Allicin inhibits cell polarization, migration and division via its direct effect on microtubules

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Allicin (diallyl thiosulfinate) is a major biologically active component of garlic that is known to inhibit cell proliferation and induce apoptosis. The effects of allicin are attributed to its ability to react with thiol groups. However, the mechanism underlying the cytostatic activity of allicin, as well as the identity of the relevant subcellular targets, are not known. In the present study, we found that the effects of allicin on cell polarization, migration, and mitosis are similar to the effects of microtubule-depolymerizing drugs such as nocodazole. Moreover, treatment of cultured fibroblasts with micromolar doses of allicin results in microtubule depolymerization in cells within minutes of its application, without disrupting the actin cytoskeleton or inducing direct cytotoxic effects. Furthermore, allicin blocks the polymerization of pure tubulin in vitro in a concentration-dependent manner, suggesting that it acts directly on tubulin dimers. Sulfhydryl (SH)-reducing reagents such as 2-mercaptoethanol and dithiothreitol abolish the effect of allicin on microtubule polymerization. Thus, allicin is a potent microtubule-disrupting reagent interfering with tubulin polymerization by reaction with tubulin SH groups. Cell Motil. Cytoskeleton 64: 321–337, 2007.

INTRODUCTION

Numerous medicinal properties of garlic are attributed to allicin (diallyl thiosulfinate), its major biologically active component [Cavallito and Bailey, 1944]. Allicin has been shown to exhibit antibacterial and antifungal properties [Cavallito and Bailey, 1944; Ankri and Mirelman, 1999; Shadkchan et al., 2004; Davis, 2005], as well as the ability to decrease the area of fatty streaks and atherosclerotic plaques, and to inhibit platelet aggregation [Mayeux et al., 1988; Abramovitz et al., 1999; Briggs et al., 2000; Gonen et al., 2005]. Moreover, allicin is known for its antiproliferative and proapoptotic effects in many cell types, including a variety of cancer cells [Zheng et al., 1997; Hirsch et al., 2000; Lea et al., 2002; Sun and Wang, 2003; Ha and Yuan, 2004; Oommen et al., 2004; Park et al., 2005].
Allicin is produced from its inactive precursor, alliin (\(\delta\)-allyl-L-cysteine sulfoxide), upon the release of the enzyme alliinase (alliin lyase; EC 4.4.1.4) from its cellular storage compartment, when garlic cloves are crushed [Stoll and Seebeck, 1951; Rabinkov et al., 1994]. Allicin can be used very efficiently as a cytostatic drug, generated from systemically administered precursor alliin and aimed directly at the cell surface, by means of cell-targeted alliinase conjugated with monoclonal antibody to appropriate cell surface receptors [Miron et al., 2003; Arditti et al., 2005]. However, once formed, allicin is a rather unstable, short-lived molecule, which disappears from the circulation within a few minutes after injection [Lawson and Gardner, 2005].

Allicin is a hydrophobic molecule that easily penetrates through biological membranes and reacts rapidly with thiol groups within cells [Rabinkov et al., 1998, 2000; Miron et al., 2000]. Most of allicin’s effects, in particular its cytostatic activity, are attributed to its sulfhydryl (SH)-modifying properties [Hirsch et al., 2000; Bianchini and Vainio, 2001]. Yet, the mechanisms whereby it blocks cell proliferation and induces apoptosis, as well as the nature of its subcellular target(s), are not known.

To elucidate its bioactivity, we treated cultured NIH-3T3 mouse fibroblasts with low subtoxic levels of allicin and examined the effect of this treatment, by time-lapse microscopy. Surprisingly, we found that the motile behavior of allicin-treated cells closely resembled the behavior of cells treated with microtubule-disrupting drugs. We then determined that treatment of these cells with allicin indeed leads to rapid depolymerization of cytoplasmic and spindle microtubules, and arrests cell division. Moreover, submicromolar concentrations of allicin block microtubule assembly from pure tubulin in vitro, suggesting that the effect of allicin on microtubules in vivo is direct.

Both alpha and beta tubulin contain cysteine residues bearing SH-groups that could potentially interact with allicin. We demonstrate herein that the inhibitory effect of allicin on tubulin polymerization in vitro can be partially abolished by SH-reducing reagents such as di-thiothreitol (DTT) or 2-mercaptoethanol (2-ME). Thus, we conclude that allicin interferes with microtubule assembly by modifying SH groups in tubulin. The novel effects of allicin on microtubule assembly provide new insights into the molecular underpinnings of allicin’s activity. We hypothesize that these antitubulin effects are also responsible for allicin’s antitumor properties.

### MATERIALS AND METHODS

#### Cells and Reagents

NIH-3T3 mouse fibroblast cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (penicillin and streptomycin) and replated by treatment with standard trypsin-EDTA solution. Tissue culture medium, antibiotics, trypsin-EDTA solution and glutamine were obtained from Gibco (Rhenium, Jerusalem, Israel), and fetal calf serum, from Biological Industries (Kibbutz Beit Haemek, Israel). For observations of microtubules in living cells, fibroblasts were transiently transfected with a plasmid encoding β-tubulin-GFP kindly provided by Dr. C. Ballestrem, University of Manchester, Manchester, UK [Ballestrem et al., 2000].

Allicin was synthesized as previously described [Stoll and Seebeck, 1950]. Allicin was then prepared by applying synthetic alliin onto an immobilized alliinase column [Miron et al., 2006] and its concentration was determined as described [Miron et al., 1998].

Nocodazole (methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-1-yl]-carbamate) and tissue culture-grade fibronectin were purchased from Sigma-Aldrich (St. Louis, MO). DTT and 2-ME were purchased from Merck (Merck KGaA, Darmstadt, Germany).

#### Antibodies and Immunofluorescence Staining

Anti-α-tubulin monoclonal antibodies (clone DM1A), rabbit anti-γ-tubulin polyclonal antibodies, FITC-labeled phalloidin, and DAPI were purchased from Sigma-Aldrich (St. Louis, MO). Secondary antibodies, Cy3-conjugated goat anti-mouse IgG and Cy5-conjugated goat anti-rabbit IgG, were obtained from Jackson Laboratories (West Grove, PA).

Cells were cultured on fibronectin-coated coverslips, fixed, and permeabilized in a solution containing 3% paraformaldehyde, 0.05% glutaraldehyde, and 0.25% Triton X-100 for 15 min at room temperature. They were then washed with PBS, and treated with NaBH4. Cell immunolabeling and staining with FITC-phalloidin and DAPI were performed as outlined in Bershadsky et al. [1996].

#### In Vitro Tubulin Polymerization

Tubulin polymerization was carried out using a tubulin polymerization assay kit (Cat. no. BK007; Cyto-skeleton, Denver, CO). Unlabeled purified bovine brain tubulin (Cat. no. TL238) was dissolved in General Tubulin Buffer (Cat. no. BST01-001) with glycerol according to the manufacturer’s protocol, and mixed with the rhodamine-labeled tubulin (Cat. no. TL331M) at a 5:1 ratio. The polymerization mixture contained a total of 2.5 mg/ml of tubulin, along with 0.4 mM GTP, and 6% glycerol in General Tubulin Buffer. A range of allicin concentrations (from 0.2 to 10 μM) was added to the polymerization solution, prior to addition of tubulin.
To initiate microtubule polymerization, the reaction mixture was warmed to 35°C, and incubated for 25 min. The polymerization reaction was then stopped by adding glutaraldehyde in General Tubulin Buffer at a final concentration of 0.5%. The samples were then uniformly diluted, dropped on ethanol-cleaned microscope slides, covered with no. 1 coverslips, and analyzed by fluorescence microscopy as described below, using a filter set for rhodamine fluorescence. Images of microtubules, polymerized after each type of treatment, were obtained and subsequently analyzed, in order to calculate the total microtubule length (see “Image Analysis” section, below).

**Video Microscopy**

For time-lapse recording of cell spreading, polarization and locomotion, as well as to analyze microtubule dynamics, cells were plated in medium containing HEPES buffer (25 mM), on glass-bottomed dishes (MatTek Corporation, Ashland, MA), coated with fibronectin (10 μg/ml). The dishes were mounted on the stage of an Olympus IX71 inverted fluorescence microscope equipped with a Box & Temperature Control Unit (Life Imaging Services, Reinach, Switzerland; http://www.lis.ch/).

Phase contrast cell images were taken using a Zeiss 20×/0.45 NA Achrostigmate objective. Examination of fluorescent microtubules in β-tubulin-GFP-transfected cells as well as in fixed, fluorescently stained specimens, were performed with an Olympus 60×/1.42 NA objective. Fluorescence microscopy equipment included a dichroic mirror, and excitation/emission filter wheels (Chroma Technology, Rockingham, VT) suitable for detecting DAPI, FITC, Rhodamine, and Cy-5. The same fluorescence microscopy set-up with a 100×/1.3 NA UplanFI objective was used for examination of microtubule assembly in vitro.

Image acquisition was carried out using a CoolSNAP HQ CCD camera (Photometrics, Tucson, AZ) mounted in the optical path of the microscope, and controlled by a DeltaVision system (Applied Precision, Issaquah, WA). Phase contrast recordings of spreading cells were performed at 2-min intervals, while fluorescence time-lapse images of cells labeled with β-tubulin-GFP were taken at 1-min intervals.

**Image Analysis**

Time-lapse phase contrast movies of cell spreading, polarization and movement were prepared using Image J software (http://rsb.info.nih.gov/ij/). Cell trajectories were traced using Prism software packages (Applied Precision); the cell position was defined according to the location of the center of the nucleus. The method enabling assessment of cell polarization is described in “Results.”

The amount of microtubule polymer produced by tubulin assembly in vitro was estimated by measuring the microtubule’s total length in 15 randomly selected fields, using the FiberScore algorithm [Lichtenstein...
Fig. 2. Effects of allicin and nocodazole on cell polarization and locomotion. (See also Supplementary Videos 1–3.) NIH-3T3 cells were plated in serum-free medium onto fibronectin-coated dishes and filmed for 12 h with phase contrast optics. Allicin (2 μM) or nocodazole (2 μM) were added to the medium 10 min after cell seeding. For each cell population (control, allicin-treated, and nocodazole-treated), videos were analyzed either (A) by examining 150–300 randomly selected cells at several time points, or (B) by following the “life histories” of 20 individual cells. (A) Percentage of polarized cells as a function of time. Error bars represent standard error of mean (SEM). While 80% of control cells were polarized 3 h after plating, the majority of allicin- or nocodazole-treated cells were nonpolarized even after 6 h. (B) Life histories of individual cells incubated in control, allicin-containing and nocodazole-containing medium are represented by lines consisting of blue and orange segments corresponding to nonpolarized and polarized cell morphology, respectively. Time point zero corresponds to the start of cell spreading. While control cells that became polarized remained in that state (upper panel), most allicin- or nocodazole-treated cells oscillated between polarized and nonpolarized shapes (middle and lower panels). (C) Red tracks represent the movement of 4 control, 4 allicin-treated and 4 nocodazole-treated cells. Unlike control cells (upper panel), allicin- and nocodazole-treated cells move nondirectionally (middle and lower panels). Scale bar: 30 μm.
et al., 2003]. The algorithm was implemented using a Priism software environment (Applied Precision).

RESULTS
Allicin Inhibits Polarization and Migration of Cultured NIH-3T3 Fibroblasts

To determine the effect of allicin on cell spreading, polarization, cell motility and cell division, we plated NIH-3T3 fibroblasts on fibronectin-coated culture dishes in serum-free medium, and monitored their behavior by time-lapse phase contrast microscopy in the presence of allicin (2 μM) or the microtubule-depolymerizing drug nocodazole (2 μM) [De Brabander et al., 1976; Mareel and De Brabander, 1978]. Both allicin and nocodazole were applied to the cells 10 min after seeding.

During the initial stages of spreading (the first 30 min after seeding), fibroblasts in both control and drug-containing medium spread radially, extending flat, circular lamellae (Supplementary Video 1). Following longer incubation periods, control, untreated fibroblasts polarize; namely, concentrate their lamellipodial activity within one or a few segments of their edge and stabilize other segments [Vasiliev, 1982, 1985; Harris, 1999].
While active, protruding lamellipodia are flat, adhere to the substrate, and are often characterized by convex contours, the stable edges are retracted, do not adhere to the substrate, and display a concave shape determined by elastic forces [Zand and Albrecht-Buehler, 1989; Bar-Ziv et al., 1999; Thery et al., 2006]. Thus, stable edges are easily recognizable by phase contrast microscopy (Fig. 1), and can be used as markers for quantitative assessment of cell polarization in culture. For purposes of quantification, we classified a cell as being “polarized” if the length

Fig. 4. Concentration dependence of allicin’s effect. Immunostaining of α-tubulin in NIH-3T3 cells (control) (A) and in NIH-3T3 cells treated for 30 min with a range of allicin concentrations: 0.2 μM (B), 0.5 μM (C), 1 μM (D), 2 μM (E), 5 μM (F), and 10 μM (G). The depolymerizing effect of allicin is already apparent at 0.2 μM, and becomes more pronounced as allicin concentrations increase. However, treatment of cells with 25 μM alliin, allicin’s precursor, for 30 min, does not produce any visible effect on microtubule organization (H). Scale bar: 20 μm.
of at least one of its stable edges exceeded the radius of its circumcircle, the smallest circle that completely contains the cell contour (Figs. 1A and 1B).

As seen in Fig. 2A, about 60% of control fibroblasts became polarized (according to the aforementioned definition) within 2 h after plating; at 6 h, the entire cell population acquired a polarized shape. Time-lapse analysis of individual cell spreading revealed that, sooner or later, each cell underwent polarization, and remained in this state for the remainder of the observa-

Fig. 5. Time course of allicin’s effect on microtubules. (See also Supplementary Video 4.) Immunostaining of α-tubulin in control cells (A) and in cells treated with 2 μM of allicin for 10 min (B), 20 min (C), 30 min (D), 1 h (E), and 2 h (F). The disruptive effect of allicin on microtubules increases with the duration of incubation. Scale bar: 20 μm.
Figure 6.
tion period (Fig. 2B, upper panel, and Supplementary Video 1).

Addition of allicin to cells strongly inhibited or slowed down their ability to polarize: the majority of allicin-treated cells were nonpolarized even 6 h after plating (Fig. 2A), while others acquired a polarized morphology following longer incubation, compared to control culture (Fig. 2B, middle panel). Moreover, these cells did not remain polarized for a long time, like control cells, but tended to oscillate between polarized and nonpolarized morphologies (Fig. 2B, middle panel, and Supplementary Video 2).

An inability to maintain a stable, polarized shape was previously shown to be characteristic of fibroblasts treated with microtubule-disrupting drugs [Vasiliev et al., 1970; Ivanova et al., 1976]. Indeed, NIH-3T3 fibroblasts treated with nocodazole demonstrated polarization defects that were indistinguishable from those observed in cells treated with allicin (Figs. 2A and 2B, lower panel, and Supplementary Video 3).

The ability of cells to successfully polarize is a critical prerequisite for their ability to undergo directional migration [Vasiliev et al., 1970; Vasiliev, 1982; Bershadskey and Vasiliev, 1993; Harris, 1999]. In the present study, we found that polarization preceded the migratory activity in control cultures (see Supplementary Video 1). Comparison of the migration trajectories of control cells with those of cells treated with allicin or nocodazole indicated that both drugs suppress directional migration [Vasiliev et al., 1970; Vasiliev, 1982; Bershadsky and Vasiliev, 1993; Harris, 1999]. It therefore appears that allicin’s effect on the actin cytoskeleton mimics that of nocodazole and other microtubule-disrupting drugs (Fig. 3).

To characterize the concentration dependence of the effect of allicin on microtubules, we treated NIH-3T3 cells with a wide range of allicin concentrations, for 30 min (Figs. 4A–4G). Some reduction in microtubule density could already be detected at doses of 0.2 μM allicin (compare Figs. 4A and 4B); increased allicin concentrations led to the gradual decrease of microtubule polymerization (Figs. 4C and 4D), so that allicin at concentrations of 2 μM or more induced essentially complete depolymerization of microtubules (Figs. 4E–4G). It is noteworthy that this concentration of allicin is lower by 50% than the concentration required to inhibit the incorporation of H3 thymidine [Hirsch et al., 2000]. In contrast, allicin’s precursor, alliin, did not produce any visible effect on microtubule organization in treated cells, even at much higher concentrations (Fig. 4H). In a similar, dose-dependent manner, allicin was found to disrupt microtubule organization in CHO-K1 hamster cells (data not shown). It should be emphasized that in all experiments presented herein, we removed serum from the medium prior to the addition of allicin, in order to avoid possible serum-dependent allicin degradation. In fact, when serum was present in the medium, the effective concentrations of allicin were somewhat higher than in serum-free medium (data not shown).

To determine the time dependence of the effect of allicin on microtubules, cells were treated with allicin for various time intervals (from 10 min to 2 h) before fixation (Figs. 5A–5F). In addition, to visualize allicin-induced microtubule disruption in a single cell, time-lapse filming of NIH-3T3 cells expressing GFP-tubulin and treated with allicin, was performed (Supplementary Video 4). Our results indicate that the effect of allicin on microtubules is very rapid (Fig. 5). Time-lapse filming of cells transfected with β-tubulin-GFP showed that cytoplasmic microtubules essentially disappeared within...
Figure 7.
several minutes following addition of the drug (Supplementary Video 4).

**Allicin Blocks Cell Division by Preventing Spindle Formation**

Time-lapse recording of the NIH-3T3 cells, described in the first section of the Results, showed that in addition to its effect on cell polarization, allicin also blocks cell division. In control NIH-3T3 fibroblasts, about 20% of the cells divide during the observation period (12 h). As expected, nocodazole treatment arrested cell division at the colchicine-mitosis (C-mitosis) stage [Rieder and Palazzo, 1992] due to its well-documented disruptive effect on microtubules [Hamilton and Snyder, 1982; De Brabander et al., 1986]. Essentially, the same behavior was observed in allicin-treated cells. Unlike controls (Fig. 6A and Supplementary Video 5), none of the allicin- or nocodazole-treated cells completed division during the 12-h observation period. The majority of these cells remained rounded in shape; formation of irregular transient pseudo-cleavage furrows was sometimes seen (Figs. 6B and 6C, and Supplementary Videos 6 and 7). Occasionally, following a lengthy period (5–6 h) in a rounded state, cells treated with either allicin or nocodazole re-spread, bypassing division (see Supplementary Video 8 for nocodazole-treated cells, and Supplementary Video 9 for allicin-treated cells). The fraction of such cells was similar for nocodazole-treated and allicin-treated cells, comprising less than 10% in each population.

To further compare the effects of allicin and nocodazole, the drug-treated fibroblasts were subjected to fluorescence staining, in order to visualize chromosomes (with DAPI), the mitotic spindle (with α-tubulin antibody), and centrosomes (with γ-tubulin antibody) (Figs. 6D–6G). Examination of the resulting images indicated that while dividing NIH-3T3 control cells demonstrated typical metaphase and anaphase figures (Figs. 6D and 6E), in both allicin- and nocodazole-treated cells, mitotic spindles were not formed, and chromosomes not only failed to organize into metaphase plates, but also appeared more condensed than those of control cells (Figs. 6F and 6G). Taken together, these results demonstrate that, within the context of its role as a microtubule-disrupting drug, allicin produces mitotic arrest.

**Microtubules Recover Slowly From Treatment With Allicin**

To study whether the disruption of microtubules by allicin could be reversed, we monitored microtubule organization for 24 h after removal of the drug (Fig. 7). After total microtubule disruption induced by incubation of cells with nocodazole or allicin (10 μM for 30 min), the cells were washed and put into the medium (serum-containing, or serum-free) without the drug. At different time points, the recovering cells were fixed and stained for tubulin. Notably, in contrast to the rapid (within less than half an hour) microtubule recovery after treatment of cells with nocodazole (Figs. 7A–7C), no recovery of microtubules was observed, even 3 h after allicin was removed (Figs. 7D–7G). Only about 4 h following allicin removal, initial recovery events could be detected. At this stage, numerous short microtubules appeared at the cell periphery (Fig. 7H). Moreover, the progress of recovery was slow: allicin-treated cells incubated for 8 h in allicin-free medium still displayed significantly sparser microtubule networks, than control cells (Fig. 7I). The newly formed microtubule arrays in these cells remained primarily located in the cell periphery (Fig. 7I). However, 24 h following allicin removal, cytoplasmic microtubules recovered completely (Fig. 7I). At this same time point, mitotic cells with normal spindle morphology could also be observed (Figs. 7K and 7L). Neither the rate nor the extent of microtubule recovery depended on the presence of serum in the medium (our unpublished observation). Thus, the effect of allicin on microtubules is reversible, but recovery is very slow, as compared to the recovery of these cells from “classic” microtubule drugs such as nocodazole.

**Allicin Inhibits Tubulin Polymerization In Vitro**

To determine whether the effect of allicin on microtubules is direct or indirect, we examined whether allicin influences the polymerization of pure tubulin in vitro. Accordingly, we used a tubulin polymerization kit (Cytoskeleton), which included purified bovine brain tubulin, purified tubulin labeled with rhodamine, and a polymerization buffer with GTP and glycerol. For our assay, unlabeled tubulin was mixed with rhodamine-labeled tubulin in the polymerization buffer. Polymerization was then allowed to proceed in the absence or presence of allicin, at concentrations from 0.2 to 10 μM.
Fig. 8. Effect of allicin on tubulin polymerization in vitro. Microtubules polymerized from fluorescently labeled tubulin under control conditions (A), and in the presence of 0.2 μM (B), 0.5 μM (C), 1 μM (D), 2 μM (E), and 5 μM (F) of allicin. Scale bar: 10 μm. (G) The averaged values of microtubule polymer amount (total microtubule length, measured as described in “Materials and Methods,”) presented as a percentage of the microtubule polymer amount under control conditions (without allicin). Error bars correspond to standard deviations. The graph demonstrates that the addition of 0.5 μM allicin to microtubules reduces the amount of polymerized tubulin by nearly fivefold. (H) Bars (from left to right) represent the amount of microtubule polymer after polymerization for 25 min in a control sample (taken as 100%), and in samples containing the same amount of tubulin together with 25 μM alliin; 2 μM alliin; 0.5 mM 2-ME; with a mixture of 2 μM alliin and 0.5 mM 2-ME; or with a mixture of 2 μM allicin and 100 μM DTT. Error bars represent standard deviations. Note that while alliin hardly affects tubulin polymerization, allicin strongly reduces it (consistently with graph G), while both 2-ME and DTT reduce the effect of allicin in a statistically significant manner (t-test; $P < 0.05$ and $P < 0.001$, respectively). (I) Addition of 2-ME leads to partial recovery of the allicin-treated tubulin ability to polymerize into microtubules. In the control sample (left bar) tubulin was allowed to polymerize for 50 min. The center bar represents the level of polymerization seen when 2 μM allicin was added to the microtubules for 25 min. The right bar represents the level of polymerization seen when tubulin was incubated with 2 μM allicin for 25 min, after which 0.5 mM 2-ME was added to the polymerization solution for an additional 25 min.
Under control conditions, numerous long microtubules (typically, about 25 μM in length) were formed (Fig. 8A). However, allicin inhibited this polymerization of tubulin in a concentration-dependent manner, reducing both the number and the length of newly formed microtubules (Figs. 8B–8F). Total microtubule length (as quantified by “FiberScore” image analysis software) dropped to around 20% of the control value, even when as little as 0.5 μM of allicin was added, and decreased further as allicin concentrations increased (Fig. 8G). It is noteworthy that the polymerization of microtubules was only slightly affected by a far greater quantity (25 μM) of alliin, allicin’s precursor (Fig. 8H).

Since the main biological activity of allicin is attributed to its reaction with thiol groups [Rabinkov et al., 1998; Hirsch et al., 2000], we examined the possibility that the effect of allicin on tubulin polymerization also depends on its SH reactivity. In agreement with previous studies [Chaudhuri et al., 2001] we found that the SH-reducing agent 2-ME inhibits, to some extent, microtubule polymerization in vitro (Fig. 8H). Likewise, DTT produced the same effect (data not shown). Nonetheless, when 2-ME or DTT were added to the polymerization solution together with allicin, they abolished allicin’s inhibitory effect (Fig. 8H). Moreover, addition of 2-ME to the tubulin–alliin mixture, in instances where polymerization was already completely blocked, triggered substantial rescue of microtubule polymerization, despite the presence of allicin (Fig. 8I). Altogether, these results indicate that allicin inhibits microtubule formation in vitro by modifying certain SH groups on the tubulin molecule, which are essential for polymerization.

DISCUSSION

In this study, we demonstrated that treatment of cultured NIH-3T3 mouse fibroblasts with allicin brings about a series of characteristic changes in cell shape and motility: Cells are unable to polarize; i.e., lose their ability to concentrate their lamellipodial activity at the leading edge, nor can they maintain stable lateral/trailing edges. In parallel, though these cells remain motile, their ability to migrate directionally is also suppressed. Such deficiencies in polarization and migratory capacity are hallmarks of microtubule system failure, as extensively documented in previous studies addressing the effects of microtubule-disrupting drugs [Vasiliev et al., 1970; Vasiliev, 1991] and kinesin antagonists [Rodionov et al., 1993] on cultured cells.

Indeed, in our experiments, phenotypic assessment of cells treated with allicin and with a known microtubule-disrupting drug, nocodazole, revealed a close similarity between the effects of both drugs on cell shape and motility. Moreover, we clearly demonstrated that at very low concentrations (0.5 μM), allicin triggers rapid depolymerization of cytoplasmic microtubules and prevents the formation of spindle microtubules, thereby blocking cell division in an abnormal, C-mitosis state, similar to nocodazole, colchicine, and other antitubulin drugs [Hamilton and Snyder, 1982; De Brabander et al., 1986; Rieder and Palazzo, 1992]. We further determined that allicin inhibits the polymerization of purified tubulin in vitro, at the same doses that produce microtubule depolymerization in vivo. Finally, the effects of allicin in vitro can be reversed by the thiol-reducing reagents DTT and 2-ME. Taken together, our results suggest that allicin is a potent microtubule-disrupting drug which interacts directly with tubulin dimers, most probably via reactions with tubulin thiol groups.

Tubulin is a multicysteine protein with 12 thiol-bearing cysteine residues in its α-subunit, and eight in its β-subunit [Roychowdhury et al., 2000; Britto et al., 2005]. While the majority of cysteine residues are highly conserved, their specific functions are poorly understood. The published three-dimensional structure of tubulin does not reveal any disulfide bonds in the tubulin dimer [Nogales et al., 1998; Lowe et al., 2001], but biochemical studies enable their detection [Chaudhuri et al., 2001]. Moreover, one or two disulfide bonds seem to be required for optimal tubulin polymerization [Chaudhuri et al., 2001], even though fully reduced tubulin is still polymerization-competent [Britto et al., 2002]. Cysteine oxidation by reactive oxygen species such as peroxynitrite anion (ONOO−) that brings about the formation of excessive disulfide bonds between the tubulin subunits, leads to dose-dependent inhibition of microtubule polymerization [Landino et al., 2002].

It was further proposed that modification of SH groups at certain cysteine residues could constitute a mechanism to fine-tune control of tubulin polymerization dynamics in the living cell [Luduena and Roach, 1991; Chaudhuri et al., 2001]. Many studies have demonstrated that microtubule assembly is sensitive to a wide variety of nonphysiological SH-oxidizing agents [Luduena and Roach, 1991]. For example, a small disulfide reagent, methyl methanethiosulphonate, which can react with any of the tubulin cysteines but does not induce formation of disulfide bonds between subunits, was recently shown to increase critical concentrations of tubulin polymerization [Britto et al., 2005]. The list of known microtubule polymerization inhibitors includes several drugs that function via reactions with tubulin thiol groups. Among these is 2-fluoro-1-methoxy-4-pentafluorophenylsulfonamido-benzene (T138067), which modifies selectively conserved Cys-239 residues shared by the β1, β2 and β4 tubulin isotypes [Shan et al., 1999]. Other SH reactive antitubulin drugs are ethacrynic acid [Xu et al., 1992; Luduena et al., 1994], which interacts with SH groups...
other than Cys-239 [Luduena et al., 1994], and natural products such as calvac acid [Gadoni et al., 1995], and cytochalasin A [Himes and Houston, 1976].

A comparison of allicin’s effects with the effects of these agents highlights allicin’s surprising effectiveness as an antitubulin drug. Both in vitro and in vivo, allicin prevents microtubule assembly, in concentrations which are much lower than the inhibitory concentrations of many other SH reagents, and are close to those of the classic antitubulins, nocodazole and colcemid. Moreover, even though allicin is shown to inhibit a broad class of thiol-containing enzymes in vitro [Rabinkov et al., 1998; Millard et al., 2003], its in vivo effect at submicromolar concentrations seems to be rather specific. In particular, it not only does not disrupt the actin cytoskeleton but, in fact, strengthens the stress fiber/focal adhesion system, in a manner similar to nocodazole. Such microtubule disruption-induced strengthening of the actin cytoskeleton is known to depend upon Rho A activity, myosin-II-driven cell contractility and integrin-mediated signaling [Danowski, 1989; Kolodney and Elson, 1995; Bershadsky et al., 1996; Liu et al., 1998]. Thus, allicin, in the low concentrations sufficient for microtubule disruption, does not seem to affect myosin-II, known to be sensitive to several other thiol reagents [Tiepold et al., 2000], nor does it impact the entire molecular machinery underlying adhesion-dependent signaling. Certain thiol groups critical for tubulin polymerization seem to be particularly prone to modification by allicin.

The slow reversibility of allicin’s effect on microtubules can be explained by the fact that it chemically modifies tubulin by a thiol-disulfide exchange reaction. Recovery therefore requires endogenous thiol-containing compounds such as glutathione which, similarly to DTT or 2-ME, can reverse allicin’s effects [Rabinkov et al., 1998, 2000]. Synthesis of new tubulin may also play some role in the recovery process. Notably, the pattern of microtubule recovery in the allicin-treated cells differs from that in nocodazole-treated cells: in the former instance, newly formed microtubules occur predominantly at the cell periphery, suggesting that their polymerization is not directed by the centrosome. The mechanism underlying microtubule system recovery after allicin treatment deserves further investigation.

Some of the garlic organosulfur compounds known to be allicin derivatives were recently found to inhibit tubulin polymerization. Allicin at room temperature is rather unstable and converts into various mono-, di-, and trisulfides, including diallyldisulfide (DADS), diallyltri-sulfide (DATS) and other compounds such as ajene (4,5,9-trithiadodeca-1,6,11-triene-9-oxide) [Bianchini and Vainio, 2001]. Furthermore, reaction of allicin with cysteine produces S-allylmercapto-cysteine (SAMC) [Cavallito et al., 1944; Rabinkov et al., 2000], one of the active ingredients of aged garlic extract. An isomer of ajene, cis-Z-ajoene, was shown to gradually depolymerize microtubules in cultured PtK2 cells, and to inhibit microtubule assembly from purified microtubule protein (tubulin and MAPs) [Li et al., 2002]. It was further demonstrated that SAMC [Xiao et al., 2003] and DADS [Xiao et al., 2005] can also depolymerize microtubules in interphase cells, cause mitotic defects, and inhibit in vitro tubulin polymerization, albeit at relatively high concentrations (100–1000 μM). Finally, DATS appears to be a potent antimicrotubule drug disrupting (at 10 μM concentrations) cytoplasmic microtubules, preventing spindle formation, and interfering with pure tubulin polymerization in vitro [Hosono et al., 2005].

Like that of allicin, the activity of the aforementioned compounds seems to depend on their reaction with tubulin cysteine residues. In fact, the inhibition of tubulin polymerization by SAMC in vitro can be partially abolished by 2-ME [Xiao et al., 2003], and DATS was shown to specifically modify Cys-12 and Cys-354 β-tubulin residues [Hosono et al., 2005]. Analysis of the modes of action of these allicin derivatives does not support the idea that allicin affects tubulin indirectly, via conversion into one of these compounds. In our experiments, allicin disrupted microtubules at much lower concentrations (0.5–1.0 μM), and far more rapidly (see Supplementary Video 4) than reported even for Z-ajoene and DATS, the most potent of its derivatives [Li et al., 2002; Hosono et al., 2005]. Further studies are required to clarify the mechanisms of such exceptional activity, as well as the specificity of allicin as a tubulin inhibitor.

Another important direction for future research is to elucidate whether the known effects of allicin arise from its effect on tubulin polymerization. In view of microtubule involvement in almost all aspects of cell regulation, the activity of allicin as a microtubule-disrupting drug may play a role in almost all of its known effects. It therefore seems plausible that allicin’s effects on T cell migration [Sela et al., 2004] and on platelet aggregation [Mayeux et al., 1988; Briggs et al., 2000] are related to its microtubule-disrupting activity, since the role of microtubules in these processes is well-recognized [Menche et al., 1980; Long et al., 2004].

Furthermore, the effects of allicin on cell proliferation and apoptosis can also be explained in terms of its microtubule-disrupting activity. We demonstrated herein that allicin arrests cells in an abnormal C-mitosis state, known for years to be induced by colchicin, nocodazole and other microtubule depolymerizing agents [Rieder and Palazzo, 1992]. It is well-documented that these drugs also cause a variety of cell cycle abnormalities, including delay in G2-M transition [Rieder and Cole, 2000] and sometimes G1 arrest [Blajeski et al., 2002]. In
some cell types, microtubule disruption ultimately leads to apoptosis [Asnaghi et al., 2004; Nagy et al., 2005; Beswick et al., 2006]. Thus, the possibility that microtubule disruption is involved in the pathways responsible for allicin antiproliferative and proapoptotic effects clearly deserves further investigation.

Finally, it is worth noting that certain microtubule-disrupting agents are important anticancer drugs [Jordan and Wilson, 1998; Zhou and Giannakakou, 2005]. Therefore, allicin’s potential as an anticancer agent may also depend on its antitubulin function. Thus, our study sheds new light on the mechanisms by which allicin exerts its effects, and on its potential therapeutic applications.

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