Comparison of the chemopreventive efficacies of garlic powders with different alliin contents against aflatoxin B1 carcinogenicity in rats

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Garlic (Allium sativum) is well known for its beneficial effects on health and particularly for its chemopreventive potential against cancer. The present study was designed to compare the chemopreventive efficacies of several garlic powders with various levels of alliin, a precursor of active sulfur compounds. For this purpose we used the medium-term hepatocarcinogenesis protocol (resistant hepatocyte powders with various levels of alliin, a precursor of active sulfur compounds. For this purpose we used the medium-term hepatocarcinogenesis protocol (resistant hepatocyte model), which allows the detection of preneoplastic foci expressing the placental form of glutathione S-transferase (GST-P) as an end-point. Rats were fed diets containing three garlic powders (5% of the diet) with various alliin contents for 3 weeks. Garlic powders were obtained from bulbs grown on soils with different levels of sulfur fertilization. During the period of garlic feeding hepatocarcinogenesis was initiated by administration of 10 i.p. injections of 0.025 mg/kg body weight aflatoxin B1 (AFB1). The rats were later submitted to 2-acetylaminofluorene treatment and partial hepatectomy, and GST-P foci were detected and quantified. Consumption of diets containing garlic powders decreased the appearance and size of hepatic GST-P foci. A strong reduction was observed in rats fed garlic containing the highest level of alliin. In addition, increased alliin content of the garlic powder was associated with a proportional decrease in the number and area of preneoplastic foci. Elsewhere, garlic powder ingestion increased hepatic ethoxysorufin deethylase, glutathione S-transferase and UDP glucuronosyl transferase activities while no modification of nifedipine oxidase activity was found. We also observed an increase in the levels of GST A5 and AFB1 aldehyde reductase. It is suggested that garlic partly exerts its anticarcinogenic effects through increasing enzymes involved in AFB1 detoxification. This study highlights the possibility of controlling the cultivation conditions to improve the chemopreventive efficacy of garlic.

Introduction

The antitumoral effects of garlic (Allium sativum) have been recorded since very early times. In ancient Egypt (1550 BC) garlic was used for the treatment of tumors. Hippocrates and Indian physicians also reported garlic as a method to reduce tumor growth (1). More recently, epidemiological studies have shown that high consumption of garlic is associated with reduced cancer risk in humans, primarily stomach and colon cancer (2). In addition, experimental studies have demonstrated the ability of garlic to reduce chemical carcinogenesis in different animal models. The protection offered by garlic was demonstrated in several tissues and was effective against a broad range of carcinogens (reviewed in 3,4). Garlic is characterized by a high content of organosulfur. In the bulb, the sulfur is primarily γ-glutamyl peptides and allylcysteine sulfides (Figure 1) (5). When the bulb is cut, chopped or squeezed, alliin, the main allylcysteine sulfoxide, is metabolized to allicin through the action of alliinase. Allicin is a self-reactive constituent and it is converted readily to more stable compounds such as polysulfides (6). Moreover, depending on the processing method applied to garlic, the amounts and the types of sulfur compounds vary significantly (5). Many studies on the anticarcinogenic effect of garlic have been performed with isolated fat-soluble compounds such as allylsulfides (7,8) or water-soluble compounds such as S-allylcysteine (9–11). Moreover, in most of the published studies on the protective effects of garlic preparations, their chemical composition with regard to sulfur compounds was poorly characterized (12–16). As the sulfur composition of garlic can vary according to environmental factors such as climate or cropping conditions (17,18), a comprehensive evaluation of the chemopreventive effect of garlic towards chemical carcinogenesis needs to take into account these environmental factors.

The present work was designed to study the preventive effects of garlic powders containing variable levels of sulfur compounds such as allyli on the development of preneoplastic foci initiated by aflatoxin B1 (AFB1) in rats. To obtain garlic with differing sulfur levels, bulbs were grown in soils with variable levels of sulfate fertilization. AFB1 was selected because it is considered to produce severe hepatotoxicity and hepatocarcinogenicity in animals but also presents a risk factor for human liver cancer (19,20). AFB1 requires metabolic activation to express its carcinogenicity. Cytochromes P450 (CYP) are primarily responsible for activation of AFB1 to the ultimate carcinogen AFB1-8,9-epoxide (AFBO) (21,22). AFBO may be conjugated enzymatically with glutathione by glutathione S-transferase (GST), which is a critical pathway for AFB1 detoxification (23). In addition, AFB1 aldehyde reductase (AFAR) can reduce the cytotoxicity of AFB1 by preventing binding of the dialdehydic form of the mycotoxin to intracellular proteins (24). It has previously been shown that garlic compounds such as allylsulfides affect AFB1 carcinogenicity (8) and reduce the formation of DNA adducts through

Abbreviations: 2-AAF, 2-acetylaminofluorene; AFAR, aflatoxin B1 aldehyde reductase; AFB1, aflatoxin B1; AFBO, aflatoxin B1-8,9-epoxide, CYP, cytochrome P450, DADS, diallyl disulfide; EROD, ethoxysorufin O-deethylase; GST, glutathione S-transferase; GST-P, placental form of glutathione S-transferase; PROD, prohexyoxorufin O-depentylase; NO, nifedipine oxidase; UGT, UDP-glucuronosyltransferase.
modulation of phase I and phase II enzyme activities (25). Consumption of garlic powder was also demonstrated to modify the activities of hepatic phase I and phase II enzymes (26). This suggests that garlic could have a chemopreventive effect against carcinogenesis initiated by chemicals which are transformed by these enzymes.

To compare the relative chemopreventive efficacies of garlic powders with different alliin contents, we used the medium-term hepatocarcinogenesis protocol (resistant hepatocyte model), adapted from Solt and Farber (27). This protocol allows the detection of preneoplastic foci expressing the placental form of glutathione S-transferase (GST-P) as an endpoint (28). Garlic powders were added to the diet during the initiation phase, i.e. before and during the administration of AFB1. To investigate the modulation of phase I and phase II enzymes through which garlic powders may exert their anti-initiating effect, we measured the activities of several CYP isoforms (1A2, 2B and 3A) and several phase II enzymes, such as GST and UDP-glucuronosyltransferase (UGT), at the end of the garlic feeding period. The amounts of CYP isoenzymes, GST A5 and AFAR were analyzed by western blotting.

Materials and methods

Chemicals

AFB1 was obtained from Sigma-Aldrich (St Quentin Fallavier, France). Biotinylated goat anti-rabbit immunoglobulin and streptavidin-alkaline phosphatase were purchased from Dako (Trappes, France). Polyclonal antibodies against rat CYP 1A1,2, CYP 2B1 and CYP 3A2 were purchased from Gentest (Woburn, MA). Polyclonal antibody against GST-P was obtained from Medical and Biological Laboratories Co. Ltd (Nagoya, Japan). Polyclonal antibody against rat GSTA5 and rat AFAR1 were kindly donated by Prof. D.J.Hayes (University of Dundee, UK). Other chemicals were of the highest quality available.

Plant materials, cultivation and preparation of garlic powders

Garlic, variety printanor, was produced the same year, in two field trials carried out in France (Crest) and in Spain (Cordoba). Sulfur fertilization was provided as dehydrated CaSO4 applied in two stages before bulb formation. Three distinct levels of CaSO4 were used: 0 and 200 kg/ha in the French field trial and 400 kg/ha in the Spanish one. Four replicates of 100 plants were planted for each CaSO4 treatment. The bulbs were harvested 6 months later, when considered mature (juice 430/°Brix). They were air dried naturally and cured until completely dry (3-4 weeks later). Afterwards the bulbs were processed as previously described (26). Briefly, the bulbs were peeled and sliced and then dehydrated in an oven (70°C for the first 2 h, followed by 65°C overnight and 60°C for the last 2 h). The dry flakes were immediately ground to a powder (<25 μm particles).

Analysis of sulfur compounds in the garlic powders

Analysis of sulfur compounds in garlic powders was performed by ion pair HPLC with UV detection. Garlic powder (1 g) was ground in 10 ml methanol/water (80/20 v/v) + 0.05% formic acid. An aliquot of the suspension was diluted five times and filtered (0.2 μm). Aliquots of 10 μl were analyzed by HPLC (29).

Animals and dietary treatments

Male SPF Wistar rats, 4 weeks old, from Janvier (Le Genest Saint Isle, France) were housed in individual stainless steel cages and maintained at 21°C, with constant humidity and with a 12 h light-dark cycle. They were maintained in accordance with the French Ministry of Agriculture guidelines for care and use of laboratory animals. Throughout the experiment they were fed a...
Chemopreventive efficacies of garlic powders

The experimental protocol design is illustrated in Figure 2. At the start of the study, 60 rats were divided into four groups of 15 rats each. These groups were designated C, S0, S200 and S400. Group C was the control group and was given a garlic-free diet. Groups S0, S200 and S400 were given the same diet containing 5% of the garlic powders produced from bulbs grown in soils fertilized with 0, 200 or 400 kg/ha CaSO4, respectively. The animals were fed for 3 weeks with these diets. During this period, initiation of carcinogenesis was performed in 10 rats of each group by 10 i.p. injections of 0.025 mg/kg body weight AFB1. Five rats of each group were not treated with AFB1 and was performed in 10 rats of each group by 10 i.p. injections of 0.025 mg/kg body weight AFB1. Five rats of each group were not treated with AFB1 and were killed at the end of the garlic feeding period (sacrifice S1). Their livers were removed, weighed and processed to obtain microsomal and cytosolic fractions. Enzyme assays (CYP and phase II) were performed on these subcellular fractions (see below). All the remaining rats were fed with the control diet for 2 weeks. They were then submitted to a selection phase for initiated hepatocytes. This consisted of supplementing the diet with 50 p.p.m. 2-acetylaminofluorene (2-AAF) for 2 weeks. In the middle of the 2-AAF treatment period a two-thirds partial hepatectomy was performed on the rats. One week after the end of the 2-AAF treatment period the rats were killed (sacrifice S2).

Liver sections and analysis of preneoplastic foci

The livers were rapidly removed and two slices ~5 mm thick were excised from the right anterior lobe. These samples were immediately frozen in liquid nitrogen and stored at −80°C. Serial cryosections ~10 μm thick were prepared from each slice and GST-P was detected by an immunohistochemical method using a streptavidin-biotin complex (30). The immuno-
histochemical method was performed using polyclonal antibodies raised against rat CYP 1A1 (diluted 1:1000), CYP 2B1 (diluted 1:4000), CYP3A2 (diluted 1:2000), GST A5 (diluted 1:3000) and AFAR1 (diluted 1:1000). For the detection of CYP isoforms, hepatic microsomes from rats treated with methylcholanthrene, phenobarbital or dexamethasone were used as positive controls. For the detection of GST A5, hepatic cytosol from male mice was used as a control since antibodies raised against rat GST A5 cross-react with the ortholog murine GST A3, which has the same electrophoretic mobility as rat GST A5 (37). Rat kidney cytosols were used as a positive control for the detection of rat AFAR1 because substantial amounts of this isoform was found in kidney (38). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and 4-chloro-1-naph- tol. The stained sheets were scanned and the intensities of individual bands were measured using Optimas image analyzer software (Media Cybernetics, USA). Comparisons were made between the blot intensities of treated rats with those of control rats. Results were expressed as a percentage of the control.

Statistical analysis

The numbers and the areas of the preneoplastic foci as well as the enzyme activities were submitted to a one-way analysis of variance followed by a Dunnett’s test to assess the difference between treated and control rats \((P < 0.05)\). Correlation analysis (Pearson’s correlation coefficient) was used to test the relationship between the alliin content of the powder and the morphometric parameters of foci (numbers and areas) as well as with the enzyme activities. Correlations were considered to be statistically significant at \(P \leq 0.05\). Calculations were computed with StatBoxPro v5 (Grimmer-Soft, Paris, France).

Results

Sulfur analysis of garlic powders

The following compounds were identified in the garlic powders: alliin, γ-glutamyl-S-allyl-L-cysteine, γ-glutamyl-S-(trans-1-propenyl)-L-cysteine and γ-glutamyl-phenylalanine (Figure 3). Among these compounds, alliin was the main component and it was quantified in the three powders (Table I). The alliin content of garlic powder S400 was 6-fold higher than the alliin content of garlic powder S0 (without sulfur fertilization).

Differences of ethoxyresorufin O-deethylase (EROD) and pentoxyresorufin O-depentyalase (PROD) activities were adapted from the method of Burke et al. (33). Nifedipine oxidase (NO) activity was determined by HPLC using a method adapted from Guengerich et al. (34). Total GST activity was measured with 1-chloro-2,4-dinitrobenzene as substrate according to Habig et al. (35). The UGT activity was determined with p-nitrophenol as substrate by the method of Mulder and van Doorn (36). All these measurements were described elsewhere (26).

Western blot immunoc assays

Microsomal or cytosolic proteins (20 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride sheets. The sheets were incubated with polyclonal antibodies raised against rat CYP 1A1 (diluted 1:1000), CYP 2B1 (diluted 1:4000), CYP3A2 (diluted 1:2000), GST A5 (diluted 1:3000) and AFAR1 (diluted 1:1000). For the detection of CYP isoforms, hepatic microsomes from rats treated with methylcholanthrene, phenobarbital or dexamethasone were used as positive controls. For the detection of GST A5, hepatic cytosol from male mice was used as a control since antibodies raised against rat GST A5 cross-react with the ortholog murine GST A3, which has the same electrophoretic mobility as rat GST A5 (37). Rat kidney cytosols were used as a positive control for the detection of rat AFAR1 because substantial amounts of this isoform was found in kidney (38). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and 4-chloro-1-naphtol. The stained sheets were scanned and the intensities of individual bands were measured using Optimas image analyzer software (Media Cybernetics, USA). Comparisons were made between the blot intensities of treated rats with those of control rats. Results were expressed as a percentage of the control.

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Food intake and growth of rats
The presence of garlic powder in the diet modified the food intake of the rats for several days. The growth of the treated rats declined during the first few days due to lower food consumption. Afterwards, the rats recovered and at the end of the experiment the weights of the rats were not significantly different in all groups (Table II). There was no statistical difference between liver weights of the different groups. One rat in the control group died during the hepatectomy.

Morphometric analysis of preneoplastic foci
The consumption of garlic powder S400 decreased the number of GST-P-positive foci by 72% and the percentage of the section area occupied by foci by 74% (Table III). The distribution of foci in size classes showed a significant reduction in the number of foci in nearly all classes (Figure 4). In the S0 and S200 groups the number of foci and their area were reduced by 20–35%, but these decreases were not significant (Dunnett’s test). Correlation analysis showed that the number of foci and their areas were inversely correlated to the alliin content of the garlic powder (Table III).

Enzymes involved in the metabolism of AFB1
EROD activity was increased from 44 to 170% in the groups S0, S200 and S400 while PROD and NO activities were not significantly modified by the garlic treatments (Table IV). Quantitative estimates of individual CYP levels were performed by western blotting (two rats per treatment). The levels of CYP 1A2, only analyzed in control and S0- and S200-treated rats, showed a slight increase. The levels of CYP 2B1 were increased over control values whereas those of CYP 3A2 were slightly modified by the different garlic treatments (Table V). GST and UGT activities were significantly increased by all the treatments (Table IV). In order to determine whether some specific GST isoenzymes were responsible for the increase in GST activity, the levels of GST A5 were examined by western blotting. GST A5 is not constitively expressed in control rat livers. It was not detected in S0- and S200-treated rats, while it appeared in S400-treated rats. The activity of AFAR was not measured. However, immunoblots carried out in hepatic microsomes with an antibody against AFAR revealed an increase in the intensities of the bands for rats treated with garlic in comparison with the control (Table V).

Discussion
This study confirms the efficacy of dietary garlic in the prevention of hepatocarcinogenesis and highlights its positive correlation with the alliin content of the garlic powders.
Several experimental studies have previously explored the protective capacity of garlic against chemical carcinogenesis. Dietary administration of garlic (0.25%) to rats significantly reduced the number of $\gamma$-glutamyl transpeptidase-positive foci induced by AFB1 (16). El-Mofty et al. showed that feeding toads with fresh minced garlic or garlic oil resulted in a marked reduction in the incidence of liver and kidney tumors induced by AFB1 (39). Other animal experiments demonstrated that oral or topical administration of garlic extracts inhibited chemically induced carcinogenesis in several species and several organs, such as colon (12), breast (10,40,41), liver (13,15), skin (14,42), buccal pouch (43) and stomach (44). All these studies stressed the role of sulfur compounds for the chemoprotective effect of garlic, but in most of these studies the exact composition of the garlic extracts was not known. Two factors may substantially modify the sulfur composition of the bulb: the plant variety and environmental factors such as climate or fertilizer (17,18). In addition, the composition of garlic extracts may vary significantly depending on the processing applied (45). For these reasons it is difficult to compare the efficacies of different garlic preparations in inhibiting experimentally induced tumors. The present study demonstrated that increased alliin content of the garlic powder is associated with a proportional decrease in the number and area of preneoplastic foci induced by AFB1. By controlling some cropping factors, it was possible to modify the alliin content of garlic. These results point to the importance of controlling the manner in which garlic is cultivated when evaluating its anticancer properties.

In this study we also investigated the mechanisms responsible for the chemoprotective properties of garlic against AFB1-induced carcinogenesis by measuring the activities and the levels of enzymes involved in the metabolism of AFB1. AFB1 is a procarcinogen requiring metabolic activation to express its carcinogenicity. AFB1 is activated to the ultimate metabolite AFBO by CYP 2C11 and CYP 3A2 in the rat (19). Other CYPs, such as CYP 1A and CYP 2B, are involved in the formation of several hydroxylated metabolites which are less genotoxic than the epoxide (21,22). Induction of phase II enzymes plays an important role in protection against AFB1 genotoxicity. Indeed, the ultimate metabolite of AFB1, AFBO, is conjugated with glutathione by GST and more specifically

![Fig. 4. Size distribution of GST-P foci in the different groups. Values are means ± SEM (n = 9 or 10 rats). *Significantly different from control (C) mean (Dunnett’s test, P ≤ 0.05).](https://academic.oup.com/carcin/article-abstract/25/10/1953/2475816)
by GST A5 (23). In addition it has been proposed that AFAR can also reduce the cytotoxicity of AFB1 by preventing binding of the dialdehyde form of AFB1 to intracellular proteins (24). In this study garlic administration slightly increased CYP 1A-mediated EROD activity while no modification of CYP 3A-mediated NO activity was observed. We also showed an increase in the activities of GST and UGT and an increase in the levels of GST A5 (only in the S400 group) and AFAR. We suggest that garlic consumption prevents AFB1 carcinogenicity by favoring the formation of AFBO-glutathione conjugate through up-regulation of GST and by increasing the formation of hydroxylated metabolites of AFB1 which can be further conjugated by UGT. This hypothesis is supported by the fact that there is a significant correlation between the reduction in the number of foci and the activities of GST and UGT and also a significant correlation with the levels of AFAR quantified by western blot (Table VI). Elsewhere, garlic could have anti-initiating effects through mechanisms other than drug metabolizing enzyme modulation. A direct protective effect cannot be excluded, as garlic compounds can react with electrophilic species and inactivate them (46–48). Garlic could also mediate its preventive effects by enhancing antioxidant status (13,49).

The present study suggests that compounds derived from alliin may be the active constituents of garlic accounting for the decreased number of foci. Diallyl disulfide (DADS) was previously shown to strongly reduce the number of noreneoplastic foci initiated by AFB1 in the rat liver (8). This reduction was related to the modulating effects exerted by this compound on the enzymes involved in the detoxification of AFB1. DADS treatment produced induction of GST, particularly GST A5, as well as induction of AFAR (25,50). Therefore garlic and DADS ingestion have similar mechanism of action in inhibiting hepatocarcinogenesis initiated by AFB1. Under our HPLC analysis conditions DADS was not detected in the garlic powder. Nonetheless, it was shown that the garlic powders had the capacity to release alliin upon contact with water (45). To our knowledge, there is no indication that alliin has chemopreventive effects in animals. We suggest that alliin could be formed in the diet or after ingestion. In a separate experiment, we identified vinylidithiins by GC-MS in the stomach of rats dosed with a mixture of garlic powder and water (unpublished results). This is indirect evidence of the presence of alliin in the rat body, since these latter compounds are formed by thermal degradation of alliin (6). Afterwards alliin could be further transformed to DADS. There is very little information on the metabolites present in rat tissues after dietary garlic ingestion. Egen-Schwind et al. (51) have identified DADS as a metabolite of alliin in perfused rat liver. DADS and other volatile sulfur compounds were detected in urine following garlic oil ingestion by a human subject (52). Therefore, DADS could be one of the active metabolites of garlic responsible for its anticarcinogenic effects. Other compounds could also be involved since alliin is transformed into a large number of compounds (6).

In conclusion, this study has demonstrated that consumption of garlic is efficient in protecting against AFB1 carcinogenesis. By controlling some cropping factors for garlic, particularly the sulfur supply, it is possible to enhance the protective effect of garlic. As garlic consumption could be an attractive strategy for chemoprevention in individuals who are exposed to dietary aflatoxins and other chemical carcinogens, additional investigations should be carried out to determine if the protective effects obtained in animals are relevant to humans.

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**References**


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