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Ajoene, a garlic compound, inhibits protein prenylation and arterial smooth muscle cell proliferation

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1 Ajoene is a garlic compound with anti-platelet properties and, in addition, was shown to inhibit cholesterol biosynthesis by affecting 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase and late enzymatic steps of the mevalonate (MVA) pathway.

2 MVA constitutes the precursor not only of cholesterol, but also of a number of non-sterol isoprenoids, such as farnesyl and geranylgeranyl groups. Covalent attachment of these MVA-derived isoprenoid groups (prenylation) is a required function of several proteins that regulate cell proliferation. We investigated the effect of ajoene on rat aortic smooth muscle cell proliferation as related to protein prenylation.

3 Cell counting, DNA synthesis, and cell cycle analysis showed that ajoene (1–50 μM) interfered with the progression of the G1 phase of the cell cycle, and inhibited rat SMC proliferation.

4 Similar to the HMG-CoA reductase inhibitor simvastatin, ajoene inhibited cholesterol biosynthesis. However, in contrast to simvastatin, the antiproliferative effect of ajoene was not prevented by the addition of MVA, farnesol (FOH), and geranylgeraniol (GGOH). Labelling of smooth muscle cell cellular proteins with [3H]-FOH and [3H]-GGOH was significantly inhibited by ajoene.

5 *In vitro* assays for protein farnesyltransferase (PFTase) and protein geranylgeranyltransferase type I (PGGTase-I) confirmed that ajoene inhibits protein prenylation. High performance liquid chromatography (HPLC) and mass spectrometry analyses also demonstrated that ajoene causes a covalent modification of the cysteine SH group of a peptide substrate for protein PGGTase-I.

6 Altogether, our results provide evidence that ajoene interferes with the protein prenylation reaction, an effect that may contribute to its inhibition of SMC proliferation.
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Abbreviations: DTT, dithiothreitol; FCS, foetal calf serum; FOH, farnesol; FPP, farnesyl pyrophosphate; GGOH, geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance liquid chromatography; MEM, minimum essential medium; MVA, mevalonate; PBS, phosphate buffered saline; PFTase, protein farnesyltransferase; PGGTase-I, protein geranylgeranyltransferase type I; SMC, smooth muscle cell

Introduction

Garlic (*Allium sativum*) has been advocated as a remedy for the treatment and prevention of a number of diseases, including atherosclerosis and cancer. However, several clinical studies conducted during the last three decades have shown contradictory lipid-lowering activity and beneficial effects on cancer incidence (Agarwal, 1996; Buiatti *et al*., 1989; You *et al*., 1989). Therapeutic actions of garlic, although still discussed, have been attributed primarily to its organosulphuric compounds, but more detailed studies on chemically defined garlic components are needed to better clarify its pharmacological properties (Agarwal, 1996). *In vitro* and *in vivo* studies have been conducted to evaluate the antiproliferative activity of garlic and its constituents. For instance, garlic extracts prepared from 1% alcoholic extraction of garlic powder, containing a mixture of organosulphur compounds, was found to inhibit [3H]-thymidine incorporation in cultured trophoblasts and endothelial cells (Sooranna *et al*., 1997). Further, development of spontaneous mammary tumours in C3H mice has been shown to be inhibited by feeding fresh garlic which contains active allinase (Kroning. 1964). Allinase acts on alliin to produce allicin, the precursor for several organosulphuric compounds including ajoene. Therefore, allicin and/or its metabolites, rather than alliin may exert the chemopreventive action of garlic (Kroning. 1964).

Ajoene (Figure 1) is a well-established antiplatelet agent, and its inhibitory effect on platelet aggregation has been extensively studied and documented both by *in vivo* and *in vitro* experiments (Apitz-Castro *et al*., 1986a, b; 1988; 1991; 1994). Further, ajoene is a potent modulator of membrane-dependent functions of immune cells, that are under control of the signal-transduction system (Romano *et al*., 1997). Beyond this action, recent evidence showed that ajoene inhibits proliferation and induces apoptosis of several cancer

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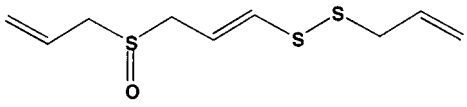

Figure 1 Chemical structure of trans-ajoene [(E)-4,5,9-trithiadodeca-1,6,11-triene-9-oxide].

cell lines, which ultimately leads to the reduction of tumour growth *in vivo* (Li *et al*., 2002). Allicin and ajoene were also reported to significantly decrease cholesterol biosynthesis in rat hepatocytes and HepG2 cells by inhibiting HMG-CoA reductase and late steps of the MVA pathway which leads to the accumulation of the precursor lanosterol (Dirsch *et al*., 1998; Gebhardt *et al*., 1994; Urbina *et al*., 1993). Mevalonate (MVA) is the precursor not only for cholesterol but also for a number of non-sterol isoprenoids, such as the farnesyl and geranylgeranyl groups, which become covalently attached to proteins that are essential for cell proliferation (Glomset *et al*., 1990; Goldstein & Brown, 1990; Grunler *et al*., 1994). Indeed, protein prenylation is required for membrane association and therefore function of several proteins involved in the regulation of cell proliferation, including p21 ras (Grunler *et al*., 1994).

Thus, given the evidence for the requirement of MVA-derived isoprenoids for cell growth, and for the ability of ajoene to interfere with the MVA pathway (Gebhardt *et al*., 1994), our study was undertaken to evaluate the effect of ajoene on SMCs proliferation as related to protein prenylation. Our results show that ajoene inhibits SMCs proliferation by interfering with the progression of the G1 phase of the cell cycle. In addition, at similar concentrations, we observed an inhibition of cholesterol biosynthesis and a decrease in the incorporation of [3H]-FOH and [3H]-GGOH into cellular proteins. *In vitro* assays for PFTase and PGGTase-I activities confirmed the ability of ajoene to inhibit protein prenylation. By reverse phase high performance liquid chromatography (HPLC) and mass spectrometry analyses, we determined the mechanism of inhibition of protein prenylation.

Methods

Materials

Eagle's minimum essential medium (MEM), trypsin ethylendiaminetetraacetate, penicillin (10,000 U ml^{-1}), streptomycin (10 mg ml^{-1}), tricine buffer (1 M, pH 7.4) and non-essential amino acid solution (100 ×) were purchased from Gibco, and FCS was from Mascia Brunelli. Disposable culture flasks and petri dishes were from Corning Glassworks, and filters were from Millipore. [2-^{14}C]-Acetate, sodium salt (58.9 mCi $mmol^{-1}$), [6-^{3}H]-thymidine, sodium salt (2 Ci $mmol^{-1}$) and molecular weight protein standards were from Amersham. Isoton II was purchased from Instrumentation Laboratories. All-*trans* FOH, was purchased from Sigma, and all-*trans* geranyl-geraniol (GGOH) was kindly provided by Prodotti Roche. SDS, TEMED, ammonium persulphate, glycine, acrylamide solution (30% T, 2.6% C) were obtained from Bio-Rad Laboratories. All-*trans* FOH [1-^{3}H] (15–20 Ci $mmol^{-1}$), all-*trans* GGOH [1–^{3}H] [50–60 Ci $mmol^{-1}$), all-*trans* [^{3}H]-FPP (20 Ci $mmol^{-1}$) and all-*trans* [^{3}H]-GGPP (20 Ci $mmol^{-1}$) were from American Radiolabeled Chemicals. Avidin-agarose was from Pierce. Cytox Dye was purchased from Molecular Probes. Ajoene (Figure 1), was obtained as previously described (Apitz-Castro *et al*., 1983), and dissolved in ethanol; control cells received the same volume of the solvent (0.5% final concentration). Simvastatin in its lactone form (Merck, Sharp & Dohme Research Laboratories) was dissolved in 0.1 M NaOH to give the active form, and the pH was adjusted to 7.4 by adding 0.1 M HCl. The solution was sterilized by filtration.

Cell proliferation and DNA synthesis

Smooth muscle cells (SMC) were cultured from the intimal-medial layers of aorta of male Sprague–Dawley rats as previously described (Corsini *et al*., 1995).

Cell proliferation was evaluated by cell counting with a Coulter Counter model ZM (Coulter Instruments) after trypsinization of the monolayers (Corsini *et al*., 1993), and DNA synthesis was estimated by nuclear incorporation of [^{3}H]-thymidine (Corsini *et al*., 1995).

Cell cycle analyses

Flow cytometry was utilized to analyse cell cycle distribution. Cells were trypsinized and centrifuged for 5 min at 1000 r.p.m. Pellets were resuspended in 0.5 ml of permeabilizing buffer (0.5 μM in 100 mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ 0.1% NP-40) containing Cytox Dye (final concentration 0.5 μM). Samples were placed in the dark for 30 min and the green fluorescence of individual nuclei was measured. Nuclear Cytox Dye fluorescence signal was recorded on the FL1 channel of a FACS scan flow cytometer (Becton Dickinson) and analysed with CellQuest software. The number of cells in G_0/G_1, S and G_2/M phases was expressed as percentages of total events (10,000 cells).

Synthesis of total sterols

The synthesis of cholesterol was determined by measuring the incorporation of radioactive acetate into cellular sterols (Corsini *et al*., 1995).

Labelling of proteins with [^{3}H]-FOH or [^{3}H]-GGOH and SDS–PAGE analyses

The prenylated proteins were analysed after incubation of the cell monolayers with [^{3}H]-FOH (10 μM) or [^{3}H]-GGOH (2.5 μM) for 5 h (see: 'Experimental protocols') (Corsini *et al*., 1999; Ferri *et al*., 2001). After this time, cell monolayers were scraped into 1.5 ml of phosphate buffered saline (PBS) containing 1 mM phenylmethylsulphonylfluoride, and the resuspended cells collected and centrifuged (14,000 r.p.m. × 3 min.). Cells pellets were delipidated with cold acetone and two extractions with chloroform/methanol (2 : 1). The delipidated proteins were solubilized in 3% SDS, 62.5 mM Tris-HCl, pH 6.8, and an aliquot (40 μg protein) was analysed by 12.5% SDS–PAGE (Corsini *et al*., 1999; Ferri *et al*., 2001). The gel was treated with Amplify (Amersham) and exposed to Kodak X-Omat-AR film at −70°C for 2–4 weeks. Fluorographic signals were analysed by densitometric scanning.

Assays for inhibition of PFT and PGGT-I by ajoene

Recombinant rat PFTase and PGGTase-I were expressed in the Sf9/baculovirus system and purified as described (Yokoyama *et al*., 1997). The standard reaction mixture for PFTase assay contains 0.75 μM (0.3 μCi) [^{3}H]-FPP and 5 μM RAS-CVIM in a total volume of 20 μl containing 30 mM potassium phosphate, 0.5 mM $MgCl_2$, 20 μM $ZnCl_2$, pH 7.7. PGGTase-I assay was carried out using 0.75 μM (0.3 μCi) [^{3}H]-GGPP and 5 μM H-Ras-CVLL as substrates. Stock solutions of ajoene were prepared in absolute ethanol, and a 1 μl aliquot was added to the reaction mixture (final concentration of ethanol is 5%). Reactions were initiated by adding rat PFTase or PGGTase-I (20 ng). After incubation at 30°C for 20 min, the reaction was terminated by adding 200 μl of 10% HCl in ethanol. The amount of the prenylated protein product was quantified by the glass fibre-filter method as described previously (Pompliano *et al*., 1992). In some cases, 50 μM biotinylated peptide substrates, biotin-lamine B peptide (biotin-GTPRASNRSCAIS) and biotin-γ6 peptide (biotin-NPFREKKFFCAIL) were used as substrates of PFT and PGGT-I, respectively. The amount of radioactivity transferred to the peptides was determined by the method using avidin-agarose as described (Yokoyama *et al*., 1991).

Analyses of products from incubation of ajoene and biotin-γ6 peptide by reverse-phase HPLC and mass spectrometry

Ajoene (50 μM) and biotin-γ6 peptide (25 μM) were incubated at 30°C for 30 min in a total volume of 200 μl containing 30 mM potassium phosphate, pH 7.7, 1 mM $MgCl_2$, 20 μM $ZnCl_2$. The sample was subjected to HPLC on a C18 reverse-phase column (Vydac 218TP52, 4.6 × 250 mm). The column was developed at a flow rate of 1 ml min with a solvent gradient using 0.06% trifluoroacetic acid in water (solvent A) and 0.054% trifluoroacetic acid in acetonittrile (solvent B): 20–80% B over 60 min. The absorbance at 218 nm was monitored.

For mass spectrometry analyses, a mixture of ajoene (500 μM) and biotin-γ6 peptide (25 μM) was incubated at 30°C for 30 min in a 200 μl mixture without buffer or salt, and an aliquot was infused into the electrospray mass spectrometer (Bruker/Hewlett-Packard Esguire).

Statistical analyses

Experimental data are expressed as mean ± s.d. The effects of the tested compounds *versus* control on the different parameters were analysed by a one-way ANOVA followed by Bonferroni test. The concentration of compounds required to inhibit 50% of cell proliferation, [^{3}H]-thymidine incorporation and cholesterol biosynthesis (IC_{50}) was calculated by linear regression analyses of the logarithm of the concentration (in micromoles per litre) *versus* logit.

Results

Effect of ajoene on arterial SMC proliferation and cell cycle progression

The antiproliferative action of ajoene was studied in rat aortic SMCs at concentrations ranging from 1 to 50 μM. In the first set of experiments, cell proliferation was measured after 3 days of growth in the presence of increasing concentrations of ajoene. Ajoene decreased SMC proliferation in a concentration-dependent manner with an IC_{50}-value of 5.7 μM (Figure 2A). In addition, ajoene inhibited [^{3}H]-thymidine incorporation in a concentration dependent manner (IC_{50} = 25.2 μM)(Figure 2B). Similar potency of the antiproliferative effect of ajoene was observed on three different strains of rat SMC (data not shown).

To further investigate the antiproliferative effect of ajoene, we performed cell cycle analyses by flowcytometry. Incubation of SMCs for 120 h with culture media containing 0.4% FCS led to the accumulation of the cells in the G1 phase (95.8%) with only a small percentage in the S phase (0.62%) (Figure 3). As expected, 20 h after stimulation with 10% FCS, the cells in S phase were increased to 5.8%, and those in G1 phase were decreased to 83.9% (Figure 3). Incubation of SMCs with ajoene led to the accumulation of cells in the G1 phase in a concentration dependent manner, with a complete inhibition at 25 μM concentration (Figure 3). Importantly, we did not detect any significant increase in the percentage of sub G0/G1 cells even after incubation with 25 μM ajoene, which indicates a specific interference with the progression of the G1 phase of the cell cycle without induction of apoptosis (Figure 3).

Effect of ajoene on the mevalonate pathway

It has previously been shown that ajoene inhibits cholesterol biosynthesis by interfering with different steps of the MVA pathway (Gebhardt *et al*., 1994). To study whether ajoene inhibits SMCs proliferation by affecting one of the steps of the MVA pathway, we evaluated the effect of this compound versus the HMG-CoA reductase inhibitor simvastatin. We

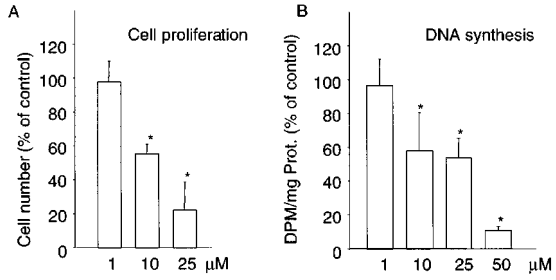

Figure 2 Concentration-dependent effect of ajoene on proliferation of rat aortic SMCs. (A) Cells were seeded (200×10^3/dish) and incubated with Eagle's MEM supplemented with 10% FCS; 24 h later the medium was changed with one containing 0.4% FCS to stop cell growth and the cultures were incubated for 48 h. At this time, the medium was replaced with one containing 10% FCS and the reported concentrations of ajoene and the incubation was continued for further 72 h at 37°C. Each bar represents the mean ± s.d.mean of three independent experiments. Inhibitor *versus* control: $*P < 0.001$ (Bonferroni test). (B) Cells were seeded at a density of 300×10^3/dish^{-1} and incubated with Eagle's MEM supplemented with 10% FCS; 24 h later, the medium was changed with one containing 0.4% FCS to stop cell growth and the cultures were incubated for 120 h. At this time, the medium was replaced with one containing 10% FCS and the indicated concentrations of ajoene. After 20 h, at 37°C, labelled thymidine was added to the medium and the incubation continued for further 2 h. Each bar represents the mean ± s.d.mean of three independent experiments. Inhibitor *versus* control: $*P < 0.001$ (Bonferroni test).

first investigated their effects on cholesterol biosynthesis and SMCs proliferation. As shown in Figure 4, ajoene strongly inhibited cholesterol biosynthesis with an IC_{50} of 1.5 μM, and at similar concentrations, it also inhibited SMCs proliferation. Under the same experimental conditions, at least 80% inhibition of cholesterol biosynthesis was necessary to induce a decrease in SMC proliferation by simvastatin (Figure 4) (Corsini *et al*., 1993). These results suggest a different antiproliferative's mechanism by the two compounds.

We have previously shown that MVA, FOH, and GGOH, counteract the antiproliferative effect on SMCs elicited by HMG-CoA reductase inhibitors (statins) but not by the protein prenylation inhibitor perillic acid (Corsini *et al*., 1995; Ferri *et al*., 2001; Raiteri *et al*., 1997). As shown in Figure 5, the antiproliferative effect of ajoene was not prevented by the addition of MVA, FOH and GGOH, suggesting that its action was directly related to the inhibition of protein prenylation.

To study whether ajoene interferes with protein prenylation, the incorporation of [^{3}H]-FOH and [^{3}H]-GGOH into cellular proteins was examined under the same experimental conditions utilized for evaluating DNA synthesis (Ferri *et al*., 2001). As shown in Figure 6, [^{3}H]-FOH and [^{3}H]-GGOH were readily incorporated into specific proteins with molecular weights ranging from 21 to 72 kDa, as reported in previous studies (Corsini *et al*., 1999; Danesi *et al*., 1995;

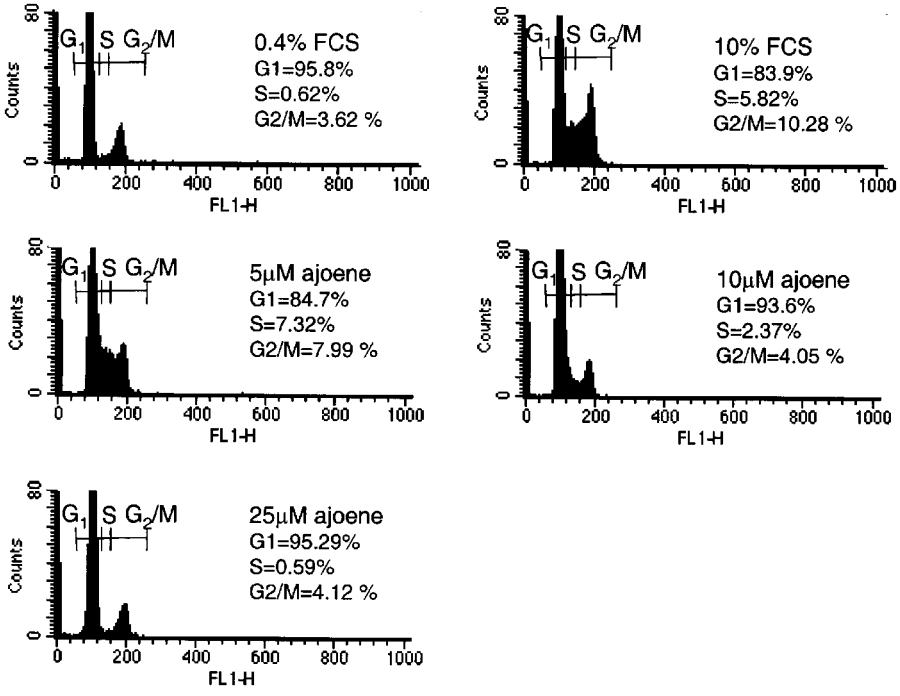

Figure 3 Effect of ajoene on the cell cycle of rat aortic SMCs. Experimental conditions are as in Figure 1B. Flow cytometry analysis of the cell cycle was performed in the presence of 0.4%, and 10% FCS alone, or in the presence of the indicated concentrations of ajoene as described in 'Methods'. FL-1: green fluorescence. The data shown are representative of two independent experiments.

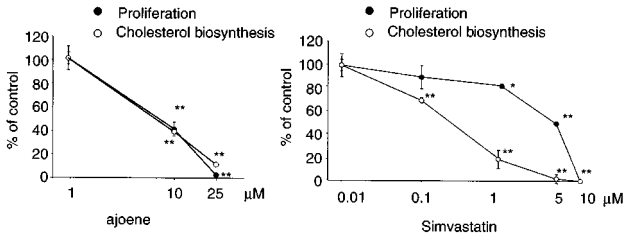

Figure 4 Effect of ajoene and simvastatin on cholesterol biosynthesis and rat aortic SMCs proliferation. Experimental conditions are as in Figure 1A [^{14}C]-Acetate incorporation was used to assay cholesterol biosynthesis, 72 h after its addition to the cells. Each point represents the mean $\pm$s.d. of triplicate dishes. The mean value for control experiments (without inhibitor) for cell proliferation was 1462×10^3 ($\pm 91 \times 10^3$) cells/dish and 19.35 ($\pm$2.37) pmol mg$^{-1} \times$h for cholesterol biosynthesis, respectively. Inhibitor *versus* control: $^*P<0.05$; $^{**}P<0.001$ (Bonferroni test). The data shown are representative of two independent experiments.

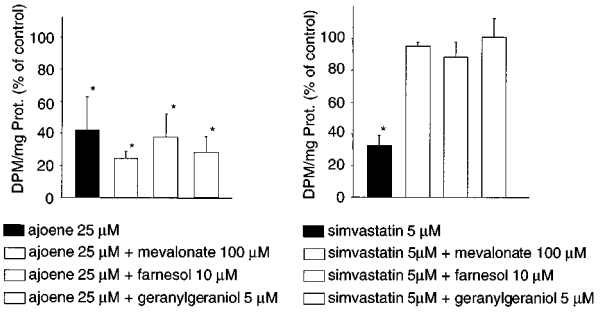

Figure 5 Effect of mevalonate and its derivatives on proliferation of rat aortic SMCs inhibited with ajoene or simvastatin. Experimental conditions are as in Figure 1B. Each bar represents the mean$\pm$ s.d.mean of three independent experiments. Bonferroni test: $^*P<0.001$, ajoene + farnesol, ajoene + geranylgeraniol, ajoene + mevalonate *versus* control (without compounds), and simvastatin *versus* control (without compounds).

Ferri *et al*., 2001). Ajoene significantly inhibited [^{3}H]-GGOH incorporation into specific low molecular weight proteins in a concentration-dependent manner (Figure 6). Although less effective, we also detected a dose dependent inhibitory effect of ajoene on [^{3}H]-FOH incorporation (Figure 6). These data suggest that ajoene might exert its antiproliferative effect by interfering with protein prenylation process.

Inhibition of PFTase and PGGTas-I activity by ajoene in vitro

To further investigate the effect of ajoene on protein prenylation, we measured the activity of PFTase and PGGTase-I using Ras-CVIM and H-Ras-CVLL as substrates, respectively, in the absence of DTT. Ajoene inhibited PFTase and PGGTase I reactions in a dose-dependent manner with IC_{50} values of 13.0 and 6.9 μM, respectively (Figure 7A). In the presence of 100 μM ajoene, which causes nearly complete inhibition of both reactions, increasing concentrations of DTT prevented the effect of ajoene, and led to even higher activity than that observed in the absence of both ajoene and DTT (Figure 7B). Similar inhibitory effects of ajoene were also observed when PFTase and PGGTase-I assays were carried out with peptide substrates biotin-lamine B peptide (biotin-GTPRASNRSCAIS) and biotin-γ6 peptide (biotin-NPFREKKFFCAIL) (data not shown).

Ajoene contains a disulphide bond that is reductively cleavable by DTT (Figure 1). Thus, our results suggest that the disulfide linkage in ajoene is an indispensable element for the ability of the compound to inhibit PFTase and PGGTase-I reactions.

HPLC and mass spectrometry analyses of the product(s) from incubation of ajoene and biotin- γ6 peptide

We next studied whether ajoene inhibits the prenylation reaction by modification of the cysteine SH group of the PGGTase-I substrate (biotin-NPFREKKFFCAIL). When a mixture of ajoene and biotin-γ6 peptide was incubated and subjected to reverse-phase HPLC analyses, we observed the formation of a new compound (retention time, 22.3 min) that separated from ajoene (20.1 min) and the peptide (19.1 min) (Figure 8A,B,C). This compound was completely removed from the mixture by treatment with avidin-agarose prior to HPLC analyses (Figure 8D) and was ninhydrin-test positive, indicating that the compound is a biotinylated peptide with modification by ajoene.

Biotin-γ6 peptide contains two lysine and one arginine residues. Electron spray mass spectrometry of this peptide (molecular mass = 1952.4) gave a major ion signal of 651.6 that corresponds to a triply charged ion ([M + 3H]/3z) of the peptide. The mass spectrum of a mixture of biotin-γ6 and ajoene showed major ion signals of 675.7, 651.6, and 781.5

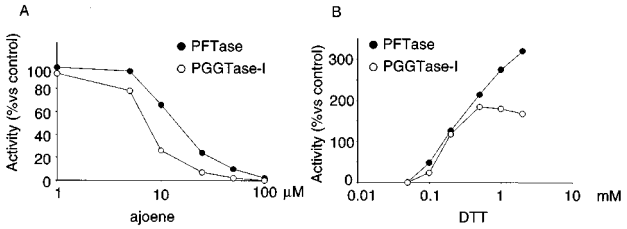

Figure 7 Inhibition of PFTase and PGGTase-I reactions by ajoene and reverse of the inhibition by DTT. (A) Rat PFTase or PGGTase-I (20 ng protein) was incubated at 30°C for 20 min with 0.75 μM (0.3 μCi) [^{3}H]-FPP/5 μM RAS-CVIM or 0.75 μM (0.3 μCi) [^{3}H]-GGPP/5 μM H-Ras-CVLL, respectively, under the standard conditions. Ajoene was tested at the indicated concentrations for inhibition of the PFTase and PGGTase-I reactions. Control levels with no ajoene (100% activity) were 11,590 c.p.m. and 3300 c.p.m., respectively, for PFTase and PGGTase-I assays. No enzyme controls (0% activity) were 120 c.p.m. and 1500 c.p.m., respectively, for PFTase and PGGTase-I assays. (B) Rat PFTase or PGGTase-I was incubated as in A in the presence of 100 μM ajoene and the indicated concentration of DTT. Activity is expressed as relative per cent to controls with no ajoene or DTT (100% activity for PFTase assay is 8710 c.p.m. and that for PGGTase-I assay is 3320 c.p.m.).

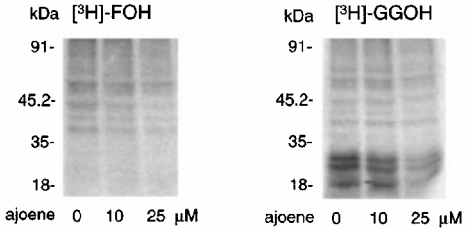

Figure 6 Concentration-dependent effect of ajoene on [^{3}H]-FOH and [^{3}H]-GGOH incorporation into proteins of rat aortic SMCs. Experimental conditions are as in Figure 1B. Quiescent cells were incubated for 20 h in a fresh medium containing 10% FCS in the presence or absence of ajoene. [^{3}H]-FOH (10 μM) or [^{3}H]-GGOH (5 μM) were added during the last 5 h of incubation. Cell pellets were delipidated and equal amounts of cell extracts (40 μg cell protein/lane) were separated by 12.5% SDS–PAGE and fluorographed. The data shown are representative of two independent experiments.

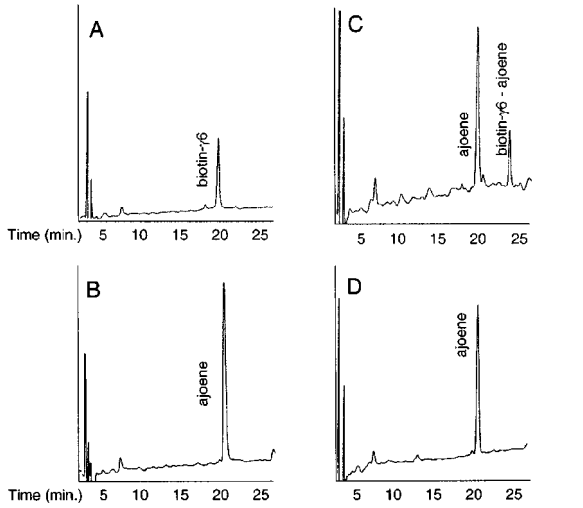

Figure 8 C18 HPLC analyses of the product from ajoene and biotin γ6 peptide. Ajoene alone (panel A), biotin-γ6 peptide (biotin-NPFREKKFFCAIL) alone (panel B), or a mixture of ajoene (50 μM) and biotin γ6 peptide (25 μM) (panel C), was incubated at 30°C for 30 min and subjected to C18 reverse-phase HPLC as described in 'Methods'. An identical mixture as panel C was also chromatographed after removal of biotinylated compounds with 100 μl of avidin-agarose gel (panel D). The elution was carried out by a gradient of acetonitrile concentrations from 20 to 80% over 60 min.

(Figure 9A). The 651.6 signal corresponds to the triply charged peptide, and the 781.5 signal corresponds to an ion of dimerized peptide ([2M + 5H]/5z) linked *via* a disulphide bridge since this signal disappeared when incubated with DTT (Figure 9B). The 675.7 signal is a triply charged ion of a compound with molecular mass of 2041.1, which is 71.7 mass unit larger than that of biotin-γ6 peptide and corresponds to the peptide covalently modified with $CH_2=CHCH_2$-S-, a portion of ajoene. The 675.7 signal was greatly reduced by further incubation with 50 mM DTT (Figure 9B), showing that this compound is the peptide attached *via* a disulphide to $CH_2=CHCH_2$-S-. The minor mass signal of 705 formed in the mixture (Figure 9A) may be a triply charged ion of a compound with molecular mass of 2112 that is 159.6 unit larger than the peptide, suggesting that the peptide may also be modified with the larger portion of ajoene $CH_2=CHCH_2S(O)CH_2CH=CH$-S-, but with about 4 fold less efficiency compared to modification with $CH_2=CHCH_2$-S-. These results suggest that the cysteine SH group of the peptide is modified with the fragments (preferentially with the smaller one $CH_2=CHCH_2$-S-) of ajoene by disulphide bond. Preferential labelling of the peptide with the 3-carbon portion of ajoene is expected, as the vinyl thiolate fragment is a better leaving group than is the allylic thiolate. This blockage of the SH group(s) of protein prenyltransferase substrates may be the mechanism by which ajoene inhibits prenyl modification of proteins in SMCs.

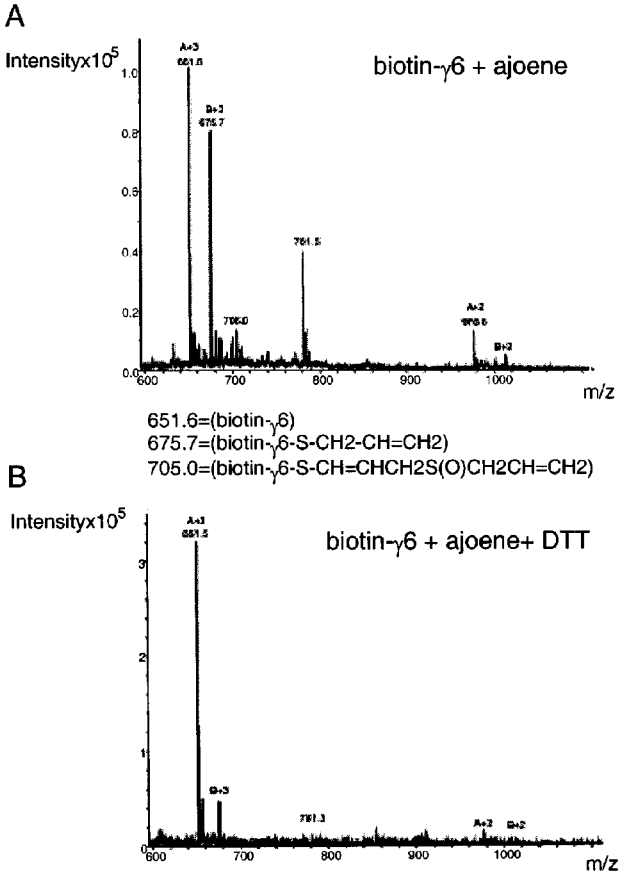

Figure 9 Mass spectrometry analyses of the product from ajoene and biotin γ6 peptide. (Panel A) The mixture of ajoene (50 μM) and biotin γ6 peptide (biotin-NPFREKKFFCAIL) (25 μM) was incubated at 30°C for 30 min and subjected to mass spectrometry analysis as described in 'Methods'. (Panel B) The same mixture as in Panel A was further incubated with 50 mM DTT at 30°C for 30 min.

Discussion and conclusion

In the present study we have evaluated the effect of synthetic ajoene, an organosulphur compound derived from garlic, on SMCs proliferation as related to protein prenylation. Incubation of SMCs with ajoene showed a dose dependent inhibition of cell proliferation and thymidine incorporation (Figure 2A and B). Cell cycle analysis also demonstrated that ajoene specifically blocks the cell cycle in G1 phase, leading to the accumulation of the cells in the G0/G1 (Figure 3).

The inhibitory effects on the G1 phase observed in cells treated with HMG-CoA reductase inhibitors (Jakobisiak *et al*., 1991), with PFTase inhibitors (Sepp-Lorenzino & Rosen, 1998), with PGGTase I inhibitors (Stark *et al*., 1998), and with a dual PFTase and PGGTases inhibitor (Ferri *et al*., 2001), suggest the involvement of specific isoprenoids metabolites in the regulation of the G1/S phase transition of the cell cycle. The addition of MVA, all-*trans* FOH and all-*trans* GGOH, precursors of prenylated proteins, restored cell proliferation inhibited by HMG-CoA reductase inhibitors (statins) (Figure 5B) (Corsini *et al*., 1993), but not by the protein prenylation inhibitor perillic acid (Ferri *et al*., 2001), further confirming a specific role of isoprenoids in regulating cell proliferation (Raiteri *et al*., 1997). Similar to perillic acid, MVA or its isoprenoids derivatives (FOH or GGOH) did not counteract ajoene-induced blockade of SMC proliferation (Figure 5A). These results suggest that ajoene might inhibit protein prenylation. In fact, analysis of the incorporation of radiolabelled all-*trans* FOH and all-*trans* GGOH into cellular proteins, demonstrated that ajoene reduces the incorporation of both prenols, with a more marked effect on [^{3}H]-GGOH. However, in contrast to perillic acid, ajoene not only affects protein prenylation, but also exerted a strong inhibition of cholesterol biosynthesis. Nevertheless, because ajoene's effect on SMC proliferation was not counteracted by MVA and its isoprenoids derivatives, and in contrast to simvastatin, was observed at concentrations similar to those required for inhibiting cholesterol biosynthesis, ajoene's action is more likely due to a decrease in protein prenylation than a decrease of HMG-CoA reductase activity (Gebhardt *et al*., 1994).

To date, three classes of enzymes, protein farnesyltransferase (PFTase) and protein geranylgeranyltransferases (PGGTase I and II) have been identified in mammals to catalyse the transfer of prenyl groups to the sulphur atom of the cysteine residue located at fourth amino acid from the carboxyl terminus of the proteins (CAAX domain; where the C is a cysteine, the two A residues are aliphatic amino acids, and the X can be methionine, serine, alanine, glutamine or leucine) (Casey & Seabra, 1996). We used an *in vitro* assay to further investigate the effect of ajoene on protein prenylation, and showed a significant inhibition of both PFTase and PGGTase-I activity by ajoene. Interestingly, the concentrations required for inhibiting SMC proliferation, PFTase, and PGGTase-I activities are similar (IC50 = 5.7, 6.9, and 13.0 μM respectively). Furthermore, a significant reduction of the incorporation of [^{3}H]-FOH and [^{3}H]-GGOH into proteins,

was observed in the presence of 10–25 μM ajoene. These data illustrate a correlation between the effect of ajoene on cell proliferation and protein prenylation.

The fact that the inhibitory effects of ajoene on PFTase and PGGTase-I activity were blocked by DTT (Figure 7B) suggests the possibility that ajoene modifies the substrate at the SH group of cysteine. Accordingly, HPLC and mass spectrometry analyses, showed the formation of adducts between the cysteine SH group of the CAAX peptide (biotin-γ6) and fragments of ajoene. These results led us to postulate that ajoene might interfere with protein prenylation by a covalent modification of the cysteine residue of protein substrates without a specific inhibitory effect on PFTase and/or PGGTase-I. The more marked inhibitory effect of ajoene on [^{3}H]-GGOH *versus* [^{3}H]-FOH incorporation into cellular proteins could be due to differences in the intracellular pool sizes of farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) (Corsini *et al*., 1999).

Although this is the first report showing the effect of ajoene on protein prenylation, our study is in agreement with the evidence that ajoene inhibits the activity of protein tyrosine phosphatases in platelets, which is a class of molecules that undergo farnesylation (Cates *et al*., 1996; Villar *et al*., 1997). It is noteworthy to mention that organosulphuric compounds derived from garlic, containing the disulphide moiety, or an active thioallyl group, have been shown to react *in vitro* and *in vivo* with cysteine to form S-allyl mercaptocysteine adducts (Weisberger & Pensky, 1958). It has also been shown that ajoene alters glutathione metabolism, (Scharfenberg *et al*., 1994), and that removal of the sulphide group of ajoene induces a 2 fold decrease in its ability to suppress collagen-induced platelet aggregation (Block *et al*., 1986).

Although the antiproliferative effects of ajoene have received little attention, a recent study shows that Z-ajoene is able to inhibit HL60 cells proliferation and tumour growth *in vivo*. The effect seems to be due to a specific inhibition of micotubules formation mediated by ajoene, and therefore a blockage of the transition of the G2/M phase of the cell cycle (Li *et al*., 2002). Although it is possible that multiple mechanisms of action can contribute to the antiproliferative effect of ajoene, the discrepancy between our results and the above study can potentially be explained by a specific effect of Z-ajoene *versus* a mixture of the isomers utilized in our study, or by the different cell type investigated. It is also important to note that, although ajoene can induce apoptosis in the human promyelcytic leukemia cell line HL-60, we did not observed hypodiploid cells in SMCs (Dirsch *et al*., 1998; Li *et al*., 2002). Different susceptibility of the cells to undergo apoptosis can account for the discrepancy between these observations, since ajoene does not induce apoptosis in peripheral blood mononuclear cells isolated from healthy humans (Dirsch *et al*., 1998).

In conclusion we show that ajoene, a synthetic garlic component, inhibits protein prenylation and SMCs proliferation. We demonstrate that ajoene, by covalent reaction, leads to covalent modification of the cysteine SH group of the CAAX motif of protein prenyl group acceptors, an effect which may contributes to the ability of ajoene to inhibit SMC proliferation.

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