidase-goat anti-rabbit antibody (KPL). Densitometry analysis was performed using ChemiDoc™ (Bio-Rad) and relative quantifications of phospho-Cdk1 and phospho-Cdk2 were performed in comparison with loading control (α-tubulin).

Statistical analysis. Data represent the mean of at least three independent experiments. Statistical analysis was performed using a Student's t-test.

Results

In vitro inhibition of phosphatase activity. Sulfur compounds naturally present in garlic and onion have been evaluated for their ability to inhibit the activities of human Cdc25 A, Cdc25 B and Cdc25 C. These enzymes are routinely expressed and purified in our laboratory, and serve as useful tools for the study of Cdc25 inhibition (20). A preliminary screening, performed with a single dose of each compound (100 μM), indicates that the highest inhibitory activities were found for the polysulfides DAS_4 and DPS_4 (Table I). The most potent inhibitory effects were observed against the purified

Table II. Inhibition of purified recombinant members of Cdc25 family with DAS_4 and DPS_4.[a]

	DAS_4		DPS_4	
	IC_{50} (μM)	SD	IC_{50} (μM)	SD
Cdc25 A	16.4	5.5	16.6	5.5
Cdc25 B	>100	-	>100	-
Cdc25 C	1.1	0.1	1.4	0.2

[a]Enzymes were exposed for 1 h at 37˚C to various concentrations of DAS_4 or DPS_4 (0-200 μM). Phosphatase activity was determined using the OMFP dephosphorylation assay. The IC_{50} values were determined using GLMTox from at least three independent experiments (19).

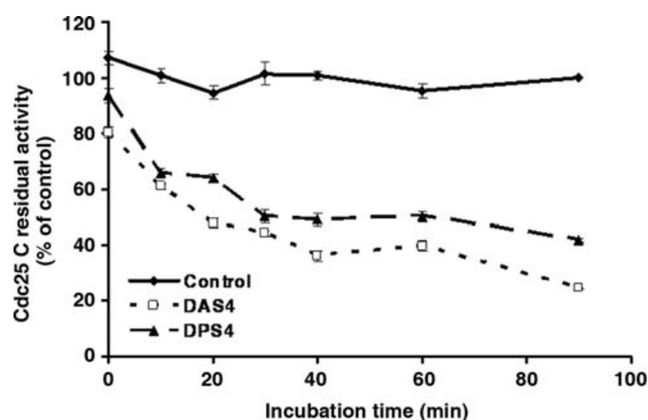

Figure 1. Reversibility assay of Cdc25 C inhibition by DAS_4 and DPS_4. Cdc25 C phosphatase was preincubated with solvent alone (control) or inhibitory concentrations of DAS_4 or DPS_4 (10 μM) for various time intervals (0-90 min). After incubation, the reaction mixture was diluted 100-fold to reach inactive concentrations of DAS_4 or DPS_4 (0.1 μM). The phosphatase activity was determined by adding the substrate OMFP in each well containing 414 ng of Cdc25 C after dilution. Data are representative of four independent experiments.

recombinant fusion proteins Cdc25 C and Cdc25 A, whereas Cdc25 B was less sensitive.

The most active compounds of this study, DAS_4 and DPS_4, were therefore chosen for a more complete evaluation of their potential. As indicated in Table II, the highest inhibitory activity was found toward Cdc25 C with IC_{50} values of 1.1±0.1 and 1.4±0.2 μM for DAS_4 and DPS_4, respectively. Concentrations required to inhibit Cdc25 A activity (IC_{50} values of 16.4±5.5 and 16.6±5.5 μM, respectively) were 10-fold higher than those used for Cdc25 C. However, the inhibitory effects were very weak toward Cdc25 B (IC_{50}>100 μM for both compounds), according to results obtained in Table I. We noted that both, allyl- and propyl-derivatives inhibited Cdc25 activity in a similar range of concentrations.

We further examined whether the inhibition achieved by these two compounds is reversible or not. Cdc25 C phosphatase was preincubated for various times (0-90 min) with solvent alone (control) or active concentrations of DAS_4 and DPS_4 (10 μM). Then, the reaction mixture was diluted to a final concentration of 0.1 μM at which the inhibitors are inactive (data not shown). If the inhibitory effects persist after dilution, the mechanism of action should be considered as irreversible (21). As indicated in Fig. 1, for DAS_4 and DPS_4, the inhibitory activity persisted after the dilution procedure in absence of reducing agent. Moreover, both compounds inhibited Cdc25 C in a time-dependent manner with a maximal inhibitory activity observed after 90 min of preincubation (25 and 42% of residual activity, respectively). These results suggest that DAS_4 and DPS_4 act as irreversible inhibitors of the Cdc25 phosphatases *in vitro*. However, polysulfides are able to react with thiols to form S-thiolated proteins, a reaction known as reversible (4). Thus, we may envisage that the inhibitory activities of DAS_4 and DPS_4 are chemically reversible in presence of reducing agents such as dithiothreitol (DTT) and glutathione (GSH). This was confirmed by additional experiments performed on purified Cdc25 A *in vitro* in presence of reducing agents (DTT and GSH). Our results indicated that the ability of the tetrasulfides to inhibit Cdc25 A was reduced in presence of DTT and GSH (data not shown).

Table III. Cytotoxic effects of DAS_4 and DPS_4 on MCF-7 human mammary cell line.[a]

		DAS_4		DPS_4	
		IC_{50} (μM)	SD	IC_{50} (μM)	SD
MCF-7	24 h	92	14	>100	
	48 h	43	12	25	4
	72 h	25	7	23	3

[a]MCF-7 cells were exposed for 24, 48 and 72 h to various concentrations of DAS_4 or DPS_4 and cytotoxicity was evaluated performing MTT test. The IC_{50} values were calculated using GLMTox (n=3).

Effect on breast cancer cell viability. The cytotoxic effects of DAS_4 and DPS_4 were first evaluated on the human mammary adenocarcinoma cell line MCF-7. As shown in Table III, DPS_4 displayed cytotoxic effects with IC_{50} of 25±4 and 23±3 μM after 48 and 72 h treatment, respectively. DAS_4 showed a concentration- and time-dependent cytotoxic activity with IC_{50} of 92±14, 43±12 and 25±7 μM after 24, 48 and 72 h treatment, respectively.

To determine the antiproliferative effects of both tetrasulfur compounds, MCF-7 cells were treated with DAS_4 and DPS_4 (25 and 50 μM) and cell growth curves (0-96 h) were plotted (Fig. 2A and B). DAS (50 μM), a non-inhibitory compound, was used as negative control, and DMSO (0.5% v/v) as a solvent control. DAS_4 and DPS_4 appeared to significantly reduce the cell growth whereas neither DAS (50 μM) nor DMSO alone showed any effect. These data suggest a cytostatic effect of both tested compounds against MCF-7 cells.

We then explored the effect of DAS_4 and DPS_4 on a second breast cancer derived cell line, Vcr-R, which presents

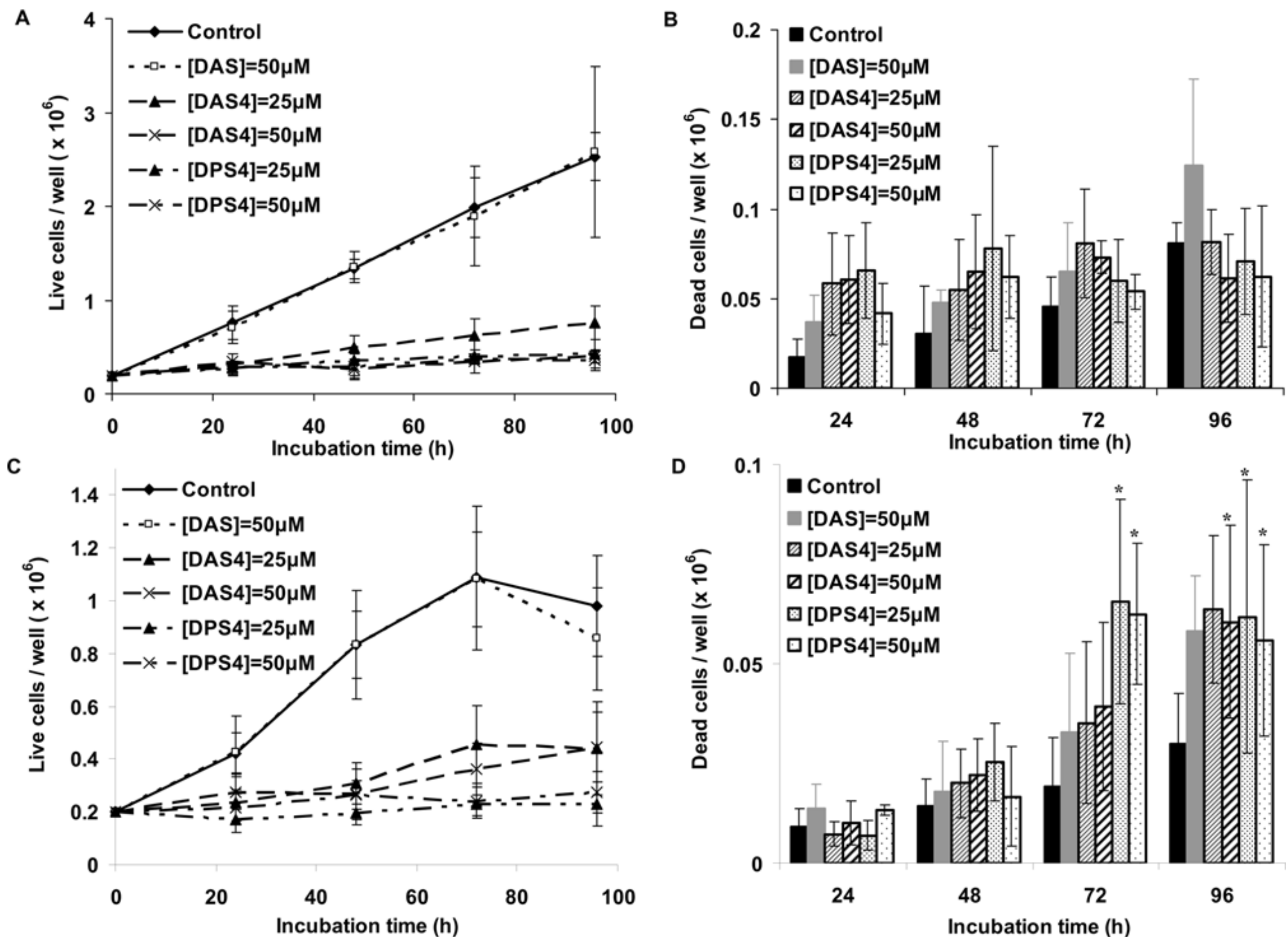

Figure 2. Effect of DAS_4 and DPS_4 on breast cancer cell growth. Cells were exposed to DAS, DAS_4 or DPS_4 and quantified by the trypan blue exclusion counting method. (A) Quantification of living MCF-7 cells. (B) Quantification of dead MCF-7 cells. (C) Quantification of living Vcr-R cells. (D) Quantification of dead Vcr-R cells. Values reported were calculated from three independent experiments.

multi-drug resistance towards vincristine, adriamycin and etoposide associated to over-expression of P-glycoprotein and glutathione S-transferase (GST) pi (14). As for MCF-7, MTT tests pointed towards a cytotoxic activity of DAS_4 and DPS_4 after 48 and 72 h treatment (data not shown). However, these effects appeared to be less dramatic when compared to MCF-7 cells (IC_{50} up to 100 μM). Vcr-R cell growth was markedly reduced in the presence of DAS_4 and DPS_4 (Fig. 2C and D).

Effect on cell cycle progression. The Cdc25 phosphatases are known to play an essential role in cell proliferation.The enzymatic *in vitro* experiments demonstrate the inhibitory potential of DAS_4 and DPS_4 towards the Cdc25 phosphatase activities (Table II) and these results suggest that these compounds could lead to a cell cycle arrest in cultured cells. The effects of DAS_4 and DPS_4 on cell cycle progression have then been determined by flow cytometry after propidium iodide staining. Compared with the control (DMSO, 0.5% v/v), MCF-7 cells displayed a G_2-M phase arrest after incubation with 50 μM DAS_4 and DPS_4 (Fig. 3A). This cell cycle blockage was observed as soon as 3 h after treatment commenced and remained up to 48 h. For example, as shown in Table IV, the cellular population at G_2-M phase was markedly increased (56 and 80%, respectively, compared to 18% for control) when DAS_4 or DPS_4 were added to the cultures for 24 h.

Flow cytometry analysis confirmed that the antiproliferative effect could also be observed on Vcr-R and was associated with a G_2-M cell cycle arrest. As shown in Fig. 3B, this inhibition appeared as soon as 3 h after treatment commenced and was most significant after 24 h of treatment with DAS_4 and DPS_4 (63 and 61%, respectively, compared to 8% for control, as described in Table IV). In contrast to MCF-7 cells, the cell cycle resumed after 48 h treatment: increases in the proportion of Vcr-R cells in the S phase (33 and 48% for treatment with 50 μM of DAS_4 and DPS_4, compared to 15% for control) were observed (data not shown).

Effect of DAS_4 or DPS_4 on tyrosine phosphorylation of Cdk1 and Cdk2 in MCF-7 cells. We finally assessed whether the G_2-M blockage, observed in MCF-7 cells treated with DAS_4 and DPS_4 (50 μM), was effectively related to the ability of both compounds to inhibit Cdc25 phosphatases in cells. The inhibition of the cellular Cdc25 phosphatases led to an accumulation of the inactive phosphorylated forms of Cdk

Table IV. Effects of DAS_4 and DPS_4 on the cell cycle.[a]

	Percent cells in					
	MCF-7			Vcr-R		
Treatment	G_0-G_1	S	G_2-M	G_0-G_1	S	G_2-M
Control (DMSO)	61	21	18	76	16	8
DAS_4 (50 μM)	38	6	56	8	29	63
DPS_4 (50 μM)	18	2	80	6	33	61

[a]Effects of DAS_4 and DPS_4 (50 μM) on MCF-7 and Vcr-R cell cycle distribution after 24 h exposure. The results are representative of two independent experiments.

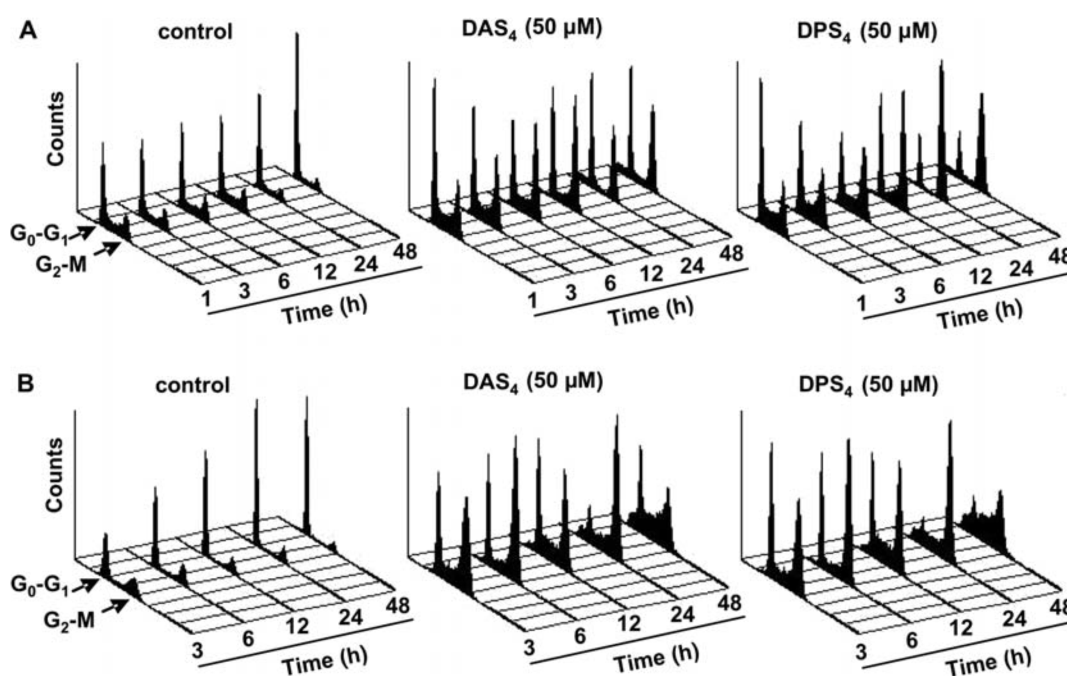

Figure 3. Effect of DAS_4 and DPS_4 on breast cancer cell cycle. (A) Representative histograms of MCF-7 cell cycle phase distribution in cultures treated with solvent alone (control), DAS_4 or DPS_4 (50 μM) for the indicated time periods. (B) Representative histograms of Vcr-R cell cycle phase distribution in cultures treated with solvent alone (control), DAS_4 or DPS_4 (50 μM) for the indicated time periods.

(cyclin-dependent kinases). Fig. 4A clearly shows that DAS_4 and DPS_4 (50 μM) led to an accumulation of the phosphorylated forms of Cdk1 and 2. After quantitative analysis, these compounds appeared to induce accumulation of p-Cdk1 (1.6- and 1.7-fold increase compared to control, respectively) and p-Cdk2 (2.1- and 2.5-fold increase, respectively) after short-time treatment of 6 h (Fig. 4B), in agreement with an inhibition of Cdc25 C and A. As shown in Fig. 4, accumulations of p-Cdk1 and p-Cdk2 did not persist for a longer incubation time (12 h) with DAS_4 and DPS_4 (Fig. 4A and C).

Discussion

Recent studies have confirmed that various organic sulfur compounds exhibit anticancer properties (22) and their efficiency was demonstrated *in vitro* and *in vivo* on various cancer types (5-8,23). These compounds appear to target different signaling pathways to inhibit cancer cell proliferation. Chemistry of the naturally occurring sulfides is very complex and mechanisms involved are not yet fully elucidated. For instance, reactive oxygen species (ROS) generation seems to be one of the antiproliferative mechanisms of such compounds in cancer cells, but ROS levels seem to be closely dependent of the cell lines tested. For example, Cerella *et al* have reported that polysulfides induced a caspase-dependent apoptosis in U937 leukemia cells by activation of pro-apoptotic Bcl-2 family members and the release of the cytochrome c into the cytoplasm without ROS production (24). In contrast, Hosono *et al* showed that DAS_3 led to the formation of ROS in colon cancer cells, causing ß-tubulin oxidation and the disruption of the microtubule network, thus affecting the formation of

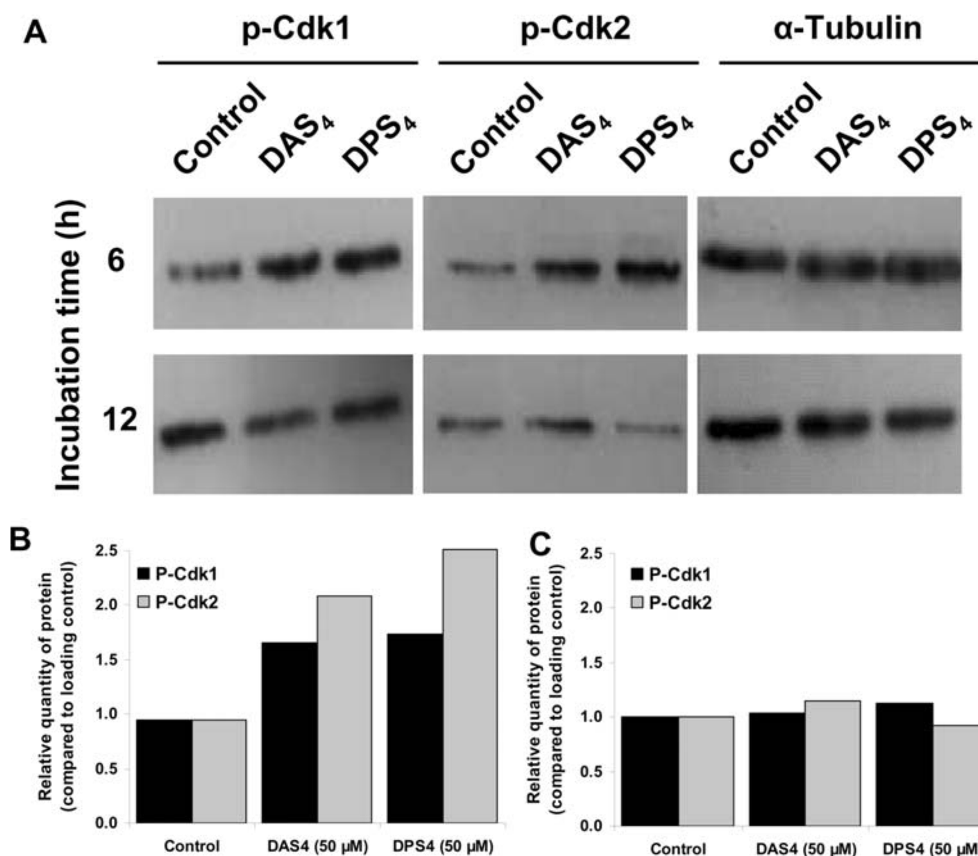

Figure 4. Effect of DAS_4 or DPS_4 on tyrosine phosphorylation of Cdk1 and Cdk2. MCF-7 cells were treated with 50 μM of DAS_4 or DPS_4 for 6 and 12 h. (A) After indicated time treatment cells were harvested and analyzed by immunoblotting. α-tubulin level was used as loading control. (B and C) Relative quantifications of phospho-Cdk1 and phospho-Cdk2 were performed in comparison with loading control (α-tubulin) after 6 h (B) and 12 h (C) treatment.

mitotic spindles, and ultimately leading to apoptosis after a G_2-M phase arrest (25). Other studies have associated Cdc25 C with the cell cycle arrest. Indeed, results obtained by Xiao *et al* indicated that DAS_3-treated prostate cancer cells exhibited a decrease in Cdc25 C protein level and an increase in its Ser216 phosphorylation, creating a binding site for 14-3-3 cytoplasmic protein (7). More recently, they have reported that the DAS_3-mediated G_2-M cell cycle arrest was associated with a down-regulation of Cdc25 C expression (26). The latter study also shows that the induction of pro-apoptotic proteins and the decrease of anti-apoptotic proteins seem to be crucial in apoptosis induction.

Other mechanisms displayed by sulfides have been reported and include the phenomenon of histone hyper-acetylation in human colon tumor cell lines (27).

In the study reported here, we investigated the effects of DAS_4 and DPS_4 in MCF-7-cultured cells, used as model of breast cancer. The analysis of MCF-7 cell growth, in the presence of both DAS_4 and DPS_4, suggests a cytostatic effect. Indeed, both compounds reduce the cell growth inducing a G_2-M phase arrest. Interestingly, in this study, we have also observed very similar effects of DAS_4 and DPS_4 in the vincristine-resistant Vcr-R cell line derived from its sensitive counterpart MCF-7. In these cells, the polysulfides exhibited cytostatic properties, with a significant cell accumulation in G_2-M phase, persisting up to 24 h. In contrast to the cytotoxic effects of polysulfides reported in the literature, we observed that few or no cell death was induced in MCF-7 and Vcr-R after treatment with DAS_4 and DPS_4 (Fig. 2B and D). One explanation could be that the doses used in our experiments were too low to induce apoptosis in these cell lines. Moreover, caspase 3, a key player of the programmed cell death mechanism appears to have a critical role in OSC-induced apoptosis (25), and its deficiency observed in MCF-7 and Vcr-R could suggest that these cells are more resistant to apoptosis compared to caspase 3 positive ones.

Our study demonstrates for the first time the *in vitro* irreversible inhibitory activity of DAS_4 and DPS_4 towards Cdc25 C and A. Interestingly, DAS_4 and DPS_4, the most potent compounds, preferentially inhibit Cdc25 C and A rather than Cdc25 B. These three isoenzymes show a highly conserved catalytic site in the C-terminal domain of the proteins where the motif (HCX_5R) is found, containing the catalytic cysteine. Our results suggest that the inhibitory potential of the organosulfur compounds studied is dependent upon the sulfur chain length (Cdc25 inhibitory activity decreases from tetra- to monosulfide). Our observations are in agreement with those reported by Wu and colleagues who found that the number of sulfur atoms in allylsulfides was correlated with the ability to inhibit the growth of the human liver tumor cells (J5) (28). One explanation for this observation is the complicated reactivity of polysulfides against proteins. Indeed, polysulfides are able to react with proteins to produce various types of modifications including oxidation, hydrophobic

interactions, or to induce radical generation, events which are able to lead to modifications of protein reactivity (4). Moreover, we found that the inhibitory activities of allyl- and propylsulfides are comparable, indicating that the presence of double bonds found in the allyl compounds seems to be not essential. Contrarily, it has been reported that the mono- and disulfides, as well as the carbon analogue of the tetrasulfide display no activity (29).

The effects observed *in vitro* with purified enzymes were confirmed in MCF-7 cells. The inhibition of the cell cycle appears rapidly (3 h) and is maintained up to 48 h. The cause leading to this early and persisting blockage of the cell progression could be due to the fact that DAS_4 and DPS_4 act as irreversible inhibitors of Cdc25 C. Although, Cdc25 C is required for entry into mitosis and to promote S phase entry in addition to the isoform A, it seems to play a significant role in the control of the G_2-M transition (10). Thus, the targeting of several Cdc25 isoforms by DAS_4 and DPS_4 probably enhances their ability to efficiently block the cell cycle.

Moreover, the rapid accumulation of p-Cdk1 and p-Cdk2 (up to 6 h) confirms the inhibitory activity of DAS_4 and DPS_4 in cells. These results are in agreement with those of Herman-Antosiewicz *et al* who observed an accumulation of p-Cdk1 in human prostate cancer cells (PC-3) after short-time treatment by DAS_3 (40 μM for 8 h). This accumulation of p-Cdk1 also appears to be related to Checkpoint Kinase 1 activation and G_2-M cell cycle arrest (30). However, these effects seem to weaken with time. An explanation for this observation could be a possible chemical reduction of organic sulfur compounds by intracellular thiols, such as reduced glutathione (GSH) as already suggested (4,31). Available data on GSH reactivity towards polysulfides and their products do not allow predicting how fast intracellular effects of such sulfides may be overcome in cells.

Finally, the biological activity of polysulfides can be explained by their ability to react with thiols, including cysteine residues in proteins, via thiol-disulfide and -polysulfide exchange. This leads to the formation of mixed disulfides, for instance in form of S-thiolated proteins and enzymes (4). Cdc25 phosphatases are potential targets of sulfur-containing compounds because of the presence of an active cysteine residue in the catalytic domain (32). Rudolph has shown that the Cdc25 phosphatases are sensitive towards redox modifications *in vitro*. Indeed, the active-site cysteine is susceptible to oxidation, which leads to protein inactivation (33). While, in our experiments, no change in ROS levels was observed after short and long time treatments with DAS_4 and DPS_4 (data not shown), we cannot exclude a direct interaction of polysulfides-enzyme in another site than the active site.

Taken together, our results, obtained *in vitro* on purified enzymes and in cell cultures, demonstrate that Cdc25 phosphatase inhibition is a mechanism by which the tetrasulfide derivatives (DAS_4 and DPS_4) may cause cell arrest in breast cancer cells. Moreover, the anticancer effects reported on both the sensitive MCF-7 cells and their resistant Vcr-R counterparts point towards promising avenues of the tetrasulfide compounds (and derivatives) as part of a wider anticancer strategy.

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