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Molecular Transduction Mechanisms of the Redox Network Underlying the Antiproliferative Effects of Allyl Compounds from Garlic[1,2]

Giuseppe Filomeni,[3,4] Giuseppe Rotilio,[3,4] and Maria R. Ciriolo[3,4]*

[3]Department of Biology, University of Rome "Tor Vergata", 00133 Rome, Italy and [4]Research Centre IRCCS San Raffaele - Pisana, 00163, Rome, Italy

Abstract

Much evidence in the last few years suggests that the antiproliferative effects of various garlic secondary metabolites in in vitro experimental systems are due to redox-based mechanisms. In particular, sulfur-containing allyl compounds have been demonstrated to generate reactive oxygen species and to modify directly the redox state of specific reactive cysteines on protein surfaces. On the basis of such properties, allyl compounds, in particular the ones present in the oil-soluble fraction of garlic extracts, can function as modulators of several redox-mediated signaling pathways related to the activation of mitogen-activated protein kinases, cell cycle, DNA repair, and cell demise. However, even though many in vitro studies have tried to dissect the mechanisms of action of garlic derivatives, research in this field is still incomplete and questions about bioavailability, biotransformation, and pro-oxidant activity are still unanswered. This review discusses recent findings on such aspects, focusing on the chemistry of allyl compounds and their preferential cellular targets as well as on related nutritional aspects.

Introduction

Garlic and other plants belonging to the *allium* specie are traditionally consumed for their peculiar taste and presumed health benefits. Pathological conditions in which this vegetable has been suggested to be valuable include thrombosis, microbial infection, lipid and carbohydrate dysmetabolism, hypertension, and cancer (1). The components that make garlic unique among vegetables are sulfur-containing molecules, the synthesis of which relies on complex chemical reactions taking place from γ-glutamylcysteine. Upon hydrolysis and oxidation, oil-soluble allyl compounds, which normally account for 0.2–0.5% of garlic extracts, such as diallyl sulfide (DAS),[5] diallyl disulfide (DADS), diallyl trisulfide (DATS), and other allyl polysulfides (2), are generated. Alternatively, it can be slowly converted into water-soluble allyl compounds, such as *S*-allyl-cysteine and *S*-allyl-mercaptocysteine (SAMC).

Some of the most convincing evidence linking garlic and/or its constituents with anticancer activity comes from preclinical studies, whereas epidemiological reports are still scarce or lacking. However, there are data reporting a lower incidence of cancer in individuals who consume garlic daily (3). This review summarizes the chemical and biological features of the most important garlic-derived allyl compounds, which have been shown to have potential antiproliferative effects.

Redox base of antioxidant cell response to garlic and garlic-derived allyl compounds

The bulk of data published before the late 1990s reported that the main effects of sulfur-containing allyl compounds rely on their antioxidant and/or detoxifying activities. For instance, the use of purified allyl sulfides or garlic extracts in combination with carcinogens decreases their mutagenic effects. However, the chemical structure and reactivity of allyl compounds rather favor a pro-oxidant activity. In fact, oil-soluble allyl compounds are the main source of disulfides and polysulfides and, due to the high intracellular abundance of reduced glutathione (GSH) and protein thiols, they can mediate thiol/disulfide exchange by determining decrease of GSH and thiolation of reactive cysteine residues on proteins (4) (**Fig. 1***A*). Whereas the former reaction induces oxidative unbalance, the latter yields reversible alterations of protein function, as demonstrated for the nonselective cation channel transient receptor potential-A1 of sensory nerve endings upon treatment with DADS (5), which underlies its pungent effects. Allyl disulfides and polysulfides can also produce reactive oxygen species (ROS) directly by reactions relying upon the homolytic cleavage of disulfide bond. This leads to the formation of allyl-(per)thiyl radicals, which can rapidly react with GSH, thus forming disulfide or polysulfide radical anions and reduce oxygen to produce ROS. Superoxide and hydrogen peroxide can be produced also as by-products of the reaction between perthiol and oxygen (e.g. O_2 bound to hemoglobin; Fig. 1*B*). Moreover, allyl sulfides from garlic, with their thiol and perthiol derivatives, might have a role in chelating both free and protein-bound metals. However, this aspect remains speculative, but it could provide evidence for additional (indirect) pro-oxidant activity of garlic-derived allyl sulfides, taking into account that also the antioxidant enzymes superoxide dismutase and catalase are metallo-proteins.

Pro-oxidants can induce antioxidant and detoxifying cell responses, phenomenon known as preconditioning, which make

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[5] Abbreviations used: Cdc25C, cell-division cycle 25C; DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, diallyl trisulfide; GSH, reduced glutathione; GST, glutathione-*S*-transferase; HDAC, histone deacetylase; JNK, c-Jun-NH_2-terminal kinase; Nrf2, nuclear erythroid factor 2-related factor 2; ROS, reactive oxygen species; SAMC, *S*-allylmercaptocysteine.
* To whom correspondence should be addressed. E-mail: ciriolo@bio.uniroma2.it.

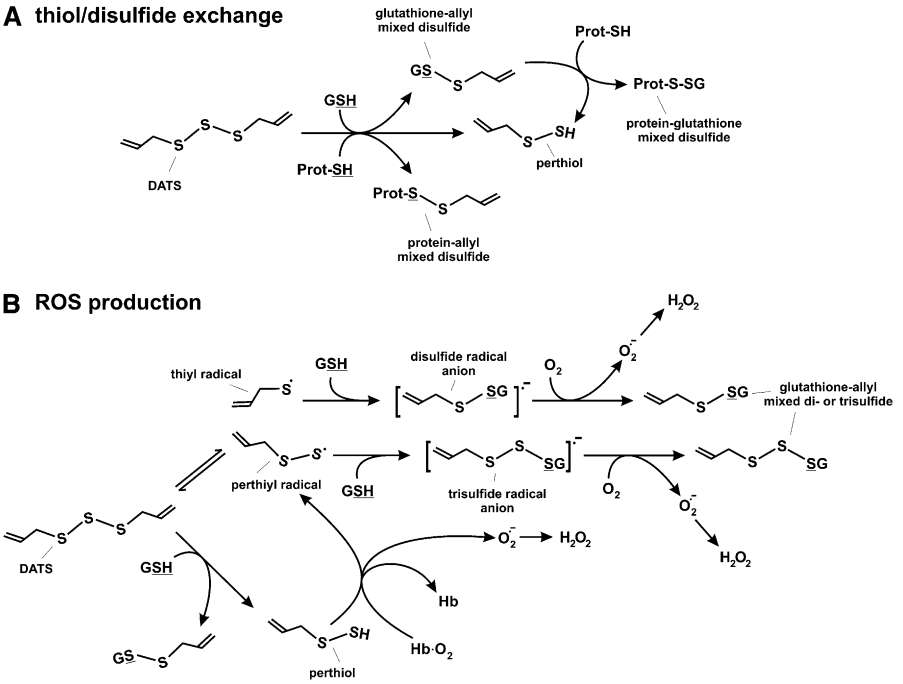

FIGURE 1 Redox chemistry of allyl sulfides. Allyl sulfides can contribute to the establishment of pro-oxidant conditions in different manners. The chemical reactions leading to protein thiolation, decrease of GSH levels, and generation of ROS are shown, taking into account DATS as representative garlic-derived allyl sulfide. (*A*) DATS can react with intracellular thiols (GSH or protein thiols, *Prot-SH*), giving rise to allyl perthiol and mixed disulfides by thiol/disulfide exchange. (*B*) DATS can undergo homolytic cleavage, producing thiyl and perthiyl radicals that, in the presence of intracellular GSH, react to form disulfide or trisulfide radical anions, respectively. The high redox potential of such species allows the direct reduction of O_2 to superoxide anion ($O_2^{\cdot -}$) and subsequently hydrogen peroxide (H_2O_2). Also, the perthiol form of DATS can react with oxygen-bound proteins (e.g. oxy-hemoglobin), thus producing ROS and perthiyl radicals.

the cell more resistant toward the occurrence of further oxidative challenges. One of the best established examples in this respect is the activation of the nuclear erythroid factor 2-related factor 2 (Nrf2), which is normally sequestered into the cytoplasm, but translocates into the nucleus in response to oxidative stress. Allyl sulfides from garlic are able to rapidly activate Nrf2, thus inducing the transcription of genes coding for enzymes of the antioxidant/detoxifying system, such as those related to GSH (6). Nevertheless, the preconditioning state implies that the oxidative stress applied to the cells should not overcome the buffer capacity of the antioxidant defense system to avoid irreversible damage of biomolecules and/or cell death. To which extent the intensity of an oxidative stimulus can switch cellular response from the preconditioning state to commitment to cell death depends on the cell type. This is consistent with the evidence that concentrations of allyl compounds needed to induce the expression of antioxidant and detoxifying enzymes depends on the cell system employed. Studies performed on hepatocytes or hepatoma cells show that high concentrations of DAS and DADS (100–500 μmol/L) induce glutathione-*S*-transferase (GST), cytochrome P450 expression, increase antioxidant enzymes, and GSH levels (7). Conversely, glioblastoma, leukemia, and neuroblastoma cells undergo apoptosis upon exposure to much lower concentrations of DAS and DADS (10–100 μmol/L) (8–10). In conclusion, the potential of garlic-derived allyl compounds to cause either cell death or preconditioning confers to these pro-oxidant molecules the capability to induce cellular antioxidant responses.

Sulfur-containing allyl compounds induce apoptosis through ROS-dependent mechanisms

The first evidence indicating that DADS yield ROS increase and cause oxidative damage, thus inducing caspase-dependent apoptosis, goes back to experiments performed in the early 2000s on human leukemia and neuroblastoma cells (8,9). It was also reported that apoptosis proceeded via the phosphorylative cascade mediated by the proapoptotic member of the mitogen-activated protein kinase family, c-Jun-NH_2-terminal kinase (JNK), upon ROS-dependent detachment of GST (10). However, the proapoptotic effectiveness of DADS was not of general application. The adenocarcinoma gastric cells did not undergo apoptosis when treated with DADS, even at high concentrations, but rather, they induced a transient arrest of cell cycle and restarted to proliferate upon buffering DADS-mediated toxicity (11). A similar behavior was also found in HepG2 cells treated with water-soluble garlic extracts (12), indicating that these histotypes had a high threshold of resistance. This was related to their capability to form reversible mixed disulfides between GSH and protein thiols and to the high expression levels of the gastrointestinal isoform of glutathione peroxidase, thus confirming the role of ROS in the induction of apoptosis or preconditioning state by allyl compounds. These biochemical features were also associated with an early and sustained translocation of Nrf2 into the nuclear compartment (G. Filomeni, S. Piccirillo, G. Rotilio, and M. R. Ciriolo, unpublished data) and with a transient inactivation of the extracellular-related kinases 1/2 (11).

Although ROS are produced by allyl sulfides, it has recently been suggested that DATS can yield generation of ROS by increasing the level of labile iron, which was associated with degradation of the ferritin light chain (13). Interestingly, it has been proposed that JNK may activate the degradation of ferritin, induce a sustained release of iron, and allow the generation of ROS by an iron-dependent Fenton reaction in the early minutes of treatment. On the basis of these results, it is likely that a sum of events can contribute to ROS production upon treatment with allyl sulfides; however, the possibility that this depends on JNK or other specific signaling pathways remains to be verified.

Sulfur-containing allyl compounds affect cell cycle by redox-dependent mechanisms

Studies by Knowles and Milner (14) demonstrated that some allyl sulfides induced cell cycle arrest in G2/M phase by decreasing the phosphatase activity of cell-division cycle 25C (Cdc25C), which resulted in the stabilization of the inactive form of cyclin dependent kinase 1. However, only recently are the molecular mechanisms of these effects being explained, with ROS causing a decrease in the level of Cdc25C and a concomitant increase of its Ser-216-phosphorylated form (15). A similar increase has been observed in response to DNA damage and it is necessary for

Cdc25C to remain sequestered within the cytosol by the interaction with 14–3-3 protein (15). Other pathways that participate in the induction of cell cycle arrest were also suggested. The cascade of events downstream of ataxia telangiectasia-mutated/Rad3 related factor and the checkpoint kinase 1 was proposed to sense double-strand break-induced DNA damage and to be responsible for cell cycle arrest in pro-metaphase (16). In this regard, we recently reported that DADS induces the phospho-activation of the double-strand break-sensitive histone H2A.x in neuroblastoma SH-SY5Y, as a consequence of DNA damage (17). Therefore, the occurrence of genotoxic stress following treatment with allyl sulfides can be assumed to be mainly ROS dependent and the alteration of DNA integrity could be responsible for cell cycle arrest.

Recently, it has been demonstrated that inhibition of histone deacetylases (HDAC) causes disruption of DNA-dependent protein kinase binding to DNA and increases the phospho-activated levels of histone H2A.x (18). DADS has been demonstrated to induce cell cycle arrest and apoptosis in human colon cancer cells by inhibiting HDAC activity (19), which eventually resulted in increased expression of the cyclin-dependent kinase inhibitor $p21^{WAF1}$. Other observations indicate that HDAC inhibition induces the expression of c-Jun either by a JNK-directed action (20) or by epigenetic control (21). This suggests that DADS and other allyl sulfides could induce JNK-dependent tumor cell death, either by means of ROS production or by inhibiting HDAC activity.

The results above indicate a causal relationship between ROS and cell cycle regulation, which could be even more strict considering the role of Cdc25C, a member of the protein tyrosine phosphatase family, which catalyzes dephosphorylation reactions by a reactive cysteine residue (Cys377). In the free enzyme, Cys377 exists as thiolate anion, the chemistry of which means for reversible regulation of the enzyme. This critical cysteine can be oxidized via direct reaction with ROS to the sulfenic acid derivative that, in the presence of excess of ROS, can be further oxidized to sulfinic acid ($-SO_2H$) (22). Alternatively, at low concentrations of ROS, the sulfenic acid derivative forms an intramolecular disulfide with the so-called backdoor cysteine (Cys330), which slows further oxidations. Although all oxidative modifications of the active-site cysteine lead to a complete loss of catalytic activity, the intramolecular disulfide specie of Cdc25C appears to be functional for the selective interaction between Cdc25C and 14–3-3 protein (23). Therefore, it is reasonable to speculate that allyl compounds from garlic modulate Cdc25C activity by a thiol-oxidizing mechanism. Interestingly, the effects of redox inactivation of Cdc25C strongly resembles those occurring upon treatment with allyl sulfides (15). On the basis of their redox chemistry, it is likely that oxidative modifications of both Cys337 and Cys330 may occur by ROS-catalyzed thiol oxidation or by thiol/disulfide exchange of the mercapto-allyl group with reactive cysteine residues. The latter modification, which has been reported to act on transient receptor potential-A1 (5), could be, in fact, effective for other proteins too, such as Cdc25C.

The role of cytoskeleton in the toxicity of sulfur-containing allyl compounds

It has been reported that SAMC, but not *S*-allyl-cysteine, is able to interfere with microtubule polymerization by inducing thiol-oxidizing–dependent disruption of the microtubule network and the formation of monopolar and multipolar spindles in mitotic human colon cancer cells (24). JNK activity has a crucial role in SAMC-induced apoptosis but only as an early phase inducer. In fact, G2/M phase arrest due to the interference of SAMC with microtubule assembly subsequently determines late-phase apoptosis, completely independent of JNK (24). These results point out that a bifurcated apoptotic pathway could be outlined after treatment with sulfur-containing allyl compounds: the first one involves the activation of a ROS-dependent JNK/c-Jun signaling; the other is associated with tubulin depolymerization, which occurs in a thiol-dependent manner and confers to the apoptogenic stimulus the feature of irreversibility. Hosono et al. (25) demonstrated that treatments with DATS induce an increase in the molecular weight of tubulin by 71.2 Da, which corresponds to the mass of the fragment of mercapto-allyl group deriving from DATS, as well as from SAMC, indicating that *S*-allyl adducts to Cys12β and Cys354β are the main event in triggering microtubule network disassembly and inducing interphase arrest.

The role envisaged for tubulin sulphydryls in allyl sulfide-mediated cytotoxicity raised the question whether microfilaments could be also affected. On the basis of the involvement of cytoskeleton in NAD(P)H oxidase activation, as well as the numerous reactive cysteines on actin mediating cell adhesion and motility, it cannot be excluded that allyl compounds may induce redox modifications also at the actin level, and hence could have a role in the induction of cell cycle arrest and apoptosis The cytoskeleton is emerging as an important player, because it could link allyl sulfide-induced damages to the mitochondrial pathway of apoptosis. Recent results indicate that JNK mediates hyperphosphorylation of Bim, a BH3-only proapoptotic member on the Bcl-2 superfamily, and that this represents one of the earliest events of garlic-induced cell cycle arrest and apoptosis (26). Indeed, Bim has been involved in JNK-dependent apoptosis, when oxidative stress induces alteration of microtubule architecture (27). On the basis of these results, we suggest that there is no strict hierarchy among the apoptotic mechanisms induced by allyl compounds, but JNK activation and cytoskeleton disassembly act in concert in inducing cell response. The decision between proliferation, arrest, or apoptosis will reasonably be the result of the relative contributions of antioxidants, mitogen-activated protein kinase equipment, and the efficiency of DNA repairing machinery (**Fig. 2**).

From cells to human: how many in vitro effects can be extrapolated to the whole organism?

The absence of straightforward and consistent results in vivo do not allow us to propose a therapeutic approach with allyl compounds in cancer treatment or to define recommended dietary intake for a successful prevention of cancer. The unfeasibility of the latter approach mainly hinges on the fact that the fixed experimental conditions used in in vitro systems cannot be reproduced faithfully in humans. Assuming that each fresh clove weighs ~2 g, we can roughly estimate an average of 10–20 mg of allyl compounds per clove. This, in theory, would be sufficient to reach blood concentrations of ~10–20 μmol/L after clove ingestion, but many factors make this hypothetical value diverge from the real blood or tissue concentrations, which are generally 1 or 2 orders of magnitude lower. The main reason for this discrepancy is the high reactivity of garlic-contained allyl compounds with intracellular thiols, which results in their metabolic transformation in less reactive molecules. In fact, no traces of DAS, DADS, and other allyl polysulfides could be detected even after 1 h from oral ingestion, and studies performed with radiolabeled DADS demonstrated that 70% of the radioactivity remained within the cytosol of hepatocytes as sulfates or other nonreactive metabolites (28).

Another feature discouraging the use of purified allyl compounds is their high toxicity. In this review, we have described the

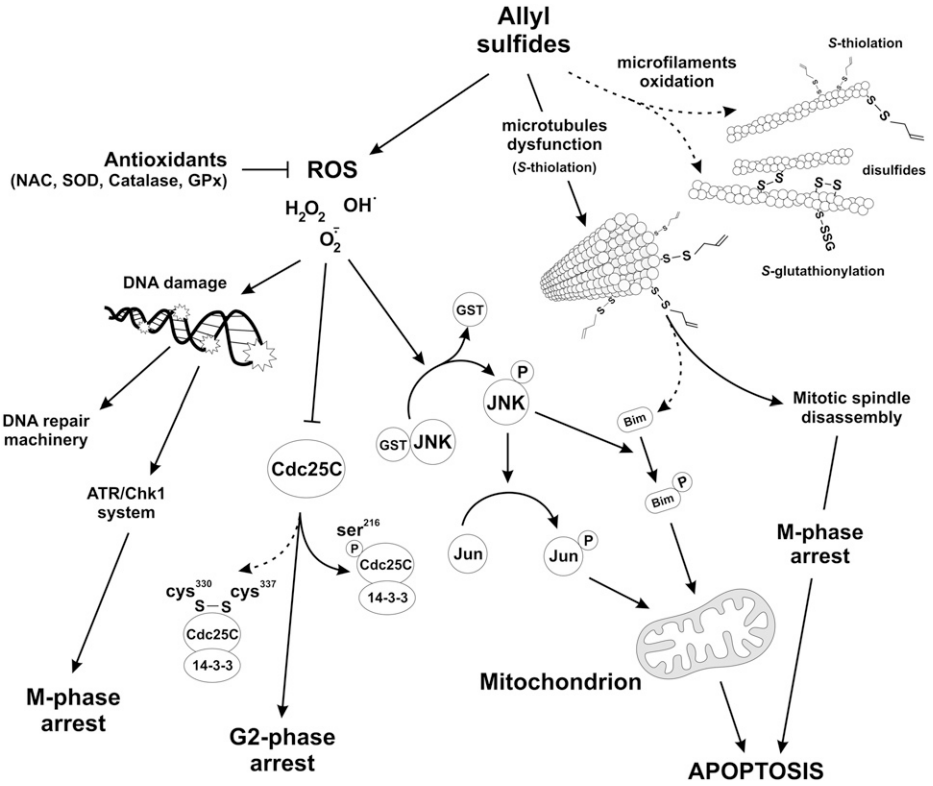

FIGURE 2 Network of redox processes underlying allyl compounds-mediated antiproliferative effects. Allyl sulfides can cause either ROS production or redox modification of specific reactive cysteines. ROS induce cell cycle arrest via both DNA damage and Cdc25C inactivation. This latter event depends on phosphorylation of Cdc25C on Ser216 and 14–3-3-dependent sequestration into the cytoplasm. Concomitantly, the ROS-dependent activation of JNK/c-Jun pathway induces apoptosis through the mitochondrial route. Thiol oxidation via the formation of a mixed disulfide between Cys12 and/or Cys354 of β-tubulin and the mercapto-allyl group of allyl sulfides is instead responsible for mitotic spindle disassembly, and finally in the induction of apoptosis. Oxidation of actin microfilaments and Cdc25C (by disulfide bonds), as well as *S*-thiolation-dependent detachment of Bim from microtubules (dotted arrows) could be additional mechanisms through which allyl compounds mediate cell cycle blockage and apoptotic cell response.

molecular bases of this characteristic mainly in replicating tumor cells, but side effects on normal tissues occur to a different extent. It has been demonstrated that raw garlic juice, as well as allicin or high doses of commercially available garlic preparations, causes severe damage to the stomach and the intestinal mucosa of rats, resulting in ulcers, shrinkage, and bleeding (2). Another aspect related to the latest knowledge available from nutrigenomic and nutrigenetic studies is the diet/genome interaction. This is essential to evaluate the selective response of each individual to dietary intake of garlic and, in turn, to modulate its effects on human health. In fact, it appears that dietary constituents affect metabolic pathways and homeostatic control by activating particular sets of genes and that the incidence of gene variants can influence such a response to nutrients. This holistic approach to the nutritional aspects of biology is still in the phase of development; however, no particular polymorphism associated with apoptotic or antioxidant genes has yet been shown to be involved in garlic-derived allyl sulfides metabolism. Only recently, it was shown that mice fed with DADS and allicin overexpressed GST in the stomach and small intestine, particularly the α and μ isoforms of the enzyme (29). It has been suggested that the mechanism responsible could involve reversible modification of protein sulphydryl groups, changes in the GSH:glutathione disulfide (GSSG) ratio, and overall alterations in cellular redox state. From these observations, it seems plausible that the in vivo metabolism of sulfur-containing allyl compounds and their effects on cancer, mostly in the initiation and promotion phases, would be strongly influenced by polymorphism of GST genes. Although information on this aspect has not been provided yet, it certainly deserves particular attention to plan an appropriate nutritional approach based on garlic consumption in cancer prevention.

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