Allium compounds, dipropyl and dimethyl thiosulfinates as antiproliferative and differentiating agents of human acute myeloid leukemia cell lines

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Abstract: Epidemiologic studies support the premise that Allium vegetables may lower the risk of cancers. The beneficial effects appear related to the organosulfur products generated upon processing of Allium. Leukemia cells from patients with acute myeloid leukemia (AML) display high proliferative capacity and have a reduced capacity of undergoing apoptosis and maturation. Whether the sulfur-containing molecules thiosulfinates (TS), diallyl TS (All_2TS), dipropyl TS (Pr_2TS) and dimethyl TS (Me_2TS), are able to exert chemopreventative activity against AML is presently unknown. The present study was an evaluation of proliferation, cytotoxicity, differentiation and secretion of AML cell lines (U937, NB4, HL-60, MonoMac-6) in response to treatment with these TS and their related sulfides (diallylsulfide, diallyl disulfide, dipropyl disulfide, dimethyl disulfide). As assessed by flow cytometry, ELISA, gelatin zymogaphy and RT-PCR, we showed that Pr_2TS and Me_2TS, but not All_2TS and sulfides, 1) inhibited cell proliferation in dose- and time-dependent manner and this process was neither due to cytotoxicity nor apoptosis, 2) induced macrophage maturation, and 3) inhibited the levels of secreted MMP-9 (protein and activity) and TNF-α protein, without altering mRNA levels. By establishing for the first time that Pr_2TS and Me_2TS affect proliferation, differentiation and secretion of leukemic cell lines, this study provides the opportunity to explore the potential efficiency of these molecules in AML.

Keywords: acute myeloid leukemia, thiosulfinate, proliferation, differentiation, matrix metalloproteinase-9

Introduction

Acute myeloid leukemia (AML) is a deadly disease, resulting from the clonal expansion and accumulation of hematopoietic stem cells arrested at various stages of development (Mason et al 2006; Plesa et al 2008). Leukemia cells are unable to undergo growth arrest, terminal differentiation and apoptosis in response to appropriate environmental stimuli, and prematurely egress from the bone marrow to disseminate into peripheral tissues (Mason et al 2006; Plesa et al 2008). The standard approach to AML remains chemotherapy, and the novel agents currently available (multidrug resistance inhibitors, farnesyltransferase inhibitors, receptor kinase inhibitors, monoclonal CD33 antibody-mediated drugs) (King et al 2007; Thomas et al 2007) show some efficacy in either monotherapy or in combination with conventional chemotherapeutic drugs such as anthracycline, cytarabine or idarubicin (Adachi et al 2004; Leone et al 2006; Thomas et al 2007). The development of new compounds directed against leukemia-specific targets is however needed to increase the cure rate in AML patients exhibiting chemoresistance and poor outcomes.

Allium vegetables (including garlic, onions, leeks, chives, and scallions) are used throughout the world for their sensory characteristics as well as their apparent health benefits (Amagase 2006; Borek 2006; Milner 2006). They exhibit antimicrobial,

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antithrombotic, antitumor, antiarthritic and hypoglycemic properties (Ackermann et al 2001; Pinto et al 2001; Khanum et al 2004; Amagase 2006; Borek 2006; Milner 2006) which are largely attributed to the presence of sulfur compounds in these plants (Le Bon et al 2000; Lanzotti 2006). For example, the major sulfur-containing molecules in intact garlic are sulfoxides, which are converted into thiosulfinates (such as Allicin) when raw garlic is cut or crushed (Amagase 2006; Lanzotti 2006). Allicin (diallyl thiosulfinate/All_2TS) (Figure 1) is rapidly decomposed to diallyldisulfide (DADS) (Figure 1), diallylsulfide (DAS) (Figure 1), diallyltrisulfide (DATS) (Figure 1) and sulfur dioxide, and therefore does not seem to be a genuine active compound of garlic (Amagase 2006). DADS and DATS exert antiproliferative and proapoptotic effects in human epithelial cancer and neuronal cell lines (Sundaram et al 1996b; Pinto et al 2001; Hosono et al 2005; Milner 2006; Xiao et al 2005, 2006). DADS induces apoptosis in the leukemic HL-60 cell line through activation of caspase-3 (Kwon et al 2002), and inhibits NO synthesis in LPS-activated macrophages (Ippoushi et al 2002). DATS also stimulates apoptosis of HL-60 cells (Zheng et al 1997) and inhibits platelet function by inhibiting platelet aggregation and Ca(2+) mobilization (Qi et al 2000). Other thiosulfinates such as dipropyl thiosulfinate (Pr_2TS) and dimethyl thiosulfinate (Me_2TS) (Figure 1) are mainly identified in onion and leek (Lanzotti 2006). Pr_2TS, Me_2TS and All_2TS are found to inhibit platelet aggregation through inhibition of calpain (Rendu et al 2001), by reaction with surface free sulfhydryls and internal thiol-containing proteins (Badol et al 2007).

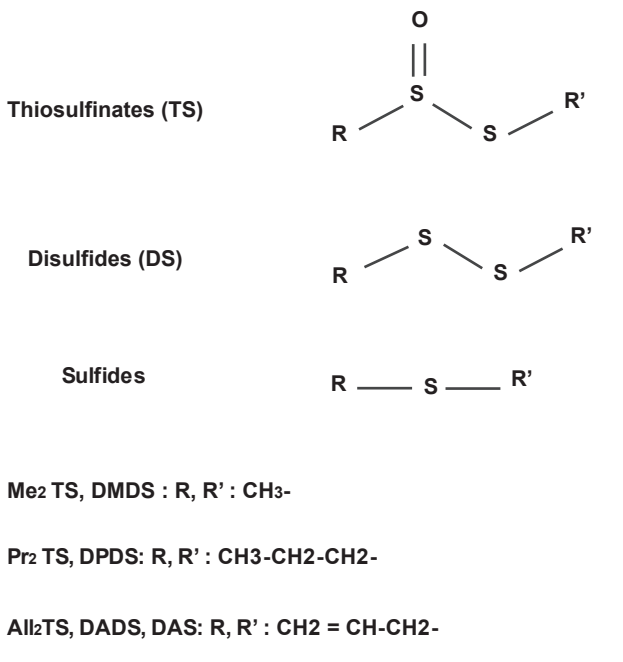

Figure 1 Structure of thiosulfinates and sulfides used in this study.
Abbreviations: DADS, Diallyl disulfide; DAS, diallylsulfide; All_2TS, diallyl thiosulfinate; DMDS, dimethyl disulfide; Me_2TS, dimethyl thiosulfinate; DPDS, dipropyl disulfide; Pr_2TS, dipropyl thiosulfinate.

Whether Pr_2TS, Me_2TS and All_2TS are able to exert chemopreventative activity against AML is presently unknown. With the above in mind, we undertook this study to evaluate the *in vitro* efficacy of these compounds on human cell lines representative of AML. We show that Pr_2TS and Me_2Ts, but not All_2TS and their related sulfides, inhibit the growth of AML cell lines and interfere with the secretion of tumor necrosis factor-α (TNF-α) and matrix metalloproteinase-9 (MMP-9). Additional studies are warranted to investigate the inhibitory activity of Pr_2TS and Me_2TS in AML patients' cells *ex vivo*.

Materials and methods

Chemicals and antibodies

Monoclonal antibodies (mAbs) (fluorescein isothiocyanate, FITC; phycoerythrin, PE) specific for CD11b (mIgG1, BEAR1), CD13 (mIgG1, SJ1D1), CD15 (mIgM, 80H5), CD44 (mIgG1, J-173), Apo 2.7 (mIgG1, 27AGA3), their isotypes and the Annexin V-FITC staining kit were obtained from Coulter/Beckman (Luminy, France). Irrelevant mouse (m)IgGs (FITC and PE) were obtained from R&D (Adbington, UK). Phorbol myristate acetate (PMA), nigericin, dimethyl disulfide (DMDS), dipropyl disulfide (DPDS), diallylsulfide (DAS), and diallyl disulfide (DADS) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Diallyl thiosulfinate (All_2TS), dipropyl thiosulfinate (Pr_2TS) and dimethyl thiosulfinate (Me_2TS) were synthesized as previously described (Auger et al 1990). Organosulfur-compounds were solubilized in water. The stability of TS was assessed by HPLC analysis (Arnault et al 2003). All-*trans* retinoic acid was provided by Ares-Serono (Geneva, Switzerland).

Human AML cell lines and cell culture

Models of AML were represented by U937 cell line (monoblastic stage, AML-M5) (Ferrara et al 2001) (American Tissue Cell Culture), HL-60 cell line (promyelocytic stage, AML-M2) (Drexler et al 1999) (American Tissue Cell Culture), NB4 cell line (promyelocytic stage, AML-M3) (Lanotte et al 1991) (a gift from Dr Lanotte, Hôpital Saint Louis, Paris, France) and MonoMac-6 (promonocytic stage, AML-M5) (Drexler et al 2004) (a gift from Dr Ziegler-Heitbrock, University of Munich, Germany). Cells were maintained in RPMI 1640 medium

supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco, Paisley, Scotland, LPS levels <0.1 ng/mL), 2 mM L-glutamine, 1 mM sodium pyruvate and 40 μg/ml gentamycin (Flow laboratories, Rockwell, MD) in a 5% CO_2 humidified atmosphere at 37 °C. Cells ($1–2 \times 10^5$/mL) were cultured in complete RPMI 1640 medium at 37 °C in the absence or presence of various concentrations of compounds (10^{-7}–10^{-4}M). After various periods of incubation, cells were collected, washed twice, and living cells (with diameters ranging from 7 to 14 μm) were counted in a cell Coulter Counter ZM equipped with a Coultronic 256 channelizer (Coulter/Beckman). Maturation of cells toward the monocyte/macrophage or granulocytic pathway was determined according to the change in morphology assessed by staining cytocentrifuged cells (using a Shandon 3 Cytospin, Thermo Electron) with the Hemacolor kit from Merck and light microscope examination, and quantification of several phenotypic markers characteristic of cell maturation into macrophages (CD11b) or granulocytes (CD15, CD13, CD44) as assessed by flow cytometry analysis (see below).

Flow cytometry analysis of cell differentiation, apoptosis and death

Intact cells were immunostained with Abs directed against CD11b, CD13, CD15, CD44 as described (Bauvois et al 2002). Analysis was performed in a FACS flow cytometer analyzer (Coulter-Beckman, Luminy, France). Values are given as percentages of positive cells and antigen relative density per cell (obtained by subtracting the peak channel number of the negative control from the peak channel number of the corresponding experimental sample). Apoptosis was determined by the binding of Annexin-V-FITC to phosphatidylserine exposed to the cell membrane, used in conjunction with propidium iodide (PI), to evaluate subpopulations of cells along the apoptotic pathway according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA extraction from treated cells and cDNA synthesis were conducted as described (Bauvois et al 2002). Specific human primers for human β2-microglobulin, MMP9, TNF-α, and vascular endothelial growth factor (VEGF), chosen according to published sequences (Westphal et al 2000; Bauvois et al 2002; Stordeur et al 2002; Parks et al 2004) were as follows: β2-microglobulin (165 bp), 5'-CAT CCA GCG TAC TCC AAA GA-3' (forward) and 5'-GAC AAGT CTG AAT GCT CCA C-3' (reverse); MMP-9 (296 bp), 5'- GGA GAC CTG AGA ACC AAT CTC-3' (forward) and 5'-TCC AAT AGG TGA TGT TGT CGT-3' (reverse); VEGF (204 bp), 5'- ACA TCT TCC AGG AGT ACC CTG ATG AG-3' (forward) and 5'- GCA TTC ACA TTT GTT GTG CTG T-3' (reverse); TNF-α (406 bp), 5'-ACC ATG AGC ACT GAA AGC AT-3' (forward) and 5'-AGA TGA GGT ACA GGC CCT CT-3' (reverse). The PCR products were visualized by electrophoresis in 2% agarose gel containing 0.2 μg/ml ethidium bromide. The β2-microglobulin gene was used to normalize the PCR products. The NIH Image 1.63 software was used for the analysis of the bands after acquisition in an Appligen densitometer (Oncor).

Measurement of MMP-9 gelatinolytic activity by zymography

Analysis of MMP-9 activity was carried out in 7.5% (w/v) SDS-polyacrylamide gels containing 0.2% gelatin (w/v) as described elsewhere (Bauvois et al 2002). Equal amounts of culture media (20 μl) were applied to the gel in Laemmli sample buffer lacking β-mercaptoethanol. Gelatinolytic activities of pro-MMP-9 (92 kDa) and mature MMP-9 (82 kDa) were detected as transparent bands on the background of Eza-blue stained gelatin. The bands were acquired with an Appligen densitometer (Oncor).

Enzyme-linked immunosorbent assays

The culture supernatant fractions from cells (10^5 cells/mL) cultured for 3 days in the absence or presence of thiosulfinates were harvested under sterile conditions and frozen before MMP-9, VEGF, and TNF-α contents were determined using commercial ELISA kits provided by R&D (Abingdon, UK). Controls included FCS-supplemented RPMI 1640 medium alone incubated under the same conditions. The concentrations were calculated after substraction of the control values. Detection level was 10 pg/mL for TNF-α and VEGF, and 1 ng/mL for MMP-9.

Statistical analysis

Values are represented as means ± SD of n independent experiments.

Results

Pr2TS and Me2TS induce growth arrest of AML cell lines

The antiproliferative potential of All_2TS, Pr_2TS and Me_2TS and their related sulfides (DADS, DAS, DMDS, DPDS) were first evaluated in the U937 cell line. Cells were cultured 3 days

in the absence or in the presence of increasing concentrations of the compounds (10^{-7}–10^{-3} M). The growth of U937 cells was inhibited by Pr_2TS (Figure 2A) and Me_2TS (Figure 2B) with IC_{50} values (half-maximal inhibitory concentrations) of 2 μM, whereas their related sulfides in the same range of concentration did not alter cell growth (Figure 2C). All_2TS, DAS, and DADS did not affect cell growth (Figure 2D) and HPLC analysis showed that All_2TS was totally degraded upon 24 h cell culture. Kinetic studies indicated also a time-dependent inhibitory effect of Pr_2TS and Me_2TS on cell growth (Figure 3A). NB4 and MonoMac-6 cell lines were also sensitive to Pr_2TS and Me_2TS (Figure 3B).

Pr2TS and Me2TS do not induce U937 cell death

We next questioned whether the inhibition of U937 cell growth by Pr_2TS and Me_2TS was associated with cytotoxicity. The criteria for cell death, measured by Cell Coulter analysis, was based on the number of necrotic cells with diameters ≤7 μm (reflecting cell shrinkage), and Figure 2A and B shows that Pr_2TS and Me_2TS tested did not induce a marked cytotoxic effect. It was next investigated whether decreased growth was related to apoptosis. To this end, we analyzed the surface binding of Annexin V, known as an early marker of apoptosis (Martin et al 1995) with simultaneous PI staining for necrotic cells. Figure 4 shows a representative experiment in which U937 cells were cultured for 3 days in the absence or presence of Pr_2TS, Me_2TS (5 μM) in comparison to the pro-apoptotic agent nigericin as a positive control. In contrast to untreated U937 cells (Figure 4A), cells treated with nigericin were strongly positive for Annexin V (L4 = 34%, Figure 4B, apoptotic cells) and for both Annexin V and PI (L2 = 40%, Figure 4B, secondary necrotic cells). U937 cells treated with Pr_2TS or Me_2TS were found weakly positive for Annexin V (L4 ≤ 14%), and for both Annexin V and PI (L2 ≤ 11%, Figure 4B and 4C). Moreover, using Apo 2.7 antibody which reacts with a mitochondrial protein exposed on cells undergoing apoptosis, we confirmed that Pr_2TS and Me_2TS did not induce U937 cell apoptosis (data not shown).

Pr2TS and Me2TS induce differentiation of AML cells towards macrophages

The capacity of U937 cells to undergo macrophage maturation was further measured by examining the

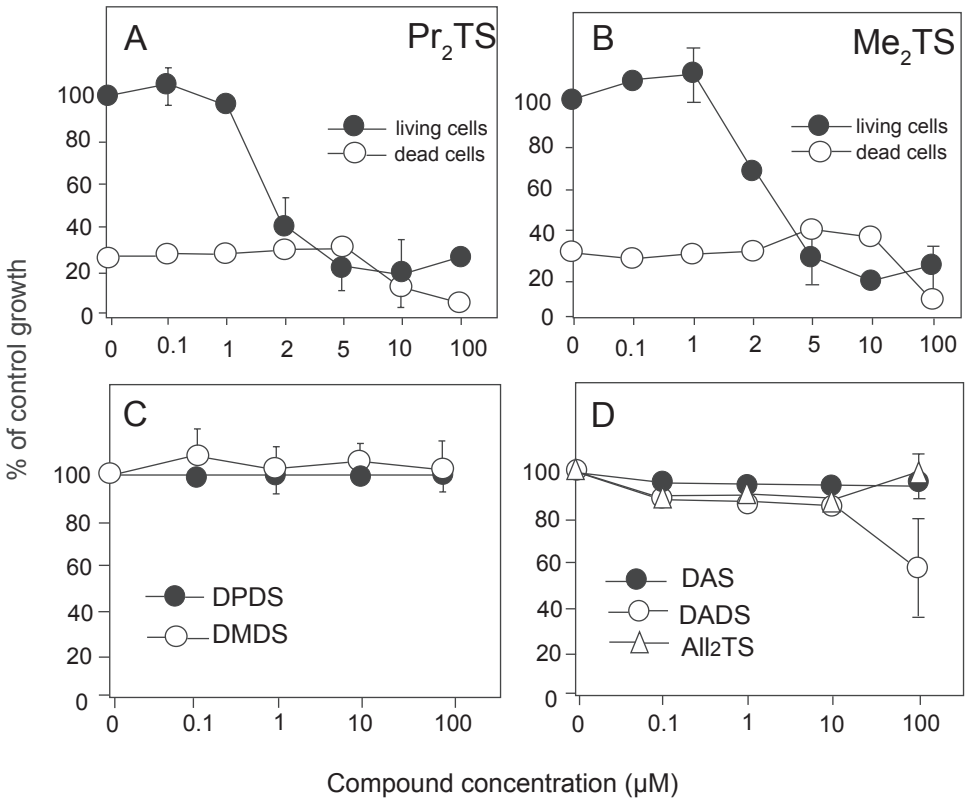

Figure 2 Dose-dependent effects of thiosulfinates and sulfides on U937 cell proliferation. Cells (10^5/mL) were cultured for 3 days in the absence (control) or in the presence of increasing concentrations (10^{-7}–10^{-4}M) of Pr_2TS (**A**), Me_2TS (**B**), DPDS (**C**), DMDS (**C**), All_2TS (**D**), DAS (**D**) or DADS (**D**). After 3 days, living cells and necrotic cells were counted with a cell Coulter as described in Methods. Results are expressed as % of untreated cells (100% = 9×10^5 cells/mL ± 10^5). Data represent the means of 2–5 independent determinations ± SD.

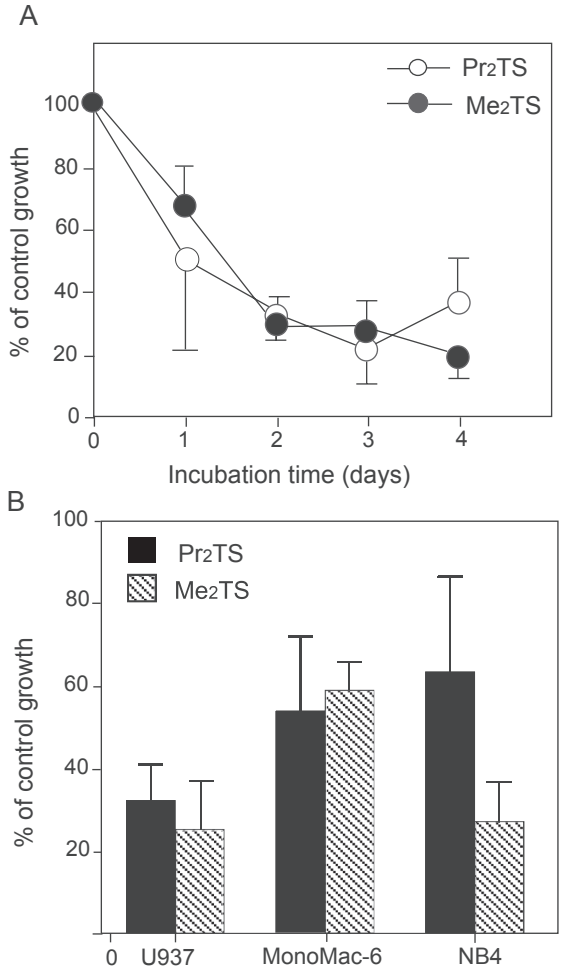

Figure 3 Time-dependent effects of Pr_2TS and Me_2TS on U937 cell proliferation; comparative effects with NB4 and MonoMac-6 cells. (**A**) U937 cells (10^5/mL) were cultured in the absence or presence of 5 μM Pr_2TS or Me_2TS for 1 to 4 days. Results are expressed as percentage of untreated cells (100% = 9×10^5 cells/mL ± 10^5). Data represent the means of 2 to 6 independent determinations ± SD. (**B**) U937, NB4 and MonoMac-6 cells (10^5/mL) were cultured in the absence or presence of 5 μM Pr_2TS or Me_2TS for 3 days (U937, NB4, MonoMac-6). Results are expressed as percentage of untreated cells. (100% = 9×10^5 cells/mL ± 10^5 for U937 and MonoMac-6; 100% = 8×10^5 cells/mL ± 10^5 for NB4). Data represent the means of n independent determinations ± SD (n = 7 to 13 for U937; n = 5 to 6 for MonoMac-6; n = 3 to 6 for NB4).

modulation of expression of the CD11b antigen and the change in morphology of stimulated cells. Enhanced CD11b expression has been associated with monocyte/macrophage differentiation (Kim et al 1991). At day 4, the block in growth of Pr_2TS- and Me_2TS-treated U937 cells was correlated with a marked enhanced expression of CD11b as compared to untreated cells (Figure 5A). In parallel, a change in morphology (increase in cell size, decrease in the nuclear/cytoplasmic ratio and apparition of vacuolisation) consistent with macrophage differentiation was observed in cells exposed to Pr_2TS and Me_2TS when compared to untreated cells (Figure 5B). As positive control, PMA-treated cells presented morphological features of cell maturation and these modifications were accompanied by the up-regulation of CD11b (Figure 5A and B). Similarly, Pr_2TS and Me_2TS induced a marked shift towards a macrophage morphology associated with the enhanced expression of CD11b in the other cell lines (data not shown).

Pr2TS and Me2TS do not induce differentiation of HL-60 and NB4 cells towards granulocytes

Additionally, we investigated whether Pr_2TS and Me_2TS could also induce differentiation part of these cells towards granulocytes. Myeloblastic cell lines such as NB4 and HL-60 can be induced to differentiate into granulocyte-like cells by all-*trans* retinoic acid (ATRA) (Collins 1987; Khanna-Gupta et al 1994). Previously described changes in antigen expression by ATRA-treated cells include the increase of CD15 in HL-60 and NB4 cells (Lanotte et al 1991; Trayner et al 1998; Barber et al 2008) and the down-regulation of CD13 and CD44 in HL-60 cells (Trayner et al 1998; Barber et al 2008). Although to a lower degree, Pr_2TS and Me_2TS (5 μM) were capable of inhibiting HL-60 cell growth (40% inhibition at day 5). Following treatment with 10 μM ATRA for 5 days, HL-60 and NB4 cells presented morphologic features of granulocytes (data not shown) associated with the increased expression of CD15 (HL-60 and NB4) and the decreased expression of CD13 and CD44 (HL-60) (Table 1). In contrast, cells treated with Pr_2TS or Me_2TS (5 μM) did not have any effect on CD13, CD15 and CD44 expression (Table 1). Together, our results indicate that Pr_2TS and Me_2TS do not induce AML cells to mature along the granulocytic lineage.

Inhibitory effects of Pr2TS and Me2TS on MMP-9 and TNF-α release by U937 and NB4 cells

As assessed by ELISA and zymography analyses of the conditioned media from day 3-cultured cells, very low amounts of matrix metalloproteinase-9 (MMP-9, 92 kDa) were produced by U937 cells and NB4 cells (<10 ng/mL). In parallel, no change in MMP-9 mRNA expression was observed to Pr_2TS or Me_2TS (from 24 to 72 h, data not shown). As described previously (Watanabe et al 1993), exposure to PMA markedly increased the levels of released MMP-9 as assessed by its gelatinolytic activity (about 10-fold increase, Figure 6A). Addition of Pr_2TS or Me_2TS (5 μM) to the culture medium resulted in a reduction of released MMP-9 activity from PMA-activated cells (Figure 6A). ELISA data

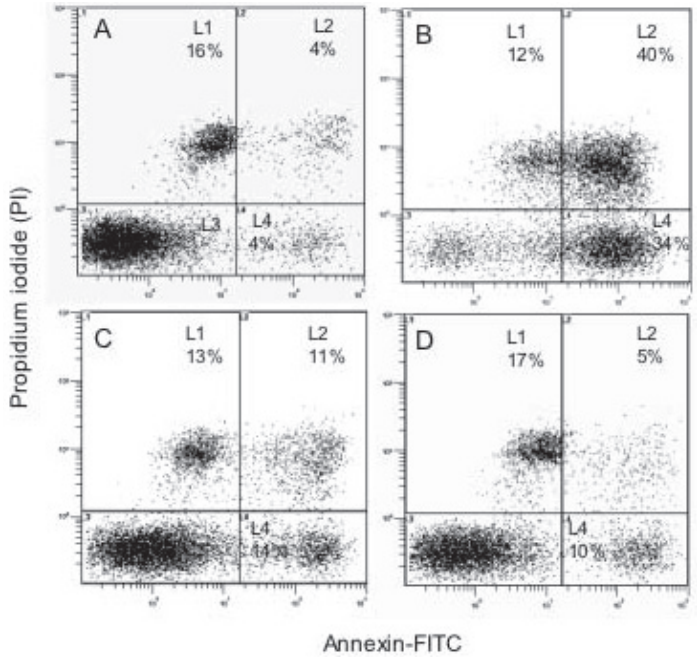

Figure 4 Representative histograms showing flow cytometry analysis of Annexin V-FITC/PI stained U937 cells. Cells were cultured in the absence (**A**) or presence of 10 μM nigericin (proapoptotic agent, positive control) (**B**), 5 μM Pr_2TS (**C**) or 5 μM Me_2TS (**D**) on U937 cells for 3 days. Results are expressed as log PI fluorescence intensity (y-axis) vs log Annexin V fluorescence intensity (x-axis).

Abbreviations: L1, necrotic cells; L2, secondary necrotic cells; L3, healthy cells; L4, apoptotic cells; PI, propidium iodide.

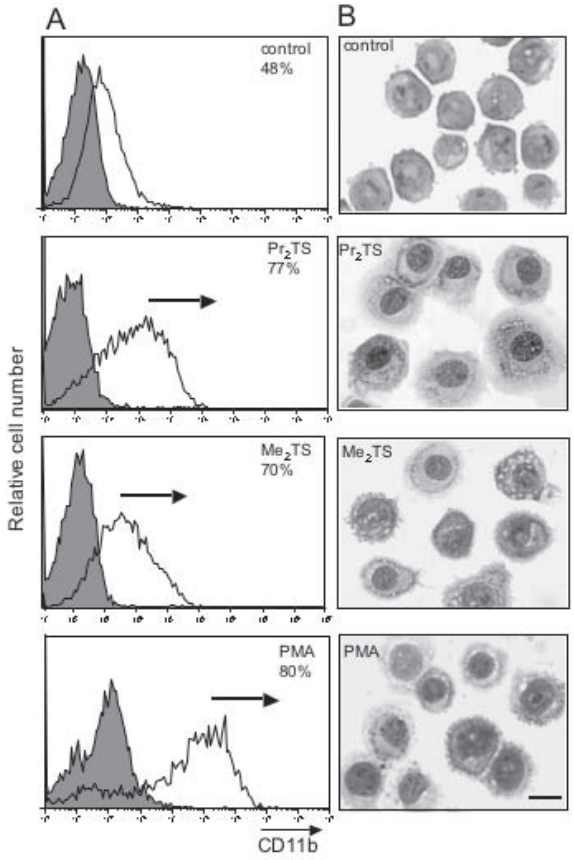

Figure 5 Differentiation effects of Pr_2TS and Me_2TS on U937 cells. Cells (10^5/mL) were cultured in the absence (control) or in the presence of Pr_2TS, Me_2TS (5 μM) or PMA (1 ng/mL) for 4 days. (**A**) Specific fluorescence intensity of CD11b antigen (black line) was detected by immunofluorescence in a Coulter flow cytometer as described in Methods. Staining of cells with a matched isotype antibody (mIgG1) served as the respective negative control (grey area). Results are expressed as relative cell number (y-axis) vs log fluorescence intensity (x-axis). Values represent percentage of positive cells. One experiment representative of four different experiments is shown. (**B**) Light microscopic morphology of U937 cells Original magnification x 600. May Grünwald stain. Scale bar, 10 μm.

Table 1 Effects of ATRA, Pr_2TS and Me_2TS on the expression of CD13, CD15 and CD44 on HL-60 and NB4 cells

Antibody antigen	Granulocyte expression	Percent of positive cells (relative density)			
		Control	ATRA	Pr_2TS	Me_2TS
1) on HL-60					
CD13	↓	82 (30)	74 (17)	89 (36)	87 (36)
CD15	↑	67 (35)	69 (84)	62 (40)	62 (27)
CD44	↓	48 (168)	42 (28)	47 (169)	48 (197)
2) on NB4					
CD15	↑	55 (17)	82 (98)	58 (26)	39 (16)

Notes: Cells cultured for 5 days in the absence (control) or presence of ATRA (10 μM), Pr_2TS or Me_2TS (5 μM) were assayed for expression of surface CD13, CD15 and CD44. Values are given as % of CD positive cells and CD relative density per cell (in brackets), up-regulation of CD15 (Lanotte et al 1991; Trayner et al 1998; Barber et al 2008); down-regulation of CD13 and CD44 (Trayner et al 1998; Barber et al 2008).

confirmed that diminished MMP-9 gelatinolytic activity resulted from the decrease in MMP-9 protein (Figure 6B). PCR analysis was used to examine the steady-state MMP-9 mRNA alteration before and after Pr_2TS or Me_2TS treatment. As shown in Figure 6C, Pr_2TS or Me_2TS did not alter the apparent levels of MMP-9 transcripts in PMA-activated cells treated for 24 h. In parallel, the amounts of TNF-α protein released from PMA-activated cells were also strongly diminished following Pr_2TS or Me_2TS treatment (Figure 7A) while the mRNA contents of TNF-α were slightly affected by Pr_2TS or Me_2TS at 24 h (Figure 7B), TNF-α mRNA/β2 mRNA ratios being decreased by 25% for Pr_2TS and 49% for Me_2TS. In both cell types, vascular endothelial growth factor (VEGF) protein and mRNA levels remained almost unchanged by Pr_2TS and Me_2TS (Figure 7A and B).

Discussion

Our present study provides new information about the influence of thiosulfinates (TS) in human malignant myeloid cell lines which are representative of acute myeloid leukemia cells accordingly to the French-American-British (FAB) classification. These are HL-60 (FAB M2), NB4 (FAB M3), U937 and MonoMac-6 (FAB M5) cell lines.

We first describe the ability of Pr_2TS and Me_2TS to inhibit in a concentration- and time-dependent manner the growth of leukemic cells *in vitro*. The comparison with their sulfide analogues (DPDS and DMDS) which do not show any influence on cell proliferation in the same range of concentration (up to 100 μM), suggests that the sulfoxide group plays a critical role in TS activity. In contrast, All_2TS as well as its sulfide analogues DAS and DADS did not affect cell growth, and such lack of effect of All_2TS was associated to its decomposition to sulfides as previously reported (Amagase 2006). In contrast to our study, allyl sulfides (from 10 to 100 μM) have been reported to reduce in culture and *in vivo* the growth rate of human cancer cell lines derived from solid tumors (Sundaram et al 1996b; Hosono et al 2005; Xiao et al 2005, 2006; Milner 2006), possibly by regulating factors involved in cellular proliferation such as NF-κB (Pinto et al 2001) or p21 (Milner 2006). Further research is required to clarify the mechanisms of inhibition of AML cell proliferation by Pr_2TS and Me_2TS. The mitogen-activated protein kinase (MAPK) pathway that proceeds from Ras, and its downstream effector Raf to MAPK kinase (MEK) and extracellular signal activated kinase (ERK), links various extracellular stimuli to proliferation, differentiation and survival. The MAPK signaling is constitutively active in blast cells of the majority of AML patients, and its blockade by selective small molecules inhibitors impairs leukemic cell proliferation (Milella et al 2001; Platanias 2003). It has also to be pointed out that the level of NF-κB is high in AML (Baumgartner et al 2002). Whether Pr_2TS and Me_2TS block NF-κB or MAPK activities is currently under investigation.

Organosulfur compounds may exhibit proapoptotic actions on various models of human cancers (Sundaram et al 1996a; Kwon et al 2002; Xiao et al 2003, 2005; Herman-Antosiewicz et al 2004; Hosono et al 2005). However, our experiments clearly indicate that Pr_2TS and Me_2TS do not induce cell apoptosis. Instead, the block in AML cell growth by Pr_2TS and Me_2TS is accompanied by differentiation of cells toward the monocyte/macrophage pathway as assessed by phenotypic and morphologic features. TNF-α is an autocrine stimulus for supporting macrophage differentiation (De Benedetti et al 1990; Kamijo et al 1990). By ELISA analysis, we showed that unstimulated cells secreted very low levels of TNF-α (2 ± 0.17 pg/mL/10^5cells) which were not altered with Pr_2TS (1.81 ± 0.17 pg/mL/10^5 cells)

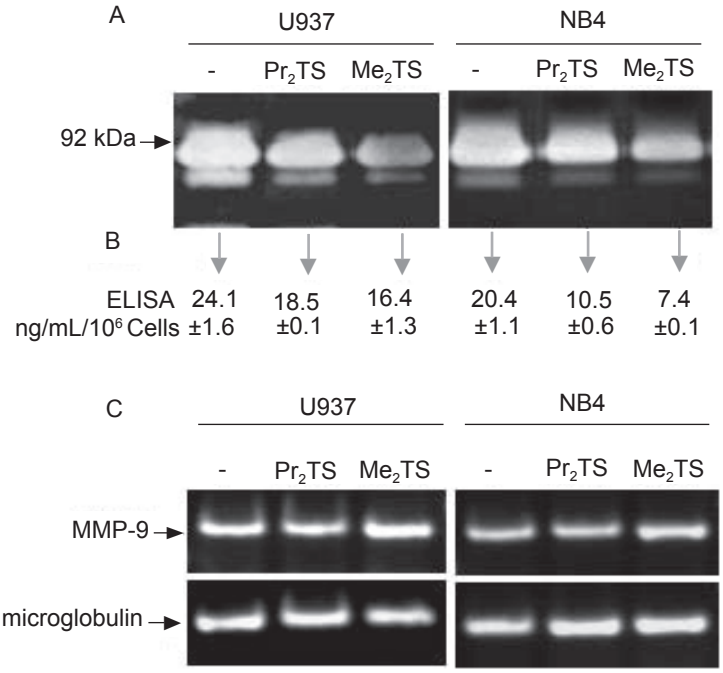

Figure 6 Effects of Pr_2TS and Me_2TS on MMP-9 expression in PMA-activated U937 and NB4 cells. U937 and NB4 cells (10^5/mL) activated with 1 ng/mL PMA, were cultured for 24 or 72 h in the absence or presence of Pr_2TS and Me_2TS (5 μM). (**A**) Analysis of gelatinolytic activity in the culture media of 72 h-treated cells. The enzymatic activity of MMP-9 was analyzed using zymography performed with equal amounts of protein loaded. Gelatinolytic activities are detected as clear bands in the gel. (**B**) MMP-9 concentrations in the culture media of 72 h-treated cells were quantified by ELISA. Values are expressed as ng/mL/10^5 cells (mean of 2 determinations ± SD). (**C**) RT-PCR analysis of MMP-9 and β2-microglobulin transcripts in 24 h-treated cells.

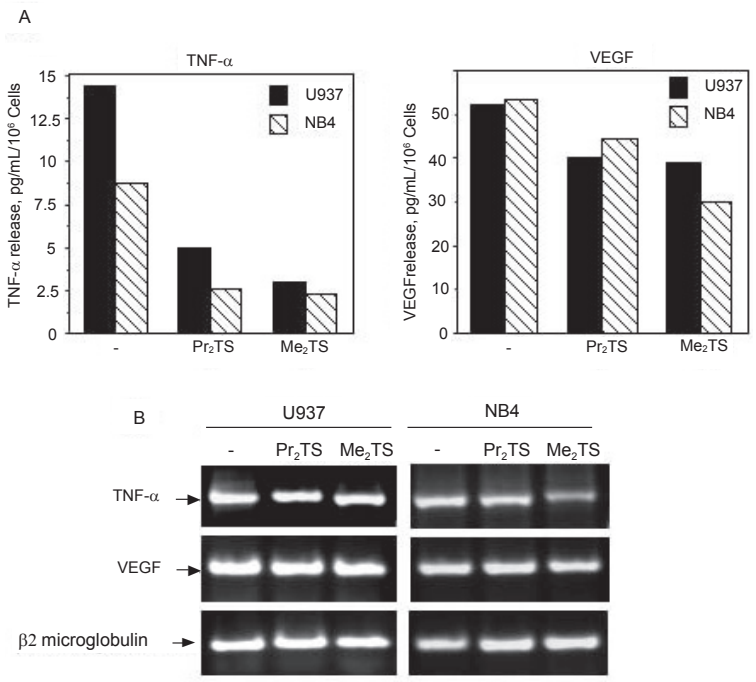

Figure 7 Effects of Pr_2TS and Me_2TS on VEGF and TNF-α expression in PMA-activated U937 and NB4 cells. U937 and NB4 cells (10^5/mL) activated with 1 ng/mL PMA, were cultured for 24 or 72 h in the absence or presence of Pr_2TS and Me_2TS (5 μM). (**A**) ELISA analyses of VEGF and TNF-α concentrations in culture media of 72 h-treated cells. Values are expressed as pg/mL/10^5 cells. (**B**) RT-PCR analysis of VEGF, TNF-α and β2-microglobulin transcripts in 24 h-treated cells.

or Me_2TS (1.94 ± 0.19 pg/mL/10^5 cells) thus indicating that endogenous TNF-α is not implicated in Pr_2TS-/Me_2TS-mediated cell maturation. The molecular mechanisms involved in regulating the balance between proliferation and differentiation in the monocytic/macrophage lineage remain poorly understood. The retinoblastoma gene product (Bergh et al 1999), interferon regulatory factors (Manzella et al 1999; Lu et al 2001) and the Ets repressor PE-1/METS (Sawka-Verhelle et al 2004) have been reported to play a critical role in the monocytic commitment. Whether Pr_2TS and Me_2TS could influence the activity of these factors remains to be determined. Differentiation therapy with retinoic acid plays an important role in treating acute promyelocytic leukemia (Jurcic et al 2007). Our data suggests that the addition of specific agents such as Pr_2TS or Me_2TS to differentiation therapy could be explored.

AML is an hematological disorder which is associated with an increased angiogenesis which disappears if complete hematological remission is achieved (Rajkumar et al 2002; Ribatti et al 2004; Longo et al 2007). Because of its capacities to degrade components of the extracellular matrix and angiogenic factors, MMP-9 can play a role in cancer progression by affecting tumor angiogenesis, tumor growth and/or metastasis (Opdenakker et al 2003; Parks et al 2004; Deryugina et al 2006). MMP-9 is synthesized as a preproenzyme, secreted as a proform (92 kDa) and processed in a 82 kDa form resulting from the proteolytic removal of a fragment of its N-terminal domain (Opdenakker et al 2003). AML leukemic cells express and release 92 kDa MMP-9 (Klein et al 2004). We show here that Pr_2TS and Me_2TS decrease MMP-9 production by activated U937 and NB4 cells. At the mRNA level, no early regulation was detectable indicating a post-transcriptional event. In connection with organosulfurs, Meyer and colleagues (2004) showed that DADS inhibit the secretion of MMP-9 by HUVEC cells while not affecting MMP-9 mRNA levels. Besides MMP-9, VEGF and TNF-α are important partners of angiogenesis (Kini et al 2001; Thomas et al 2001; Ribatti et al 2004). In addition to its angiogenic properties, VEGF may serve as an autocrine/paracrine growth factor for leukemia cells (Fiedler et al 1997; Rajkumar et al 2002). TNF-α stimulates various NF-κB target genes that modulate cell survival and proliferation, tumor metastasis and angiogenesis (Braun et al 2006; Cilloni et al 2007). Among NF-κB target genes are MMP-9 and Bcl-2 whose proteins levels are enhanced in AML (Mayo et al 2000; Braun et al 2006). A previous study showed that DADS inhibited the production of IL-1β and TNF-α by blood monocytes stimulated with lipopolysaccharide (Keiss et al 2003). Our results indicate that Pr_2TS and Me_2TS inhibit TNF-α production by U937 and NB4 cells at the post-transcriptional level, without affecting VEGF expression. Whether Pr_2TS and Me_2TS inhibit the synthesis or the release of MMP-9 and TNF-α remains to be determined.

In conclusion, this work provides first evidence that Pr_2TS and Me_2TS may exhibit antitumor activity against AML cell lines by affecting cell growth, differentiation and secretion of factors involved in tumoral processes. Our data suggest that these TS are promising compounds for improving AML therapy. A very recent study has demonstrated selective apoptosis of childhood pre-B acute lymphoblastic leukemia cells in vitro by DATS and ajoene (Hodge et al 2008) emphasizing the potential of Allium for possible treatment of human leukemias. Further studies are required to validate our observations with these TS in AML patients' cells.

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