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Efficacy of allicin, the reactive molecule of garlic, in inhibiting *Aspergillus* spp. *in vitro*, and in a murine model of disseminated aspergillosis

Yona Shadkchan[1], Einav Shemesh[1], David Mirelman[2], Talia Miron[2], Aharon Rabinkov[2], Meir Wilchek[2] and Nir Osherov[1]*

[1]Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel; [2]Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel

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***Objectives*: The evaluation of allicin, the biologically active compound responsible for the antimicrobial activities of freshly crushed garlic cloves, in inhibiting *Aspergillus* spp. *in vitro* and in a murine model of disseminated aspergillosis.**

***Methods*: Pure allicin was prepared by reacting synthetic alliin with a stabilized preparation of the garlic enzyme alliinase. We tested the *in vitro* efficacy of pure allicin against 31 clinical isolates of *Aspergillus* spp. using a microdilution broth method and following the NCCLS guidelines (document M-38P). Subsequently, the *in vivo* efficacy of allicin was tested in immunocompetent mice infected intravenously (iv) with *Aspergillus fumigatus* conidia. Allicin (5 mg/kg body weight) was administered iv once daily for 5 days post-infection or orally (po) (9 mg/kg body weight) for 5 days pre-infection and 10 days post-infection. No ill effects were observed in allicin-treated uninfected mice.**

***Results*: The *in vitro* MICs and MFCs of allicin were between 8 and 32 mg/L, indicating that allicin in its pure form may be an effective fungicide *in vitro*. Time–kill studies indicate that allicin exerts its fungicidal activity within 2–12 h of administration *in vitro*. Allicin treatment significantly prolonged survival of infected mice ($P < 0.01$) from mean survival time (MST) = 7.7 days in untreated mice to MST = 21.3 and 13.9 days for allicin iv and po treated mice, respectively. Allicin iv treatment led to a significant ($P < 0.001$) 10-fold reduction in fungal burden in *A. fumigatus* infected mice as evaluated by quantitative fungal cultures of kidney tissue samples.**

***Conclusions*: These favourable results, despite the short half-life of this compound *in vivo*, support further studies of controlled sustained release or more prolonged administration of allicin as a treatment for aspergillosis.**

Keywords: antifungal treatment, susceptibility testing, mouse models

Introduction

Allicin (diallylthiosulphinate) is one of the active compounds of freshly crushed garlic (*Allium sativum*). Allicin possesses a variety of biological activities such as antimicrobial, anti-inflammatory, antithrombotic, anti-atherosclerotic, serum lipid lowering and anticancer activities.[1–5] Allicin is produced by an enzymic reaction when raw garlic is crushed or injured. The enzyme, alliinase, stored in a separate compartment in garlic, combines with a compound called alliin in raw garlic and produces allicin. The antimicrobial mode of action of allicin is thought to be the inhibition of thiol-containing enzymes in the microorganisms. In the amoebic parasite *Entamoeba histolytica*, allicin was found to strongly inhibit cysteine proteases, alcohol dehydrogenases and thioredoxin reductases.[6] The antifungal activity of garlic extracts has been observed *in vitro* against *Cryptococcus neoformans*,[7] *Candida*[8] and *Aspergillus* spp.[9,10] The potential of allicin as an antifungal agent *in vivo* has not been clearly illustrated to date. Davis *et al.*[7] showed that the cerebrospinal fluid of two patients infected with *C. neoformans* and treated with a commercial

*Correspondence address. Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Tel-Aviv, Israel. Tel: +972-3-640-9599; Fax: +972-3-640-9160; E-mail: nosherov@post.tau.ac.il

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garlic extract has anti-*C. neoformans* activity. Ghannoum[11] demonstrated that an aqueous garlic extract diminished adherence of *C. albicans* to buccal epithelial cells. In Asia, *A. sativum* (garlic)-derived preparations are used alone or with amphotericin B to treat human systemic fungal infections and cryptococcal meningitis. *In vitro* synergy was demonstrated when both compounds were combined against *C. neoformans*.[12]

Invasive aspergillosis (IA) is an emerging, highly lethal infection of the immunocompromised host.[13] Even with the best antifungal drugs, mortality is extremely high, reaching 94%.[14] In this report we demonstrate the inhibitory effect of pure allicin on the growth of *Aspergillus* spp. *in vitro*, and in a murine model of IA.

Materials and methods

Allicin preparation

Allicin was produced by passing the synthetic substrate alliin [(+)*S*-2-propenyl L-cysteine *S*-oxide] through an immobilized alliinase column.[15] The concentration of allicin in the solution was regularly confirmed by sample analysis using high-performance liquid chromatography.[3] Allicin was kept in a dark tightly closed flask at 4°C and remained stable during all periods of the experiment (>3 months).

In vitro assays

The *in vitro* efficacy of pure allicin was tested against 31 clinical isolates of *Aspergillus* spp. [*A. fumigatus* (n = 13), *Aspergillus niger* (n = 6), *Aspergillus flavus* (n = 6), *Aspergillus terreus* (n = 6)] by a microdilution broth method and following NCCLS guidelines (document M-38P). The minimal fungicidal concentration (MFC) was the lowest drug concentration resulting in a 99.9% loss of viability as assessed by plating. MICs and minimal effective concentrations (MECs) were calculated as the lowest drug concentration resulting in complete inhibition of hyphal growth and lowest drug concentration resulting in aberrant hyphal growth,[16] respectively. Values were assessed after 48 h of incubation in standard 96-well sterile flat-bottomed polystyrene plates (Corning, USA). Control strains (*A. fumigatus* strain AF293, *A. niger* strain ATCC 16404) were tested in every experiment as internal controls. Each well received 100 µL of the diluted drug concentrations. Dilutions were made in RPMI 1640 medium containing 0.165 M MOPS buffer at pH 7.0 (RPMI/MOPS medium). Conidial inocula were counted using a haemocytometer, prepared at a concentration of 2.5×10^4 cfu/mL in RPMI/MOPS medium and added at 100 µL/well (final volume of each well, conidia and allicin, 200 µL).

For time–kill studies, representative strains Afm-13, An-1, At-3 and Afl-3 were grown as described above, in the presence of 16 mg/L allicin. This concentration was selected because it is at or 2-fold above the MFC determined for these strains in the microdilution broth method described above. After 1, 2, 4, 8, 12 and 24 h conidia and germlings in culture were removed by scraping, serially diluted in 2-fold dilutions and plated on Sabouraud agar plates. Colonies were counted after incubation for 48 h at 37°C. The MFC was calculated as the drug concentration that resulted in a 99.9% loss of viability after 1–24 h in the presence of allicin as compared with untreated viable spore counts at 0 h. Control measurements of untreated conidia over time cannot be accurately defined using this method because of clumping and the formation of a mycelium, while all the conidia grown in the presence of allicin did not undergo clumping and were amenable to plating and counting of colonies.

Murine model of aspergillosis

For the murine model of aspergillosis, female ICR mice, 4–6 weeks old, were inoculated intravenously via the lateral tail vein with *A. fumigatus* strain AF293 (Afm-13) using an inoculum of 5×10^6 freshly harvested conidia/mouse. Three means of administration, intraperitoneal (ip), intravenous (iv) and oral (po) were tested for allicin. For ip administration, allicin was diluted in saline and injected at 1.25, 2.5 and 5 mg/kg/day. The two higher concentrations caused discomfort to the animals and were discontinued. The lower concentration was ineffective when administered once daily for 5 days starting 1 h post-infection (data not shown). For iv administration for the dose-range experiment, allicin was administered iv once daily for 5 days starting 1 h post-infection, at 1.25, 2.5, 5 mg/kg body weight/day, 0.2 mL/mouse, diluted in saline, with 10 mice per group. Higher concentrations of allicin administered iv (9 mg/kg/day) caused discomfort to the animals and were discontinued. For po treatment, a regimen of 5 mg/kg/day for 5 days post infection was ineffective (data not shown). We therefore tested a modified version of the allicin po regimen described previously for the treatment of hyperlipidaemic rabbits;[3] allicin was administered po at 9 mg/kg body weight/day, 0.2 mL/mouse, diluted in saline and 20 mice per group, for 5 days pre-infection and 10 days post-infection. Amphotericin B (AMB) was also tested as a comparative standard. AMB was administered ip once daily for 5 days, starting 1 h post-infection at a concentration of 0.5 mg/kg/day, 0.2 mL/mouse, with 10 mice per group. Infection was followed up for 28 days and evaluated in terms of mortality.

For studies of tissue burden, two groups of 20 mice were infected with *A. fumigatus* strain AF293 using an inoculum of 5×10^6 freshly harvested conidia/mouse. One group was mock treated with saline iv, and the other was treated with allicin iv at 5 mg/kg/day for 5 days as described above. Two randomly selected mice were sacrificed from each group on days 2, 4, 7, 10 and 14 after infection. From each mouse, both kidneys were aseptically removed, homogenized separately in 1 mL of saline and cultured for quantitative analysis in serial 10-fold dilutions as described previously.[17] Permission for the animal experiments described in this study was granted by the institutional care and use committee of the Faculty of Medicine, Tel Aviv University.

Statistical analysis

Statistical analysis of mouse survival was performed by the log rank test using the Graphpad Prism 4 software package (Graphpad Software Inc., San Diego, CA, USA). P values of <0.05 were considered significant in these analyses. Error bars denote standard deviation.

Results

In vitro studies

The mean MECs and MICs obtained for each of the *Aspergillus* spp. at 48 h were: *A. fumigatus* (n = 13), 4 and 8 mg/L; *A. flavus* (n = 6), 16 and 16 mg/L; *A. niger* (n = 6), 16 and 32 mg/L; and *A. terreus* (n = 6), 8 and 16 mg/L, respectively. In pilot experiments, allicin exhibited fungicidal activity against all four *Aspergillus* spp. tested at MIC concentrations. The mean MECs, MICs and MFCs of allicin for *A. fumigatus* were ~2- to 4-fold lower than for *A. niger*, *A. flavus* and *A. terreus*.

Time–kill study results show that conidia grown from strains *A. fumigatus 13*, *A. niger 1*, *A. terreus 3* and *A. flavus 2* displayed a loss in viability after 8–12 h of incubation in the presence of 16 mg/L allicin (Figure 1).

In vivo studies

We tested three routes of administration for allicin, ip, iv and po:

(i) ip treatment caused discomfort to the animals and was not effective (data not shown).

(ii) iv treatment: control infected mice receiving saline iv showed 100% mortality [mean survival time (MST) = 7.2 days]. Mice treated

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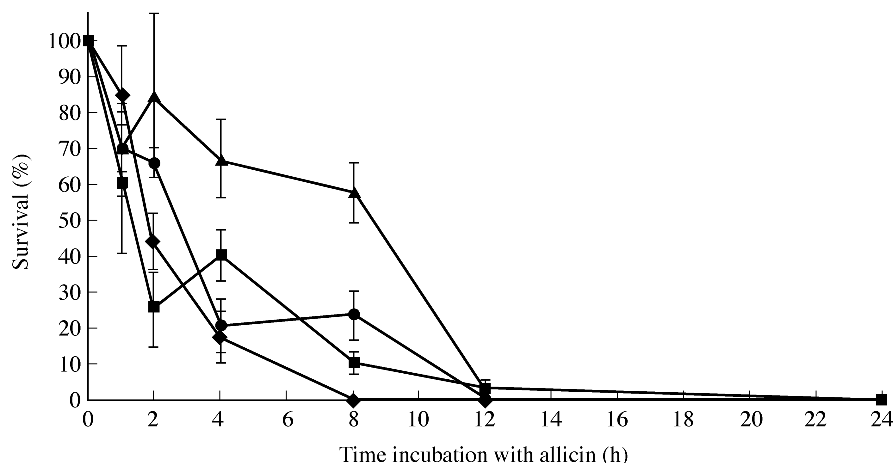

Figure 1. Time–kill curve of *A. fumigatus* (Afm-13) (circles), *A. niger 1* (An-1) (diamonds), *A. terreus 3* (At-3), (squares) and *A. flavus 3* (Afl-3) (triangles) conidia incubated in the presence of 16 mg/L allicin. The results are representative of two independent experiments performed in triplicate. Error bars denote ±S.D.

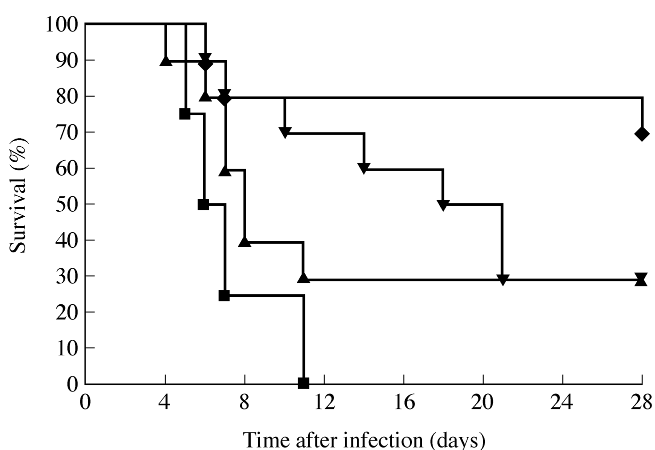

Figure 2. Survival curves for mice infected with *A. fumigatus* strain AF-293 (Afm-13) and treated with various doses of allicin iv: squares, saline-treated control; triangles up, allicin at 1.25 mg/kg; triangles down, 2.5 mg/kg; diamonds, 5 mg/kg. Each group contained 10 mice.

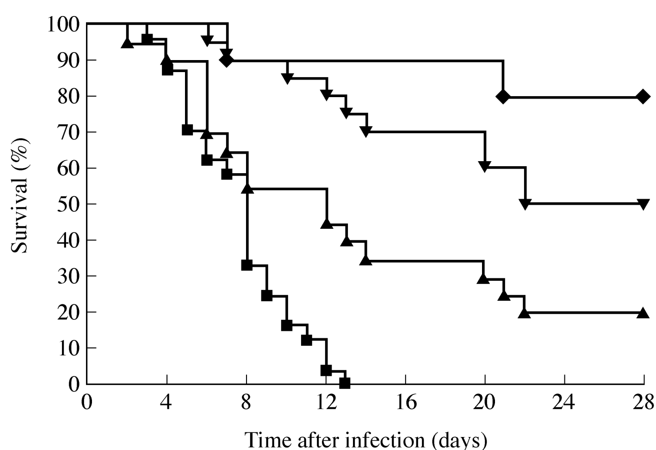

Figure 3. Survival curves for mice infected with *A. fumigatus* strain AF-293 (Afm-13) saline treated (squares) (n = 20), treated with allicin iv, 5 mg/kg/day (triangles down) (n = 20); allicin po, 9 mg/kg/day (triangles up) (n = 20) and amphotericin B (AMB) ip, 0.5 mg/kg/day (diamonds) (n = 10).

with allicin iv at 1.25, 2.5 and 5 mg/kg/day gave survival rates of 30% (MST = 13.5 days), 30% (MST = 18.1 days) and 70% (MST = 23.7 days), respectively (Figure 2). Survival time was significantly increased in mice treated with 2.5 and 5 mg/kg/day of allicin compared with the saline-treated mice (P < 0.01, 0.005, respectively). The allicin iv regimen at 5 mg/kg/day was subsequently retested in a larger group of animals and compared with the 'gold standard' treatment with amphotericin B (Figure 3). Control infected mice (20 animals) receiving saline iv, showed 100% mortality (MST = 7.7 days). The survival curve for mice treated with allicin iv (5 mg/kg/day, 20 animals) showed 50% survival (MST iv = 21.3 days, P < 0.01 compared with the controls). Treatment with amphotericin B (0.5 mg/kg/day, 10 animals) gave an 80% survival rate (MST = 25.1 days, P<0.005 compared with the controls) (Figure 3). Treatment with amphotericin B was significantly more effective than treatment with allicin iv (5 mg/kg/day) (P < 0.05).

(iii) po treatment: mice (n = 20) were treated with allicin po at 9 mg/kg/day, for 5 days pre-infection and 10 days post-infection, based on a modified version of the allicin po regimen described previously for the treatment of hyperlipidaemic rabbits[3]. This treatment gave a survival rate of 20% (MST = 13.9 days), significantly better than that of control untreated mice (P < 0.01) (Figure 3), but significantly below the efficacy of allicin iv treatment for 5 days at 5 mg/kg/day (P < 0.05).

We selected the most effective treatment regimen, allicin iv at 5 mg/kg/day, for further analysis of fungal burden. Two groups of mice (n = 20) were infected. One group was mock treated iv with saline, and the second was treated with allicin iv at 5 mg/kg/day for 5 days. On days 2, 4, 7, 10 and 14, two mice from each group were randomly selected and sacrificed. Fungal burden was determined as described in Materials and methods. Renal colony counts of mice infected with AF293 and mock treated with saline showed a dramatic increase in fungal load during the 2 weeks of infection (Table 1). Renal fungal load measured on days 7, 10 and 14 post-infection in mice treated with allicin (5 mg/kg/day, iv) was reduced ~10-fold as compared with the saline-treated mice (P < 0.001 compared with the controls).

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Table 1. Kidney fungal load for mice infected with *A. fumigatus* strain AF-293 and treated with allicin iv, 5 mg/kg/day or untreated (treated with saline)

Day after infection	cfu/g kidney (± S.D.)	
	untreated	treated
2	3671 ± 511[a]	2632 ± 177
4	7476 ± 675	1867 ± 479
7	17 968 ± 1265	3562 ± 235[b]
10	22 343 ± 3274	1656 ± 801[b]
14	208 906 ± 27 183	3312 ± 857[b]

[a]Average kidney fungal load of two mice sacrificed at each time point.
[b]$P < 0.001$ (Student's *t*-test).

Discussion

Little is known about the potential *in vivo* activity of components purified from natural herbs in the treatment of fungal infections. In this report we tested the efficacy of pure allicin, the biologically active compound responsible for the antimicrobial activity of freshly crushed garlic cloves in: (i) the killing of clinical isolates of *Aspergillus* spp. *in vitro*; (ii) the treatment of a murine model of disseminated aspergillosis.

Previous reports describing the *in vitro* efficacy of allicin against fungi were compromised by the fact that they used allicin that was produced from freshly crushed garlic cloves, which contains many additional untested compounds.[1,8,9,11,12] The potency and efficacy of such extracts were variable and difficult to reproduce reliably. In this report, we used pure allicin, prepared by passing the synthetic substrate alliin through an immobilized alliinase column. The allicin prepared by this method is chemically well defined; it is quantified by analysis and gives reliable, reproducible results. We show that pure allicin possesses effective (8–32 mg/L) and rapid (8–12 h) fungicidal activity against *Aspergillus* spp. *in vitro*. Isolates of *A. fumigatus* (responsible for >80% of IA infections) were notably more sensitive to allicin: the MFC for 12 of 13 isolates was 8 mg/L or lower. Our results are within the range of those seen previously for *Aspergillus* spp. using allicin extracts.[9]

Importantly, allicin is toxic to mammalian cells in culture at significantly higher concentrations (>60 mg/L) than those shown for *Aspergillus* spp.[2] This difference in sensitivity between fungal and mammalian cells may be explained by the higher concentrations of glutathione which the mammalian cells possess, protecting them from the thiolation activity of allicin.[6,18]

We show, for the first time, that allicin can significantly reduce mortality, prolong survival and reduce fungal load in mice infected with *A. fumigatus*. The rate of killing of the fungus was shown by us to be very rapid and though the half-life of allicin in blood, solvents and simulated physiological fluids has been reported to be rather short (50 min)[19] some of the degradation products, such as allyl mercaptan, may provide additional antifungal activity.[4,10] Nevertheless, even the most effective allicin treatment was not as successful as treatment with AMB, necessitating more study to find a better treatment modality. The *in vivo* antifungal activity of allicin could be improved perhaps by controlled sustained release using prolonged iv infusion. Targeted production of allicin at the site of *Aspergillus* infection may also hold promise. Such a targeted production was recently achieved against cancer cells by the injection of a conjugate consisting of a monoclonal antibody that was chemically ligated to the enzyme alliinase, followed by injection of the substrate alliin.[20] A similar approach could further increase the efficacy of this compound in the treatment of aspergillosis.

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