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Synthetic polysulfane derivatives induce cell cycle arrest and apoptotic cell death in human hematopoietic cancer cells

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ABSTRACT

Natural polysulfanes including diallyltrisulfide (DATS) and diallyltetrasulfide (DATTS) from garlic possess antimicrobial, chemopreventive and anticancer properties. However these compounds exhibit chemical instability and reduced solubility, which prevents their potential clinical applicability. We synthesized six DATS and DATTS derivatives, based on the polysulfane motif, expected to exhibit improved physical and chemical properties and verified their biological activity on human leukemia cells.

We identified four novel cytotoxic compounds ($\mathbf{IC_{50}}$ values: compound **1**, 24.96 ± 12.37 μM; compound **2**, 22.82 ± 4.20 μM; compound **3**, 3.86 ± 1.64 μM and compound **5**, 40.62 ± 10.07 μM, compared to DATTS: $\mathbf{IC_{50}}$: 9.33 ± 3.86 μM). These polysulfanes possess excellent differential toxicity, as they did not affect proliferating mononuclear blood cells from healthy donors.

We further demonstrated ability of active compounds to induce apoptosis in leukemia cells by analysis of nuclear fragmentation and of cleavage of effector and executioner caspases. Apoptosis was preceded by accumulation of cells in G2/M phase with a pro-metaphase-like nuclear pattern as well as microtubular alterations. Prolonged and persistent arrest of cancer cells in early mitosis by the benzyl derivative identifies this compound as the most stable and effective one for further mechanistic and *in vivo* studies.

1. Introduction

Polysulfanes occur naturally in plants and microorganisms, where they fulfill a variety of biological functions (Anwar et al., 2008; Block, 1992; Block et al., 2010; Capasso, 2013; Jacob et al., 2003). Diallyltrisulfide (DATS) and diallyltetrasulfide (DATTS) from garlic form part of the plant's defense against microorganisms and repel a range of potential predators. It is therefore not surprising that these compounds also exhibit a wide spectrum of biological activity, which includes chemopreventive, antioxidant, redox-modulatory, antimicrobial and even cytotoxic, pro-apoptotic effects. Indeed, there is increasing evidence for selective activity of various polysulfanes against certain cancer cells, whilst normal cells appear to be largely unaffected (Anwar et al., 2008; Cerella et al., 2012, 2009; Kelkel et al., 2012; Munchberg et al., 2007; Scherer et al., 2009; Schneider et al., 2011a,b).

Biological activity associated with polysulfanes such as DATS and DATTS, but also with related compounds, such as the pentasulfane varacin (2-(6,7-dimethoxy-1,2,3,4,5-benzopentathiepin-9-yl)ethanamine, found in the marine ascidian genus *Lissoclinum*), may be explained by the fact that these compounds react *via* so-called thiol/polysulfane reactions which modify cysteine residues in peptides, proteins or enzymes that are particularly reactive, abundant or accessible (*e.g.* reduced glutathione (GSH), tubulin) (Fry and Jacob, 2006; Jacob, 2006; Jacob et al., 2012). These oxidative modifications may seriously affect pivotal cellular processes and may ultimately lead to apoptosis.

Cancer cells exhibit a higher sensitivity towards these agents. First, impairment of cell proliferation generally ascribed to sulfur compounds inevitably affects cells with a high proliferation rate like cancer cells. Besides, oxidative modifications may occur preferably in cancer cells, which commonly display alterations in their redox state, consisting in elevated basal levels of Reactive Oxygen Species (ROS) and/or diminished levels of GSH (Jamier et al., 2010). These cells are fairly sensitive towards further redox changes and therefore are more likely to undergo apoptosis in the presence of a pro-oxidant redox modulator when compared to normal cells.

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Polysulfanes able to attack the 'cellular thiolstat' therefore represent a promising lead for the development of rather selective cytotoxic agents, which in the future may be useful in the treatment of a range of diseases, including cancer (Gruhlke and Slusarenko, 2012; Jacob, 2011; Jacob and Ba, 2011; Jacob et al., 2012; Montenarh and Saidu, 2012; Saidu et al., 2013; Winyard et al., 2011).

Some of the most promising and readily accessible natural polysulfanes, such as DATS and DATTS, however, have certain disadvantages. From a chemical or pharmaceutical perspective, these compounds are oils, only sparingly soluble in or miscible with water, chemically not particularly stable at room temperature and, above all, these compounds exhibit an unpleasant odor (Anwar et al., 2008; Block et al., 2010; Gruhlke and Slusarenko, 2012; Jacob, 2006; Kelkel et al., 2012; Marut et al., 2012; Munchberg et al., 2007; Viry et al., 2011). These unfavorable aspects seriously limit practical applications, such as the use of these compounds in clinics and/or as food supplements.

To overcome these inherent problems associated with naturally occurring polysulfanes, we have therefore investigated a number of newly synthesized derivatives. They retain the biologically active polysulfane motif and show improved properties, such as lack of smell, powdery consistency, better storage properties and solubility. In one case, we have also integrated an ester bond to explore the possibility of pro-drug activation (Czepukojc et al., 2013).

2. Materials and methods

2.1. Synthesis

Chemical reagents were purchased from Sigma–Aldrich-Fluka (Darmstadt, Germany) and used without further purification (unless stated otherwise). The compounds were stored at −30 °C and the integrity and purity was monitored during storage. Thin layer chromatography (TLC) was performed using Merck aluminum backed plates on Silica gel 60 F_{254} (Darmstadt, Germany). Silica gel 60 (40–63 μm, Fluka) was used for column chromatography. Deionised, MilliQ water (resistance ⩾18 MΩ cm^{-1}) was used for the electrochemical experiments. ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance 500 spectrometer [500 MHz (^1H) and 126 MHz (^{13}C)] in $CDCl_3$, $CDCl_3$ containing 5% DMSO-d_6 and in acetone-d_6. Chemical shifts are reported in δ (ppm), expressed relative to the solvent signal. Coupling constants are in Hertz (*J* Hz). HRMS were recorded with a Finnigan MAT 95 spectrometer using the CI technique.

2.2. Synthesis of 1,4-bis(2-ethoxyethyl)-trisulfane (1)

Freshly distilled 1-bromo-2-ethoxyethane (150–155 °C) (2.2 ml, 3.06 g, 20 mmol), $Na_2S_2O_3$ 5 H_2O (6.20 g, 25 mmol) and Na_2S (0.78 g, 10 mmol) in 20 ml H_2O/ethanol 1:1 (v:v) were heated under reflux for 3 h. Afterwards, ethanol was evaporated under reduced pressure, the mixture was washed with petroleum ether and mixed with Na_2S (0.78 g, 10 mmol) in 20 ml H_2O. After stirring the mixture for 45 min at room temperature, the aqueous phase was washed 3× with petroleum ether. The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate (1:1) as eluent. 1 was obtained as a yellow oil in 34% yield (1.65 g, 6.8 mmol). R_f = 0.65 (Petroleum ether/EtOAc, 1:1). ^1H NMR (500 MHz, acetone-d_6) δ 3.65 (t, *J* = 6.5 Hz, 4H, –SCH_2CH_2–), 3.49 (q, *J* = 7.0 Hz, 4H, –OCH_2CH_3), 2.91 (t, *J* = 6.5 Hz, 4H, –SCH_2CH_2–), 1.14 (t, *J* = 7.0 Hz, 6H, CH_3). ^{13}C NMR (126 MHz, acetone-d_6) δ 70.42 (–SCH_2CH_2–), 67.6 (–OCH_2CH_3), 40.7 (–SCH_2CH_2–), 16.3 (CH_3). HRMS (CI) *m/z* calcd for $C_8H_{18}O_2S_3$ $[M]^+$ 242.0469; found 242.0472.

2.3. General procedure for the synthesis of polysulfanes (2, 3, 4) (Derbesy and Harpp, 1994)

A mixture of mercaptane (RSH, 1 eq) and pyridine (1 eq) in diethyl ether was added dropwise over a period of 30 min to a solution of sulfur monochloride (S_2Cl_2) in diethyl ether at −78 °C. After the reaction mixture was stirred at −78 °C for another 30–60 min, a solution of a second equivalent of mercaptane (RSH, 1 eq) and pyridine (1 eq) in diethylether was added over a period of 30 min. Subsequently, the reaction mixture was stirred at −78 °C for another 1 h. Then the mixture was allowed to warm to room temperature. The organic phase was washed subsequently with water, 1 M NaOH (with the exception of compound 4, which was washed with 0.1 M HCl because of the presence of an ionizable COOH group), water and brine. The organic phase was dried over Na_2SO_4, filtered and evaporated to dryness. The resulting product was purified by silica gel column chromatography.

2.3.1. 1,4-Bis(2-ethoxyethyl)-tetrasulfane (2)

Following the general procedure for the synthesis of tetrasulfanes, compound 2 was obtained from 2-ethoxyethylmercaptan (2 × 640 mg, 6.0 mmol) after purification by flash chromatography (petroleum ether/EtOAc = 8:2) as a yellowish oil in 95% yield (1.56 g, 5.68 mmol). R_f = 0.57 (petroleum ether/EtOAc 5:1). n_D^{20} = 1.561. ^1H NMR (500 MHz, $CDCl_3$) δ 3.76 (t, *J* = 6.6 Hz, 4H, –SCH_2CH_2–), 3.54 (q, *J* = 7.0 Hz, 4H, (–OCH_2CH_3), 3.14 (t, *J* = 6.6 Hz, 4H, –SCH_2CH_2–), 1.21 (t, *J* = 7.0 Hz, 6H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 68.6 (–SCH_2CH_2–), 66.5 (–OCH_2CH_3), 39.0 (–SCH_2CH_2–), 15.1 (CH_3). HRMS (CI) *m/z* calcd for $C_8H_{18}O_2S_4$ $[M]^+$ 274.0190; found 274.0185.

2.3.2. 1,4-Dibenzyltetrasulfane (3)

Following the general procedure for the synthesis of tetrasulfanes, compound 3 was obtained from benzyl mercaptan (2 × 1.24 g, 10.0 mmol) and after recrystallization from petroleum ether as a yellow solid in 91% yield (2.83 g, 9.11 mmol). R_f = 0.24 (petroleum ether). mp 49–50 °C (from petroleum ether). ^1H NMR (500 MHz, $CDCl_3$) δ 7.18–7.27 (m, 5H, Ar*H*), 4.09 (s, 2H, CH_2Ar). ^{13}C NMR (126 MHz, $CDCl_3$) δ 136.2 (C_i of phenyl), 129.5 (C_m of phenyl), 128.6 (C_o of phenyl), 127.7 (C_p of phenyl), 43.6 (CH_2Ar). HRMS (CI) *m/z* calcd for $C_{14}H_{14}S_4$ $[M]^+$ 309.9978; found 309.9969.

2.3.3. 3,3′-Dipropanoic acid tetrasulfane (4)

Following the general procedure for the synthesis of tetrasulfanes, compound 4 was obtained from 3-mercaptopropanoic acid (2 × 1.59 g, 1.31 ml, 15.0 mmol) and after purification by flash chromatography (dichloromethane/MeOH = 95:5) as a yellow amorphous solid in 89% yield (3.68 g, 13.4 mmol). R_f = 0.19 (petroleum ether/EtOAc + 1% MeOH 1:1). mp 127–129 °C. ^1H NMR (500 MHz, $CDCl_3$ + 5% DMSO-d_6) δ 2.96 (t, *J* = 7.1 Hz, 4H, –SCH_2–), 2.57 (t, *J* = 7.1 Hz, 4H, –CH_2COOH). ^{13}C NMR (126 MHz, $CDCl_3$ + 5% DMSO-d_6) δ 172.6 (COOH), 33.5 (–CH_2COOH), 33.4 (–SCH_2–). HRMS (CI) *m/z* calcd for $C_6H_{10}O_4S_4$ $[M]^+$ 273.9462; found 273.9490.

2.3.4. Procedure for the synthesis of diethyl-3,3′-dipropionate-tetrasulfane (5)

3,3′-Dipropionic acid tetrasulfane (400 mg, 1.46 mmol), 6 drops of H_2SO_4 (98%) and one spatula of anhydrous $MgSO_4$ in abs. ethanol (20 ml) were stirred at room temperature overnight. Afterwards, ethanol was evaporated and the residue was dissolved in dichloromethane (30 ml). The organic phase was washed with water (50 ml), 0.1 M NaOH (50 ml) and again with water (2 × 50 ml). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product (404 mg, 1.22 mmol, 84%) was purified by flash chromatography on silica gel using a gradient of petroleum ether to petroleum ether/ethyl acetate (9:1) as eluent. **5** was obtained as a yellow oil in 70% yield (338 mg, 1.02 mmol). R_f = 0.16 (petroleum ether/EtOAc 9:1). n_D^{20} = 1.556. ^1H NMR (500 MHz, $CDCl_3$) δ 4.17 (q, *J* = 7.2 Hz, 4H, –OCH_2CH_3), 3.19 (t, *J* = 7.2 Hz, 4H, –SCH_2–), 2.82 (t, *J* = 7.2 Hz, 4H, –CH_2COO–), 1.28 (t, *J* = 7.1 Hz, 6H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.3 (*C* = O), 60.9 (–OCH_2CH_3), 33.9–34.1 (–S–CH_2–, –CH_2COO–), 14.2 (CH_3). HRMS (CI) *m/z* calcd for $C_{10}H_{18}O_4S_4$ $[M]^+$ 330.0088; found 330.0107.

2.3.5. Synthesis of 2,5-bis(propylthio)-1,4-dithiane (6)

Compound **6** was not known in the literature and hence was synthesized for the first time. Propanethiol (9.68 mmol, 737 mg, 0.88 ml) was added to a solution of dithiandiacetate (1.30 g, 4.84 mmol) and $ZnCl_2$ (660 mg, 4.84 mmol) in CH_2Cl_2 under argon atmosphere and stirred at room temperature until the reaction was complete as monitored via TLC (this took several days). The organic phase was washed three times with water and once more with brine. After drying the organic phase over Na_2SO_4 and evaporation of the solvent, the crude product (1.25 g, 4.80 mmol) was obtained as colorless crystals in 99% yield. The product was purified by recrystallisation from CH_2Cl_2. R_f = 0.78 (petroleum ether/EtOAc 8:2). Cis/trans-isomers: ^1H NMR (500 MHz, $CDCl_3$) δ 4.05–3.99 (m, 2H, –S–*CH*(CH_2)S–), 3.28 (dd, 1H, -SCH2CHS-), 3.26 (dd, 1H, -SCH2CHS-), 3.22 (dd, 1H, -SCH2CHS-), 3.05 (dd, 1H, -SCH2CHS-), 2.74 (dd, 1H, –$SCH_2C_2H_5$), 2.72 (dd, 1H, –$SCH_2C_2H_5$), 2.63 (dd, 1H, –$SCH_2C_2H_5$), 2.62 (dd, 1H, –$SCH_2C_2H_5$), 1.72–1.57 (m, 4H, –$CH_2CH_2CH_3$), 1.00 (t, *J* = 7.4 Hz, 6H, –CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ 45.8, 45.8 (–SCH(CH_2)S–), 36.6, 34.9 (–SCH_2CHS–), 33.9, 33.8 (–$SCH_2C_2H_5$), 23.0, 22.9 (–$CH_2CH_2CH_3$), 13.5, 13.4 (–CH_3). (ESI^+) *m/z* calcd for $C_{10}H_{20}S_4$ $[M + H]^+$ 269.04; found 269.0.

2.3.6. Calculation of the log*P* values

Theoretical log*P* values were calculated by the ALOGPS 2.1 Program. The log*P* calculated is the average value that is obtained from different software programs. The standard deviation indicated therefore occurs due to slightly variable values calculated by the programs included in the ALOGPS 2.1 algorithm.

2.4. Cell culture

Human histiocytic lymphoma U937 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 Medium (Lonza, Verviers, Belgium) supplemented with 10% (v/v) of fetal calf serum (FCS, Lonza) and 1% (v/v) of Pen/Strep Amphotericin B (Lonza). Cells were maintained at 37 °C in 5% CO_2 and passaged every two days to ensure a logarithmic phase of growth. 300,000 cells/ml cells were seeded one day before the experiment in 10% FCS-RPMI 1640 medium. The next day, the appropriate

concentration of 5×10^5 cells/ml was obtained in the medium with 0.1% or 10% FCS and the resulting suspension of cells was treated subsequently with compounds. Healthy peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat from the healthy donors (kindly donated by the Red Cross, Luxembourg, Luxembourg) after dilution in Ficoll, as previously described (Juncker et al., 2011). After isolation PBMCs were kept at a density of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) of FCS and 1% (v/v) of antibiotics up to 24 h. Then, PBMCs were treated similar to U937 cells. All experiments were performed in culture medium containing 0.1% of FCS.

2.5. Cell viability assay

Cells were treated with selected compounds at concentrations of 0.5, 1, 5, 10, 25, 50 and 100 μM. The viability of the cells was determined after 24 h treatment by: (i) Cell Titer Glo luminescent cell viability assay kit (Promega, Leiden, Netherlands); (ii) the colorimetric XTT (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) assay (Roche, Luxembourg, Luxembourg) according to the manufacturer's instructions. Three replicate tests for each concentration were carried out. For Cell Titer Glo assay, an equal volume (i.e., 50 μl) of the reaction buffer was added to the medium containing U937 cells (untreated or treated with the selected compounds) in a 96-well ELISA plate. The mixture was shaken on a rocking platform for 10 min in the dark at RT, before proceeding to the detection of the luminescence signal, proportional to the amount of ATP present, by using an Orion Microplate Luminometer (Berthold, Pforzheim, Germany). For XTT assay, the enzymatic reaction was performed in a 96-well ELISA plate in the dark at 37 °C and 5% CO_2 for 4 h after mixing 100 μl of the treated cells and 50 μl XTT reaction solution. After this incubation period, formazan formation by mitochondrial enzymes was quantified spectrophotometrically at 490 nm (Spectracount 96-well-plate photometer, Packard, San Diego, CA, USA).

2.6. Analysis of apoptosis

At the times of treatment indicated, cells were incubated with the DNA-specific dye Hoechst 33342 (1 μg/ml) for 20 min, then the nuclear fragmentation was assessed using a fluorescence microscope (Leica D-M, Lecuit, Luxembourg, Luxembourg). Percentages of apoptotic cells were estimated by counting at least 300 cells in at least 3 random fields with apoptotic features over the total (Kelkel et al., 2012).

2.7. Cell cycle analysis

Cells (5×10^6 cells incubated with polysulfanes for 4, 8, 16, and 24 h) were harvested by centrifugation (7 min at 1300 rpm) and washed twice with cold phosphate-buffered saline (PBS). After removing the supernatant, the cells were fixed by adding 500 μl of ice-cold 70% ethanol and incubated for at least 30 min on ice. Afterwards, cells were spun down (2,000 rpm, 7 min), washed twice with PBS and the pellet was resuspended in 500 μl PBS containing 1 μg/ml of propidium iodide (Sigma–Aldrich, Bornem, Belgium) followed by 100 μg/ml RNase A (Roche) and incubated for 20 min at room temperature in the dark. After incubation, the cell cycle distribution was analyzed by flow cytometry using a FACScalibur Flow Cytometer (Becton–Dickinson, San Jose, CA, USA). Events were recorded (10,000 events/sample) using the CellQuest software (http://www.bdbiosciences.com/features/products/display_product.php?keyID=92). Data were analyzed further with the FlowJo 8.8.7 software (Tree Star, Inc.).

2.8. Immunoblotting

2.8.1. Protein extraction

After incubation with relevant compounds at the indicated time points, cells (at least 2×10^7 cells/per sample) were collected, centrifuged (1300 rpm, 7 min) and washed twice with PBS. To obtain whole cell extracts, cells were lysed using M-PER® (Mammalian Protein Extraction Reagent; Pierce, Erembodegem, Belgium) supplemented with a protease inhibitor cocktail (Complete®, Roche), 1 μM phenylmethylsulfonyl fluoride (PMSF; Sigma), phosphatase inhibitor Phospho-Stop® (Roche; Prophac SARL, Howald, Luxemburg), 5 mM sodium fluoride and 10 mM sodium *ortho*-vanadate (Sigma–Aldrich, Bornem, Belgium)). The mix was put on a shaker with vertical agitation for 15 min at +4 °C. After lysis, cell debris was removed by centrifugation (15 min, 15,000g). The supernatant was collected, aliquoted and stored at −80 °C until use. The protein concentration was determined according to the Bradford protein assay method using Bio-Rad® reagent (Bio-Rad Laboratories, Nazareth Eke, Belgium).

2.8.2. Western Blot analysis

20 μg protein of total cell extracts were separated by using a sodium dodecyl sulfate-(SDS)-polyacrylamide gel (SDS–PAGE; 10% acrylamide separating gel, 4% acrylamide stacking gel) after denaturing samples with boiling (according to the method of Laemmli). After electrophoresis, proteins were transferred to PVDF membranes (GE Healthcare, Roosendaal, The Netherlands). Membranes were blocked in PBS-Tween (0.1%) with 5% of dry milk for 1 h at room temperature or at +4 °C overnight. The membrane was washed with PBS-Tween and incubated with the following primary antibodies diluted in PBS-Tween with 5% dry milk or in PBS-Tween with 5% BSA (for caspase-8): mouse anti-caspase-3 (Santa Cruz Biotechnology, Boechout, Belgium), mouse anti-caspase-7, mouse anti-caspase-8 and rabbit anti-caspase-9 (Cell Signaling, Leiden, The Netherlands), mouse anti-cyclin B1 (Upstate Biotechnology, Placid, NY USA) used in the dilutions of 1:1000. The washing step was repeated with PBS-Tween and the membrane was incubated with specific HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), with the following dilutions: 1:4000 for anti-caspase-3, -7 and -8, 1:5000 anti-caspase-9. After washing with PBS-Tween, the signals of specific immunoreactive proteins were visualized using the Amersham ECL Plus Western Blotting Detection System Kit (GE Healthcare Life Sciences) and the Image Quant LAS 4000 mini (GE Healthcare Life Sciences).

2.9. Immunostaining

5×10^6 cells (5×10^5 cells/ml) were incubated with different compounds for 8 h. At the end of the incubation, cells were collected, centrifuged (1000 rpm, 7 min), and washed with PBS. Cells were fixed and permeabilized using the BD Cytofix/Cytoperm Kit® (Becton Dickinson, Erembodegem, Belgium) according to the manufacturer's instructions (Kelkel et al., 2012). Briefly, the pellet was first incubated in 200 μl Cytofix/Cytoperm solution on ice for 20 min in the dark. After washing with Cytowash solution (2400 rpm, 7 min), samples were resuspended in 60 μl of Cytowash solution containing the antibody against phospho-histone H3 (Ser10) (rabbit, polyclonal; Upstate Biotechnology, Lake Placid, NY, USA); anti-α-tubulin (mouse, monoclonal; Calbiochem), diluted 1:30 and incubated at +4 °C overnight on the shaker. After washing twice with Cytowash (2400 rpm, 7 min), incubation with the secondary antibody (8 μg/ml, room temperature for 30 min) was carried out on a shaking platform (rabbit Alexafluor 568 or mouse Alexafluor 488; Invitrogen/Molecular Probes, Merelbeke, Belgium). The pellet was resuspended in 100 μl Cytowash and cells were finally counterstained with 1 μg/ml Hoechst 33342 to monitor nuclear morphology by fluorescence microscopy (Olympus, Hamburg, Germany). The images were analyzed/elaborated using Cell^M software (Olympus Soft Images Solutions GmbH, Germany).

2.10. Statistical analysis

Data are presented as means of at least three independent experiments ±SD. Student's *t* test was used to determine statistically significant differences between the respective mean values and significant changes are highlighted (n.s. not significant; $^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Selection and synthesis of polysulfanes

Based on previous reports of biological activity associated with DATS and DATTS, we selected tri- and tetrasulfane derivatives for this investigation. As the tetrasulfane DATTS is generally more active than the trisulfane DATS, we focused our studies mostly on tetrasulfane derivatives. The chemical structures of the selected compounds are shown in Fig. 1. The rationale of choice of these compounds was the expected improved solubility in aqueous media (*e.g.* the acid analogue **4**), reduced volatility and odor, powdery consistency (instead of oils) and suitable lipophilicity to cross membranes in accordance with Lipinski's Rule of Five. It should be noted from the outset that the compounds selected, synthesized and tested represent only a first and rather limited selection of possible derivatives, which, nonetheless, is representative of common pharmaceutical design features, and is sufficient to explore biological activity, selectivity and basic mode(s) of action.

For the synthesis of the tetrasulfane derivatives, the classical procedure by Derbesy and Harpp has been adapted (Fig. 1) (Derbesy and Harpp, 1994): here the appropriate thiol-bearing side-chain reacts with sulfur monochloride (S_2Cl_2) in a one-step reaction to generate the symmetrical tetrasulfane structure. This method avoids formation of any major side products (such as disulfides, trisulfanes), enables straightforward purification of the desired product and generates acceptable yields (see Section 2). Employing this method, DATTS analogues originally selected and a number of already known reference compounds, such as dibenzyltetrasulfane (**3**), were synthesized successfully. For comparison,

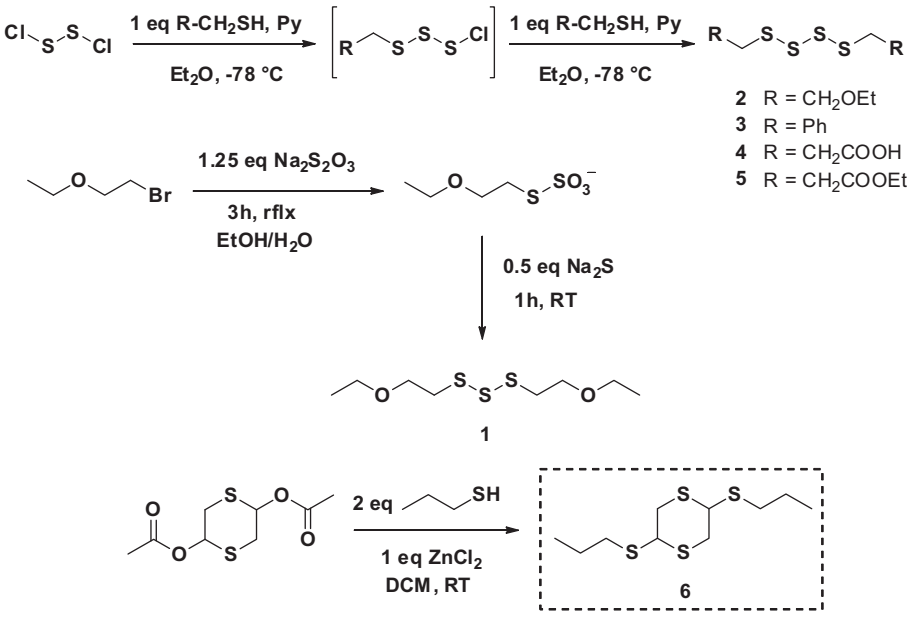

Fig. 1. General method employed for the synthesis of tri- and tetrasulfane derivatives based on previous literature reports. Chemical structures of compounds are shown.

one trisulfane analogue, *i.e.* compound **1**, was also synthesized following the general procedure reported by Milligan et al. and was subsequently used together with the naturally occurring trisulfane DATS as a trisulfane benchmark (Milligan et al., 1961).

3.2. Comparative analysis of the selective cytotoxic potential of polysulfane derivatives against hematopoietic cancer cells vs. normal peripheral blood mononuclear cells

We assayed the biological activity of our panel of selected synthetic derivatives of DATS and DATTS in human monocytic lymphoma U937 cells that we used previously to characterize the mechanisms of action of DATTS (Cerella et al., 2009; Kelkel et al., 2012). Results were compared to peripheral blood mononuclear cells (PBMCs) obtained from human volunteers as healthy control cells.

Cells were treated with 7 concentrations of each compound ranging from 0.5 to 100 μM (see Section 2). Table 1 reports the IC_{50} values after 24 h of treatment obtained by XTT assay. Data show that the two ether analogues of DATTS and DATS, (*i.e.* **2** and **1**, respectively), as well as compound **5** (the ester analogue of DATTS) exhibit cytotoxic activity against U937 cells. The acid derivative **4** and compound **6** are barely active within the range of concentrations tested and were excluded from further mechanistic investigations. Finally, the benzyl derivative **3** is the most active among all compounds investigated, even more active than DATTS itself.

While compounds **1**, **2**, **3** and **5** are active against leukemia cells, they show virtually no cytotoxicity against PBMCs (Table 1). This selective cytotoxicity of the synthetic polysulfane derivatives against cancer cells is in line with results obtained for DATS and DATTS in previous studies (Cerella et al., 2009; Kelkel et al., 2012).

Table 1

Compound	IC_{50} (μM) U937	IC_{50} (μM) PBMCs
DATTS	9.33 ± 3.86	>100
1	24.96 ± 12.37	>100
2	22.82 ± 4.20	>100
3	3.86 ± 1.64	>100
4	>100	>100
5	40.62 ± 10.07	>100
6	>100	>100

3.3. Active DATTS/DATTS derivatives induced apoptosis in hematopoietic cancer cells

Similarly to DATTS, new derivatives induced nuclear alterations corresponding to apoptotic cell death. Fig. 2A reports a kinetic analysis of apoptosis assessed by observation of nuclear morphology of Hoechst-stained cells after 24 h treatment with the selected compounds at IC_{50} concentrations. Percentages of apoptosis are in line with the reduction of cell viability obtained by XTT assay. Here, cells treated with the most active polysulfanes show significantly increased levels of apoptosis, which becomes most significant after 16 h.

Induction of apoptosis triggered by the most active polysulfanes was further investigated by Western Blot analysis of the cleavage of initiator (caspase-8 and -9) and executor (caspase-3 and -7) caspases. Initiator caspases were activated by these compounds as early as 2–4 h and massively from 8 h of treatment onwards. As expected, cleavage products of the effector caspases appear consistently later, mainly after 8–24 h of treatment (Fig. 2B).

3.4. Cytotoxic potential of DATTS/DATTS derivatives correlated with cell cycle alterations and cell accumulation in early mitosis

Next, we turned our attention to the biochemical processes, which are triggered by these compounds. First of all, we ascertained the impact of polysulfane derivatives on cell cycle (Fig. 3A and Suppl. Table 1). Our results indicate that the most active compounds, *i.e.* **1**, **2**, **3**, and **5** cause an accumulation of cells in G2/M, similar to DATTS which peaks at 8 h treatment, followed by a consistent accumulation of cells in the subG1 phase after 8 h. Concomitantly, cyclin B1 protein accumulates between 8 and 16 h of treatment with selected compounds (Fig. 3B). These findings link the action of the most active polysulfanes studied here to cell cycle arrest in G2/M phase and subsequent induction of apoptosis.

We have recently identified tubulin as a direct target of DATTS (Kelkel et al., 2012), besides other cell cycle regulators, such as

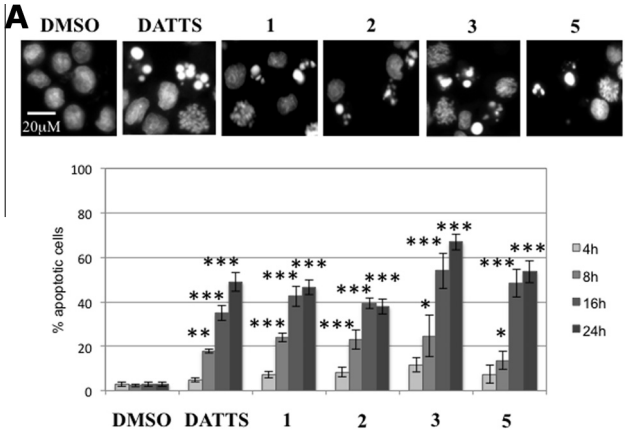

Fig. 2. Active polysulfanes trigger apoptosis in cancer cells. U937 cells were treated with indicated polysulfanes used at a concentrations corresponding to their IC_{50} values as estimated by XTT assay. (A) Analysis of apoptosis by nuclear fragmentation after Hoechst staining and fluorescence microscopy observation in U937 cells treated for 24 h with selected compounds (top) and quantification of the percentage of accumulating apoptotic cells after indicated treatment times (bottom) confirms the cytotoxic potential of **1**, **2**, **3** and **5** compared to DATTS. Apoptotic cells appear mixed with cells blocked in pro-metaphase stage (dotted staining). The mean of three independent experiments ±SD is reported and significant changes are highlighted ($^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$). (B) Western-Blot analysis of cleavage of effector caspase-8 and-9 and executor caspase-3 and-7 confirms the activation of caspase-dependent apoptosis for the most active compounds identified (**1**, **2**, **3** and **5**). C24 designates untreated control cells after 24 h of culture. Etoposide (VP16; 100 μM, 3 h of incubation) was used as positive control. Quantification of the bands corresponding to the cleaved fragments normalized for β-actin are shown. One of three independent experiments with similar results is shown.

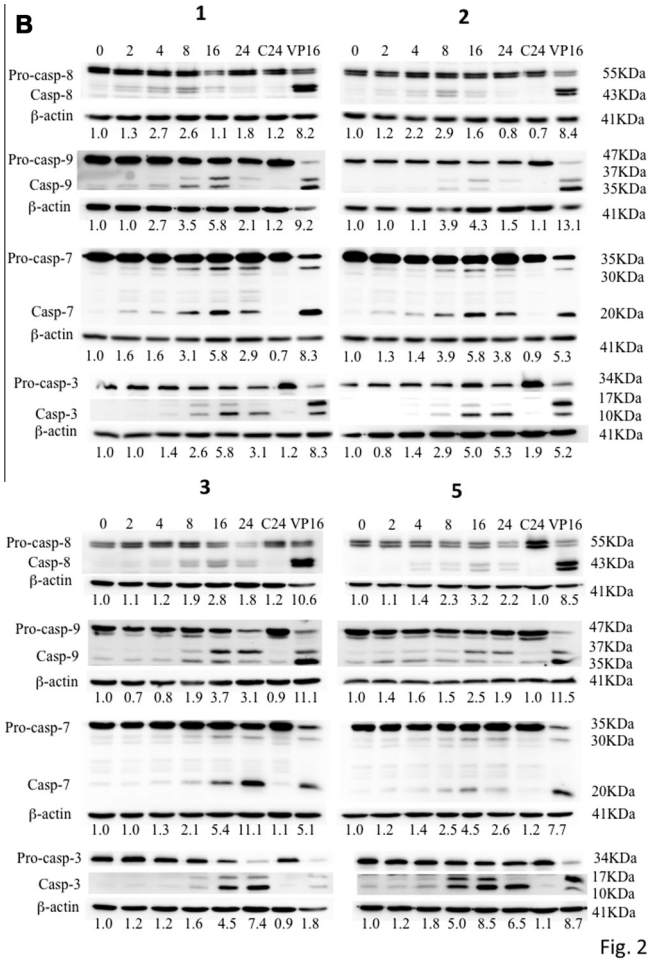

Fig. 2 (*continued*)

cdc25 phosphatases (Viry et al., 2011). Therefore, we investigated the effects of the active polysulfane derivatives (**1**, **2**, **3** and **5)** on the tubulin network. U937 cells treated with synthesized polysulfane derivatives at their corresponding IC_{50} values were immunostained with an antibody recognizing the phosphorylated form of histone 3 (H3P), whose accumulation indicates cells attempting or undergoing division and an antibody recognizing α-tubulin to visualize the microtubule network; finally, samples were counterstained with Hoechst to monitor nuclear morphology (Fig. 3B). Results show that compounds **1**, **2**, **3** and **5**, promoted accumulation of nuclei with a typical prophase-like pattern of chromatin condensation in cells, confirming accumulation of cells in early mitosis. Concomitantly, the spindle apparatus in treated cells was not formed. Instead, tubulin oligomers and polymers appeared randomly scattered throughout the cell (Fig. 3C).

As a functioning microtubule network and spindle formation are essential for mitosis, their disruption may corroborate the hypothesis that cells treated with the polysulfanes can no longer efficiently complete mitosis, experience cell cycle arrest and ultimately trigger apoptosis (Montenarh and Saidu, 2012).

4. Discussion

Our previous results confirmed that trisulfanes and tetrasulfanes are reactive agents, which exhibit an interesting biological activity. This activity is characterized by a considerable cytotoxicity against cancer cells, whilst normal cells are usually less affected. This combination of efficiency and selectivity is not limited to natural polysulfanes, such as DATS and DATTS from garlic. Here, we identified a panel of synthetic derivatives, which maintain reactivity based on the polysulfane 'pharmacophor' with improved solubility and lack of smell.

Results obtained with U937 cells point towards a comparable activity of all polysulfanes studied, confirming the notion that the tri- or tetrasulfane motif (and less so the side-chains) is the key to biological activity. Nonetheless, there are some interesting differences in the IC_{50} values measured, not only in U937 cells, but also in HCT 116 human colon cancer cells and in human prostate adenocarcinoma cell derived LNCaP cells (Results not shown). It seems that the allyl compounds DATTS and DATS exhibit a comparable activity, which is higher than the one of the saturated propyl-analogues present in onions. This observation is not new: allyl derivatives of natural sulfanes are more active than the corresponding propyl analogues (Anwar et al., 2008). One explanation for the 'allyl effect' is based on electronic considerations: allyl groups are electron withdrawing and hence may render the sulfur–sulfur bonds more electrophilic, and therefore also more reactive. Alternatively, the allyl group provides an additional site for nucleophilic attack at the γ-carbon and, under certain conditions, also enables or promotes the release of biologically active H_2S from the (poly-)sulfane core. In fact, some authors even consider H_2S release as the major mode of (bio-) chemical activity associated with such compounds (Benavides et al., 2012, 2007; Kimura, 2011).

Our observations confirm the higher activity of polysulfanes with partially unsaturated side chains. They also point towards an electronic effect as underlying cause (and against H_2S release as the sole cause of activity), as the benzyl residue is electron withdrawing, but unable to facilitate an attack at the γ-carbon, which is required for this kind of H_2S release.

Although speculative at this time, we may consider the possibility that polysulfanes become activated by oxidation. Here, tetrasulfanes may react with H_2O_2 forming chemically highly unstable

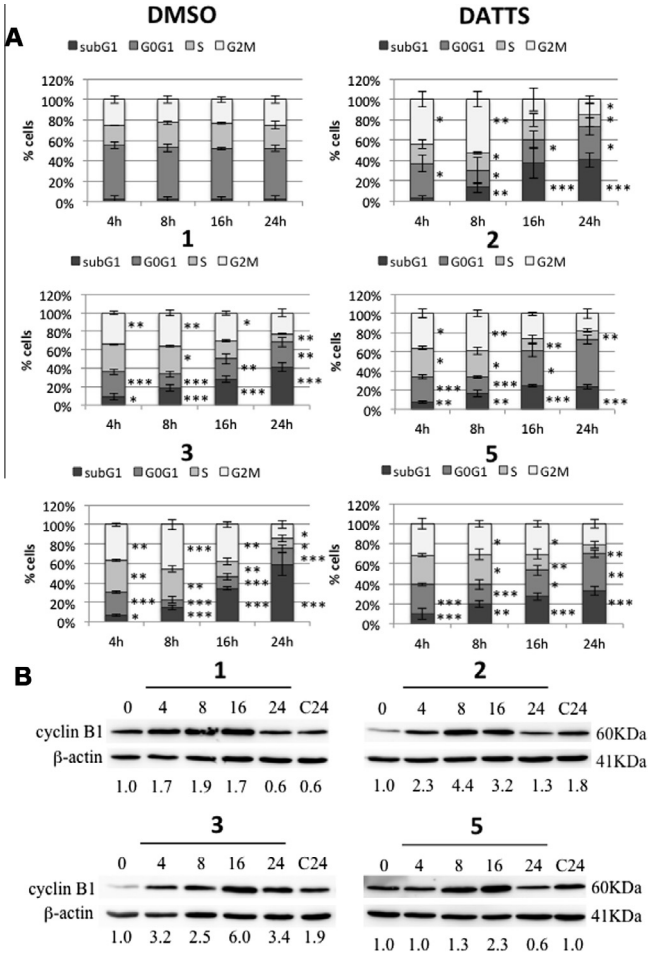

Fig. 3. Compounds 1, 2, 3 and 5 cause an inhibition of the cell cycle in G2/M leading to early mitotic arrest within 8 h of treatment. U937 cells were treated with polysulfanes used at their IC_{50} values. (A) Cell cycle analysis performed at 4, 8, 16 and 24 h. An initial increase of cells in the G2/M with subsequent accumulation in the subG1 phase is visible, a cellular response similar to the one observed previously for DATTS. The results are the mean of three independent experiments +/- SD and significant changes with respect the untreated cells (top-left panel) are highlighted ($^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$). (B) Western-blot analysis of cyclin B1 at 4, 8, 16 and 24 h for the selected compounds **1**, **2**, **3** and **5**. C24 designates untreated control cells after 24 h of culture. Quantification of bands corresponding to the cleaved fragments normalized for β-actin are shown. One of three independent experiments with similar results is shown. (C) As a marker of cells committed to early mitosis, phosphorylation of histone H3 (H3P) in treated cells (8 h of treatment) was assessed after immuno-staining of U937 with a specific antibody recognizing phosphorylation occurring at Ser-10. Percentages of H3P-positive cells were quantified by fluorescence microscopy; values are reported in the corresponding panels (mean of three experiments). Concomitant staining of U937 with Hoechst showed acquisition of a pro-metaphase-like chromatin pattern by H3P-positive cells. Cells were immuno-stained with an antibody specific for α-tubulin in parallel to monitor alterations in the microtubular (MT) organization. Similar to DATTS, **1**, **2**, **3** and **5** disarranged the MT network. One of three independent experiments is shown.

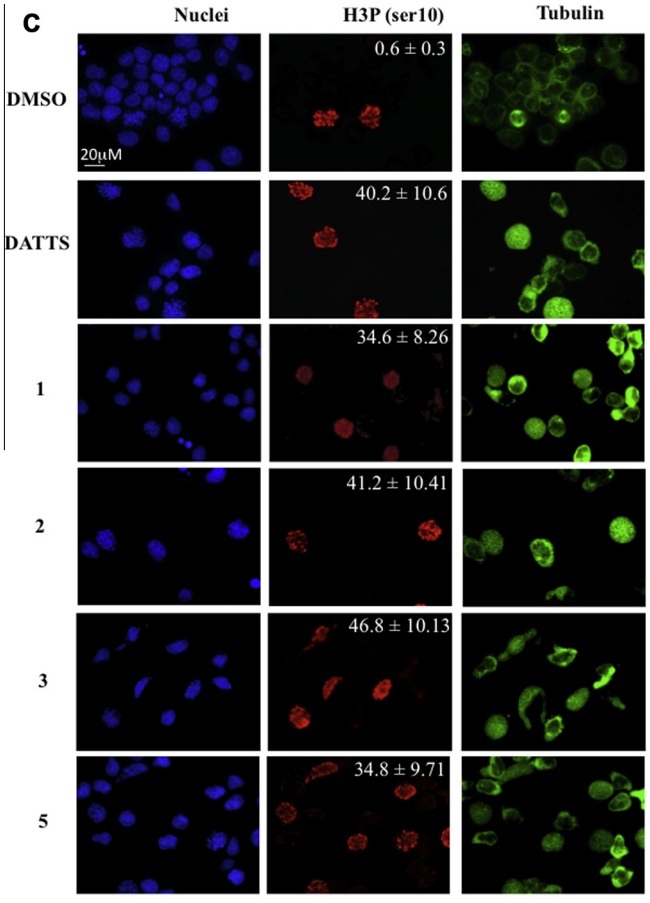

Fig. 3 (*continued*)

sulfoxide-containing species such as RSSS(O)SR. Cope-elimination involving the alkyl chain may subsequently result in a sulfenic acid (RSOH) which is known to react extraordinarily effective and fast, yet also selective with thiol groups. Indeed, a similar chemistry involving Cope-elimination has recently been reported for the garlic compound allicin (Amorati et al., 2012). It may also explain why the allyl and benzyl derivatives are more active compared to the alkyl ones.

Besides the importance of the allyl or benzyl moieties, the studies also show that lipophilicity is an important parameter. Here, the uncharged ether and ester, *i.e.* **2** and **5**, are more lipophilic (calculated log*P* = 1.99 ± 0.95 and 2.22 ± 0.99, respectively) and, at the same time, also more active than the acid (**4**). These results may not be entirely unexpected, as the deprotonated acid (calculated log*P* = 0.66 ± 1.08 for the protonated and 0.38 ± 0.94 for the deprotonated form) may be unable to penetrate the cell membrane at physiological pH. Interestingly, **4** also resembles certain bolaamphiphilic molecules which show increased self-assembly or firm attachment to proteins. This similarity might also explain reduced activity or bioavailability, which is currently under investigation.

Ultimately, the ester **5** may be the most suitable compromise between the active, oily ether and the less active, solid acid, as far as handling and activity are concerned. If such an ester can be activated by esterases, or is 'locked up' and hence enriched inside cells by liberation of the more hydrophilic acid, needs to be shown.

In 2005, Hosono and colleagues have shown that Cys-12β and Cys-354β in tubulin form prime targets of DATS, and are *S*-thiolated (*i.e.* modified to *S*-allymercaptocysteine) despite the obvious presence of an excess of GSH (Hosono et al., 2005). We have recently shown that tubulin is a direct target for the action of DATS and DATTS (Kelkel et al., 2012). The present study further suggests tubulin alterations as a general mechanism for anti-cancer activities of sulfur compounds specifically reactive towards thiols, such as polysulfanes of natural and synthetic origin. Indeed, alterations in the tubulin network may occur *via* modification of its cysteine residues and this event may be sufficient to prevent an orderly polymerization required for the assembly of the microtubuli essential for rapidly dividing cells (Kelkel et al., 2012; Seki et al., 2008). This model may also imply that sulfur compounds – in contrast to conventional microtubule altering agents – do not (have to) target or bind to specific domains on the tubulin proteins. Consequently,

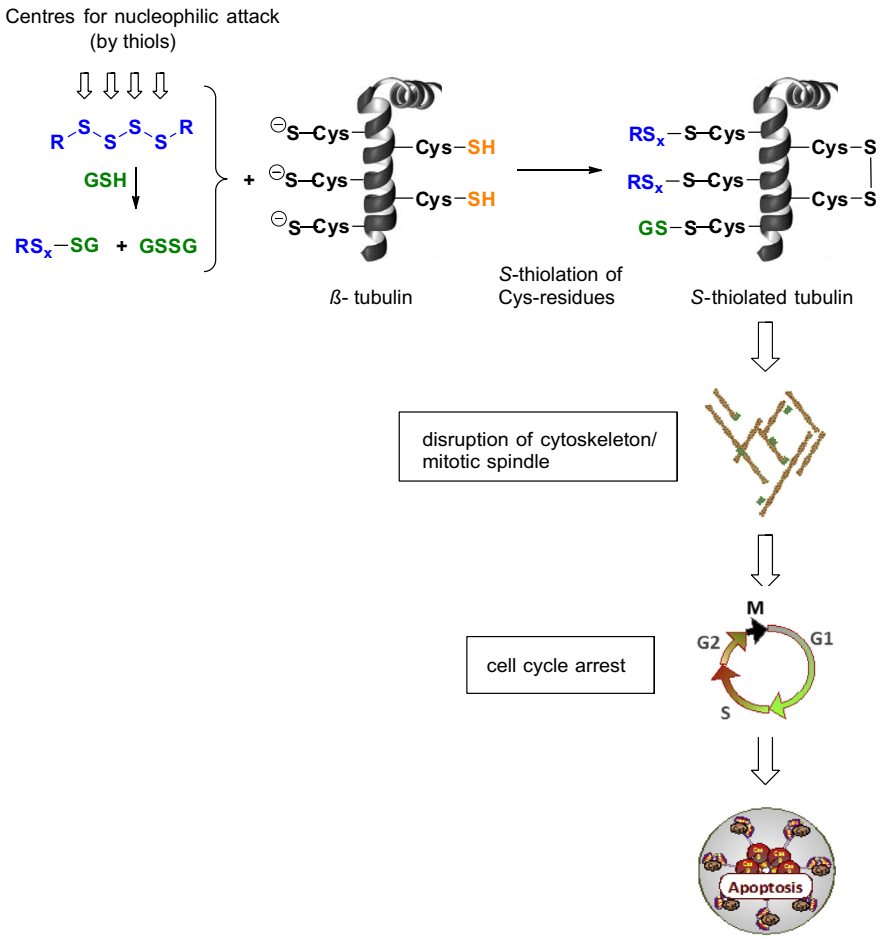

Fig. 4. Schematic overview of a prospective mode of action. Tetrasulfanes react with a range of cellular thiols *via* thiol/polysulfane exchange. As tubulin is highly abundant, its thiols form a prime target for such modifications. Once modified, tubulin is unable to form the mitotic spindle required for cell division, leading to cell cycle arrest and subsequent induction of apoptosis. One must note, however, that this scheme depicts just one possible cellular pathway explaining the experimental findings, without necessarily excluding alternative mechanisms involving various different cellular signaling and response pathways.

these agents might be able to target all isoforms of tubulin, even the ones normally resistant to taxols or *Vinca* alkaloids (Kavallaris, 2010). Besides, sulfur compounds may easily cross cellular membranes and should not be affected by common phenomena of drug resistance related to the intracellular import/export.

The inability of tubulin to properly form microtubuli and the nuclear spindle prevents orderly mitosis and may therefore explain why such polysulfanes cause cell cycle arrest in G2/M, accumulation in the subG1 phase and ultimately cell death *via* caspase activation and induction of apoptosis. Moreover, further experimental evidence is required to explain the reduced biological activity of compounds **1**, **2** and **5** compared to DATTS and compound **3**. A decrease in G1/G0 phase is detected after 8 h of treatment. Subsequently, U937 cells re-accumulate in G1/G0, manifesting a yet unexplored ability to partially bypass the alterations initially arresting the cells in early mitosis.

Apoptosis induced by polysulfanes is paralleled by cleavage of all major effector (caspase-8 and -9) and executor caspases (caspase-7 and -3) similarly to DATTS (Suppl. Fig. 1). Analysis of the pattern and timing of caspase cleavage may reveal some differences, where cleavage of caspases appears mildly evident at earlier treatment times with *i.e.*, compound **1** and **2** in contrast to compound **3** and **5** and DATTS (Suppl. Fig. 1), where this cleavage is simultaneous to caspase-9 and executor caspase-3 and -7 cleavage as early as 8 h treatment. In DATTS-induced cell death, we have previously identified the intrinsic pathway as determinant and occurring even in the absence of BH3-interacting domain death agonist (Bid) truncation (Cerella et al., 2009). Further investigations are required to elucidate the meaning of this difference that seems to rely on the different chemical structure of the compounds. We cannot exclude additional targets of compounds **1** and **2** that may contribute to trigger caspase cleavage at earlier time points. Caspases might conceivably be a potential target for modification by polysulfanes. However, commitment to apoptosis occurs after 8 h of treatment when massive accumulation of cells exhibiting apoptotic nuclear fragmentation appear, supporting a general dominating mechanism triggering cell death.

Our studies map out one particular pathway, which may lead from the thiol/polysulfane exchange at tubulin directly to apoptosis. Nonetheless, additional intracellular targets of polysulfanes remain to be explored. These targets would further contribute to the modulation of cell death commitment. Moreover it remains to be elucidated whether tubulin is also modified in healthy cells, and, if not, why such modifications occur preferably in cancer cells. Here, it is possible that the cytosolic level of GSH plays a decisive role in protecting tubulin thiols against oxidative modification and/or is pivotal for reversing oxidative thiol modifications. Furthermore, one must bear in mind that there may be numerous other signaling cascades, either pro- or anti-apoptotic, which may also be affected by compounds such as DATTS and related polysulfanes. Eventually, the sequence and extent by which each cascade is activated may decide about the ultimate fate of the cell.

In conclusion, our studies showed that it is possible to use the tri- and tetrasulfane motif in compounds as a promising pharmacophore to attack cultured cancer cells effectively and selectively. Selectivity may seem surprising considering the simplicity of the compounds but may be explained by a specific reactivity with (abundant) cellular thiols, which in turn can lead to cell cycle arrest and apoptosis. Fig. 4 gives a schematic overview of a prospective mode of action.

As the synthesis of these natural product analogues is fairly straightforward, it is now possible to generate a vast arsenal of tri- and tetrasulfane analogues available for biological testing. Indeed, the synthesis of the tetrasulfanes proceeds *via* the reaction of two thiol equivalents with one S_2Cl_2 equivalent, and therefore one may even consider synthesis of some asymmetrically substituted derivatives by employing a 1:1 mixture of two different thiols in the reaction. Similarly, recent reports of various biologically active trisulfanes also bode well for future studies as trisulfanes may also be synthesized with comparable ease. Furthermore, polysulfanes beyond the tetrasulfane (such as penta- and hexasulfanes) may also be considered, yet chemical stability seems to decrease with increasing sulfur–sulfur chain length and may negate further gains in activity.

Future studies need to consider the chemistry and biochemistry of polysulfanes in considerably more detail. Various questions remain, from the chemistry of these compounds, to the cellular target(s) and ultimately also to their pharmacological properties (metabolic stability, tissue distribution, bioavailability, etc.). Here, tailor-made polysulfanes with improved chemical stability, solubility and bioavailability are required. At the same time, the thiol/polysulfane interaction as an avenue for (selectively) modifying tubulin needs to be explored further. While higher polysulfanes may be unsuitable, more active di-, tri- and tetrasulfanes, as well as selenium- and tellurium-based agents may be worth considering.

Since the formation and resolution of the tubulin network is a key step in cell division (whose disruption can lead to apoptosis rather than necrosis), tubulin has become a major target of anticancer drug research. Most of the compounds presently considered are rather unselective and generally toxic. Moreover, cancer cells develop resistance mechanisms as conventional agents require specific binding domains, which are not conserved in all tubulin isoforms and are often notably absent in isoforms over-expressed in most aggressive and advanced forms of cancer. Hence the possibility of direct, covalent interactions of these agents with tubulin, and their ability to easily cross cellular membranes, turn polysulfanes into a promising class of compounds certainly worth considering further in the future in anticancer research and drug development.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.10.020.

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