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Aspirochlorine: A Highly Selective and Potent Inhibitor of Fungal Protein Synthesis

FEDERICA MONTI†, FRANCA RIPAMONTI, STEPHEN P. HAWSER†
and KHALID ISLAM†,*

Lepetit Research Center,
Via R. Lepetit 34, 21040 Gerenzano (Varese), Italy

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Aspirochlorine, a compound belonging to the gliotoxin family of compounds, exhibits antifungal and antibacterial activity but its mechanism of action remains unknown. In this study we show that aspirochlorine inhibits the pathogenic fungus *Candida albicans* by acting on fungal protein synthesis. The compound selectively inhibits cell-free protein synthesis when using a *C. albicans* system, but does not inhibit this synthesis *in vitro* when tested with bacterial and mammalian systems. Moreover, in intact *C. albicans* cells, aspirochlorine inhibits protein synthesis but does not inhibit chitin, DNA or glucan synthesis though at high concentrations some inhibition of RNA synthesis is observed. By contrast, in intact *Bacillus subtilis* cells, aspirochlorine did not inhibit protein, DNA, or cell wall synthesis though it significantly inhibited RNA synthesis. Furthermore, using heterologous systems (mammalian ribosomes and *C. albicans* cytosolic factors) the data suggest that the inhibitory action of aspirochlorine is not exerted through a direct interaction with *C. albicans* EF-1 or EF-2.

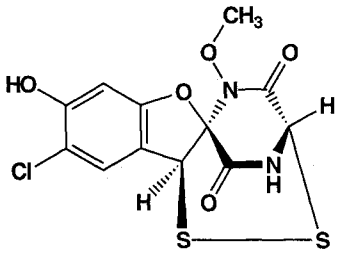
Aspirochlorine has been separately isolated from microbial fermentation broths of *Aspergillus* species by different groups and the structure of aspirochlorine has been determined by both NMR and X-ray crystallography, shown to be identical with that of oryzachlorine[1] and A 30641[2~5]. The compound belongs to the epipolythiodioxopiperazines (ETPs) class which consist of a large group of fungal toxins notably gliotoxin[6]. Gliotoxin, and several ETPs, are known to exhibit multiple activities including antifungal, antibacterial, antitumor, antiviral and immunomodulatory[6~9]. ETPs have been shown to inhibit viral RNA-directed RNA and DNA polymerases as well as RNA synthesis in HeLa cells[9]. More recently, it has been shown that gliotoxin causes oxidative damage to plasmid and cellular DNA which may be responsible for the cytotoxic effects of the compound in eukaryotic cells[10].

Aspirochlorine has been shown to exhibit good activity against several yeasts[1,2]. In addition, the compound has been reported to be active against *Staphylococcus aureus*[2] and to be active against newcastle disease virus[1]. Furthermore, aspirochlorine has been shown to be active against Ehrlich ascites tumor in mice, suggesting that the compound is bioavailable *in vivo*[1]. The lethal toxicity of the compound in mice is about 100 mg/kg[2], which is significantly lower when compared with polyenic antifungal agents such as amphotericin B (4 mg/kg). However, the mechanism of action of aspirochlorine particularly as regards its antifungal and antibacterial activity remains to be elucidated. By analogy with gliotoxin and other ETPs it is likely that aspirochlorine affects RNA or DNA to exert its antiviral and anti-tumor activity[6,9].

During the course of a screening programme, aimed at isolating molecules exhibiting inhibitory activity in protein synthesis systems, we repeatedly isolated aspirochlorine from the fermentation broths of *Aspergillus*. In this paper we now show that aspirochlorine, but

† Present Address: Hoechst Marion Roussel, Route De Noisy 102, Romainville Cedex, F-93235, France.

Fig. 1. Structure of aspirochlorine.



not other mycotoxins or gliotoxin, selectively inhibits fungal protein synthesis. Moreover, aspirochlorine does not inhibit either mammalian or bacterial protein synthesis. Additional studies suggest that aspirochlorine may inhibit fungal protein synthesis by acting on an essential cytosolic factor. An abstract of this work has recently been presented[11].

Results

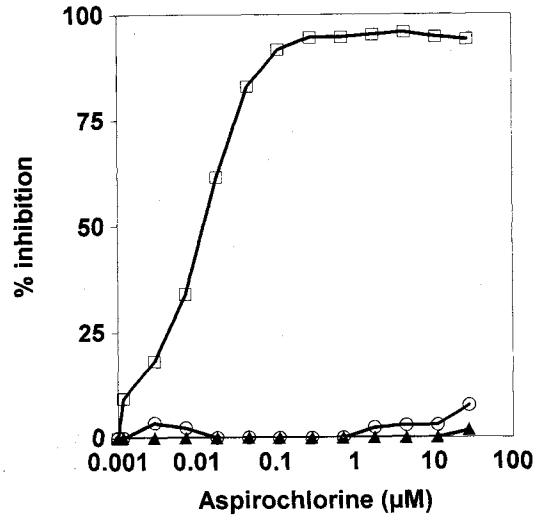
Isolation and Identification

A. flavus GE 49710 was identified in our screening programme as a producer of an inhibitory activity against *C. albicans* cell-free protein synthesis. Fermentation of this strain, isolation and structure elucidation of the active chemical entity showed that it was identical to the previously described compound aspirochlorine, also know as oryzachlorine or A-30641 (see Fig. 1).

Poly(U)-Directed Poly(Phe) Synthesis

The effect of aspirochlorine on the cell-free protein synthesis systems from *C. albicans*, *E. coli* and rabbit reticulocyte was examined. As shown in Figure 2, aspirochlorine exhibited a dose-dependent inhibition of *C. albicans* cell-free synthesis with an IC_{50} (the amount of compound required to inhibit protein synthesis by 50%) of about 11 nM and complete inhibition was observed at about 100 nM. By contrast, aspirochlorine failed to inhibit bacterial (*E. coli*) or rabbit (similar results were also obtained when using a cell-free protein synthesis system from rat liver; see below) protein synthesis systems even at high concentrations of aspirochlorine (> 100 μM; Fig. 2). Anisomycin and fusidic acid, known inhibitors of eukaryotic protein synthesis[12,13], efficiently inhibited both mammalian and fungal protein synthesis (Table 1). Purpuromycin, known to inhibit prokaryotic and eukaryotic protein synthesis[14,15], inhibited all three systems. On the other hand, three inhibitors of bacterial protein synthesis, namely kirromycin, pulvomycin and MDL 62,879[16], efficiently inhibited the bacterial system but did not inhibit the eukaryotic protein synthesis (Table 1). Taken together, these data would suggest that aspirochlorine specifically inhibits fungal protein synthesis but does not affect bacterial or mammalian protein synthesis.

Fig. 2. Effect of aspirochlorine on cell free protein synthesis systems from *C. albicans* (□), *E. coli* (▲) and rabbit reticulocyte (○).



Aspirochlorine was added to each system at time 0 at final concentrations of 0.03 nM to 30 μM. After 30 minutes the reaction was terminated by the addition of cold TCA and the incorporation into TCA-precipitated material determined by liquid scintillation counting.

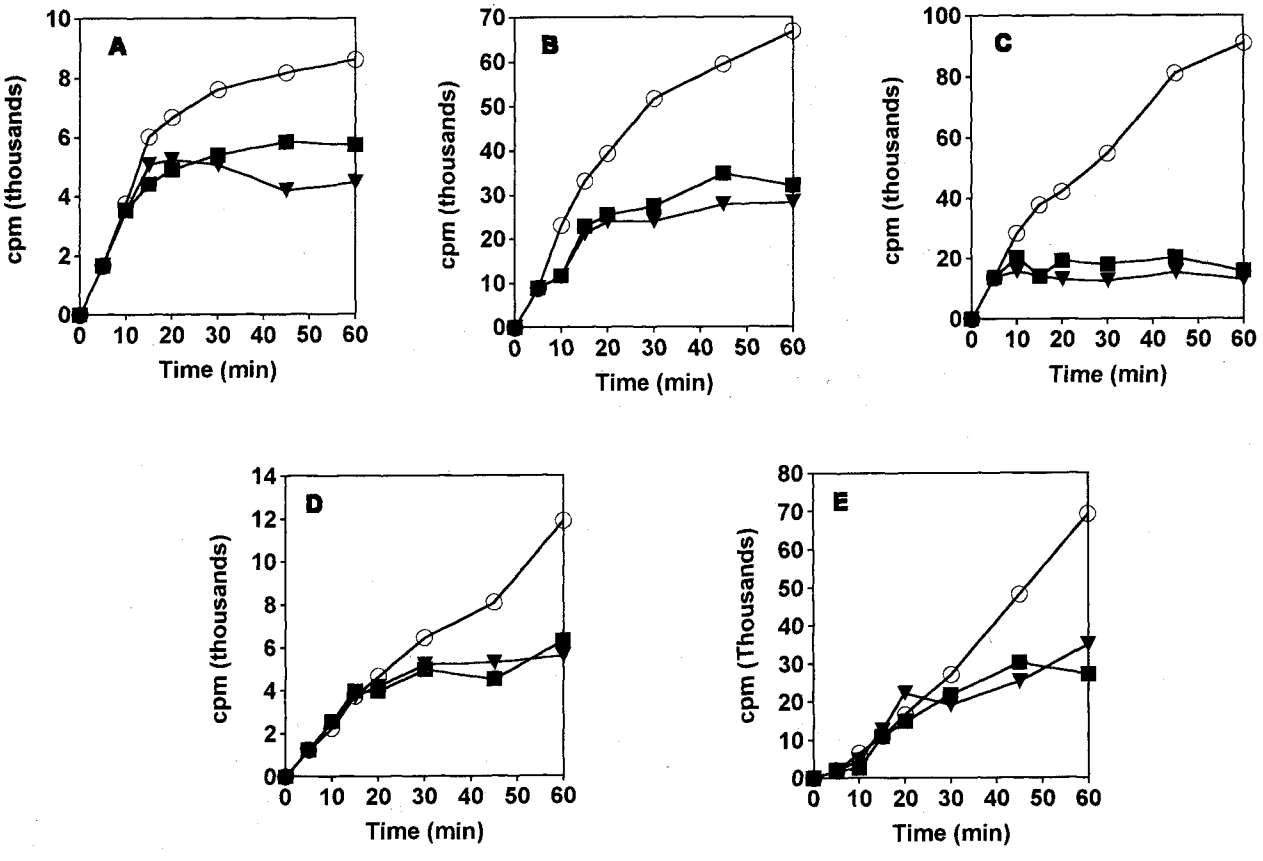
Macromolecular Syntheses

In view of the observations that aspirochlorine exhibited a selective inhibitory activity against *C. albicans* protein synthesis and showed good antifungal activity (MIC of 0.25 μg/ml; see also[1,2]), we decided to further investigate the effects of aspirochlorine in macromolecular syntheses using intact *C. albicans* cells. The time course of DNA, RNA, protein, chitin and glucan syntheses were examined in the absence and after addition

Table 1. Effects of different inhibitors on cell-free protein synthesis.

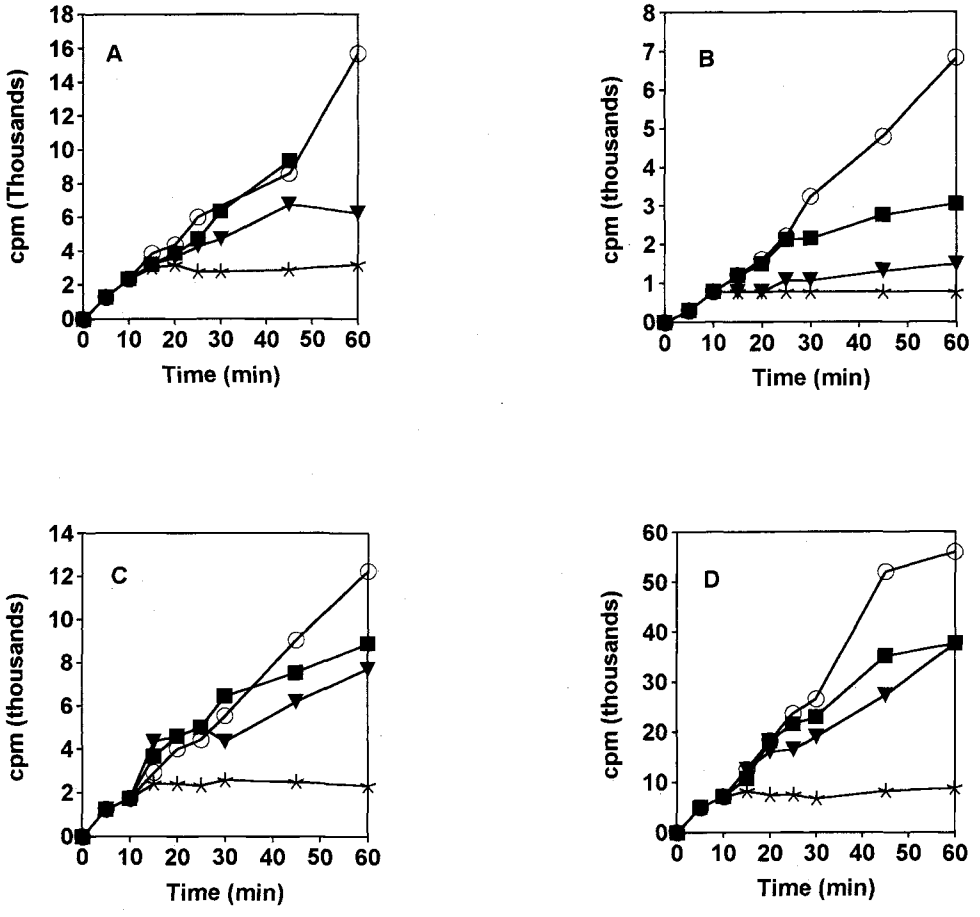
Inhibitor	Site of action	IC_{50} (μM)		
		Candida albicans	*Escherichia coli*	Rabbit
Kirromycin	Elongation factor Tu	>100	0.125	>100
MDL 62,879	Elongation factor Tu	>100	0.40	>100
Pulvomycin	Elongation factor Tu	>100	1	>100
Thiostrepton	Elongation factor G	>100	0.60	>100
Fusidic acid	Elongation factor G	6	>100	40
Anisomycin	Ribosome	0.75	>100	7.50
Purpuromycin	tRNA aminoacylation	10	35	5
Aspirochlorine	Unknown	0.01	>100	>100

Fig. 3. Effect of aspirochlorine on macromolecular syntheses by intact *C. albicans* cells.
(A) DNA, (B) RNA, (C) protein, (D) glucan and (E) chitin.



The reaction was initiated by the addition of appropriate precursors (see Materials & Methods) and after 5 minutes either buffer (○) or aspirochlorin was added at final concentrations of 10 μg/ml (■) or 50 μg/ml (▼). At the indicated times aliquots were removed, the reaction terminated and the incorporation determined by scintillation counting.

Fig. 4. Effect of aspirochlorine on macromolecular syntheses by intact *B. subtilis* cells.
(A) DNA, (B) RNA, (C) protein and (D) cell wall.

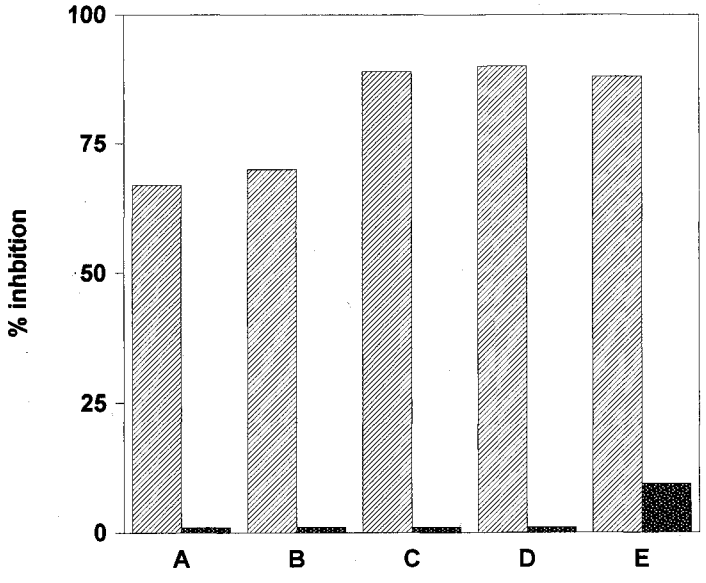


Buffer (○) or aspirochlorine was added 10 minutes after initiation of each reaction at final concentrations of 80 μg/ml (■) or 200 μg/ml (▼) and each reaction run for a total of 60 minutes. Control experiments (*) employed the following compounds: Nalidixic acid for DNA synthesis, 50 μg/ml; rifampicin for RNA synthesis, 0.2 μg/ml; chloramphenicol for protein synthesis, 25 μg/ml; teicoplanin for cell wall synthesis, 5 μg/ml.

of aspirochlorine using appropriate precursors (see Experimental). The initial rate of DNA, chitin and glucan synthesis in the absence of aspirochlorine was similar to that observed after the addition of aspirochlorine (see Fig. 3A, D and E), and a decrease in the syntheses was observed only at longer time-periods. By contrast, the addition of aspirochlorine resulted in a rapid inhibition of the initial rate of protein synthesis when compared to the synthesis in the absence of aspirochlorine (Fig. 3C). In addition, a reduction in RNA synthesis was also observed (Fig. 3B), although it was not so marked as that on protein synthesis (*cf.* Fig. 3B and C). Aspirochlorine was also used in dose-dependent experiments whereby it was tested over a concentration range of 1～100 fold its MIC on *C. albicans*. Typically, aspirochlorine efficiently inhibited the rate of protein synthesis at concentrations close to the MIC and had no effect on the rates of synthesis of DNA, glucan or chitin at up to 100-fold the MIC, an effect on the rate of RNA synthesis was observed only at 20～40 fold the MIC.

In view of the antibacterial activity of aspirochlorine we also investigated its effects on macromolecular synthesis in *B. subtilis*. As shown in Figure 4, aspirochlorine typically failed to inhibit protein, DNA or cell wall syntheses in comparison to the corresponding positive controls, namely nalidixic acid (DNA), teicoplanin (cell wall) and chloramphenicol (protein). However, aspirochlorine did exhibit a significant inhibitory activity

Fig. 5. Effect of aspirochlorine on cell free protein synthesis in reconstituted heterologous and homologous systems.



Aspirochlorine was added at a final concentrations: A = 0.014 μM, B = 0.028 μM, C = 0.278 μM, D = 2.777 μM and E = 27.777 μM to the following systems: homologous *C. albicans* system (▨) consisting of S-100 *C. albicans* (17 μg) + *C. albicans* ribosomes (4.98 pmol); heterologous system (■) consisting of S-100 *C. albicans* (17 μg) + rat ribosomes (5 pmol). The total counts incorporated after 30 minutes incubation were determined.

against bacterial RNA synthesis (Fig. 4) indicating that its antibacterial activity *in vitro* may be due to inhibition of RNA synthesis.

Reconstituted Systems

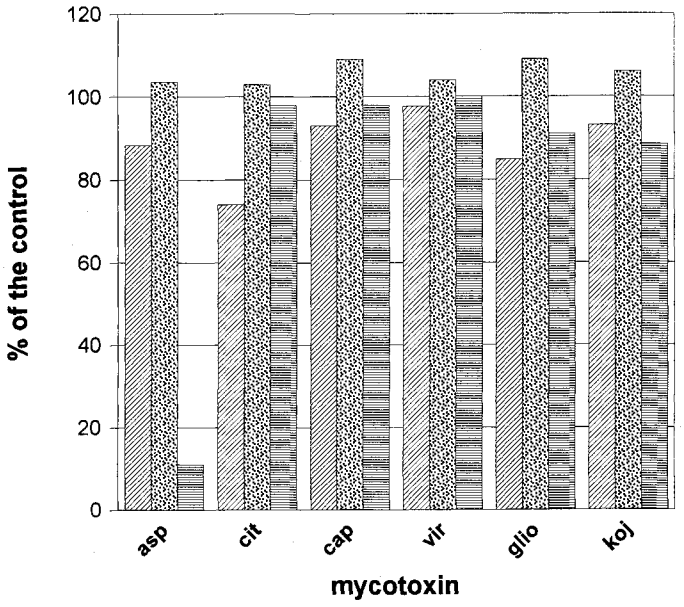
We also examined the action of aspirochlorine on heterologous and homologous reconstituted systems. It is known that elongation factors (EF) EF-1 and EF-2 from *C. albicans* can functionally substitute those from their mammalian counterparts[17]. However, fungal protein synthesis is known to require an additional EF, namely EF-3[18,19]. Indeed, in a heterologous reconstituted system consisting of ribosomes from *C. albicans* and mammalian S-100 no poly(U)-directed poly(Phe) synthesis was observed, indicating that the ribosomes were free of EF-3. The homologous system reconstituted with *C. albicans* ribosomes and *C. albicans* S-100 exhibited protein synthesis activity and this activity was efficiently inhibited by 10 ~ 20 nM aspirochlorine (Fig. 5). By contrast, a heterologous system where mammalian ribosomes were reconstituted with *C. albicans* S-100 exhibited poly(U)-directed poly(Phe) synthesis but the synthesis was not inhibited by aspirochlorine (Fig. 5) even at high concentrations (30 μM).

The effects of gliotoxin and other mycotoxins were also examined in such systems. As shown in Fig. 6, these mycotoxins had little or no effect either in the reconstituted *C. albicans* system or in the heterologous system consisting of mammalian ribosomes and *C. albicans* S-100. Similarly, these mycotoxins failed to inhibit the reconstituted homologous mammalian system. These data would, therefore, suggest that the mycotoxins do not inhibit cell-free protein synthesis in either the fungal or the mammalian systems (Fig. 6).

Discussion

Aspirochlorine, a member of the ETP class of compounds, exhibits antifungal and antibacterial activity but its mechanism of action has not elucidated. Our studies show that it specifically inhibits fungal protein synthesis but does not inhibit protein synthesis in bacteria

Fig. 6. Effects of different mycotoxins on reconstituted homologous and heterologous protein synthesis systems.



The effects of the different agents (10 μg/ml) on protein synthesis were assessed in the following protein synthesis systems: (▨), rat ribosomes (18 pmol) + S-100 rat; (▩) rat ribosomes (18 pmol) + S-100 *Candida* (17 μg); (▤) *Candida* ribosomes (4.98 pmol) + S-100 *Candida* (17 μg). Key: asp, aspirochlorine; cit, citrinin; koj, kojic acid; glio, gliotoxin; vir, viridin. The bars represent the percentage inhibition of each system in comparison to the control.

or higher eukaryotes. Inhibition of fungal protein synthesis by aspirochlorine is dose-dependent with a potent IC_{50} of about 10 nM (Fig. 2 and Table 1). Studies on macromolecular syntheses in whole cell assays employing *C. albicans* and *B. subtilis* strongly suggest that the antifungal and antibacterial activities of aspirochlorine are attributable to the inhibition of protein (Fig. 3) and RNA synthesis (Fig. 4), respectively.

We compared the ability of aspirochlorine to inhibit fungal protein synthesis with that of different compounds which are known to inhibit protein synthesis. For example, we used kirromycin, MDL 62,879 and pulvomycin which specifically inhibit bacterial protein synthesis by interaction with EF-Tu[16], anisomycin and fusidic acid which inhibit both fungal and mammalian protein synthesis[12,13], and purpuromycin which inhibits both eukaryotic and prokaryotic protein synthesis[14,15]. In contrast to these compounds, aspirochlorine specifically and selectively inhibited fungal protein synthesis (Table 1). Furthermore when compared with the other inhibitors which all exert their action in the micromolar ranges, the fungal system appears to be exquisitely sensitive to aspirochlorine with an IC_{50} in the nanomolar range. Moreover, this action of aspirochlorine appears to be quite different when compared with other mycotoxins and gliotoxin which do not exhibit any inhibitory activity on eukaryotic protein synthesis under these conditions (Fig. 6).

It is known that protein synthesis in yeast and mammalian cells requires elongation factors, EF-1 and EF-2, and that EF-1 and EF-2 from *C. albicans* can functionally substitute those from their mammalian counterparts[17]. Furthermore, it is also known that fungal protein synthesis also requires EF-3 which is widely distributed and highly conserved in fungi though is absent in bacteria and higher eukaryotes[20]. In fact, EF-3 is known to be essential for cell viability since disruption of its gene is lethal to the organism[18,19]. We have exploited this information in order to further examine the putative mechanism by which aspirochlorine inhibits fungal protein synthesis. Indeed, aspirochlorine inhibited protein synthesis in a reconstituted system consisting of *C. albicans* ribosomes and *C. albicans* S-100 but failed to inhibit protein synthesis in the het-

erologous system consisting of mammalian ribosomes and *C. albicans* S-100 (Fig. 5 and 6). These data would suggest that aspirochlorine does not directly affect the activities of either *C. albicans* EF-1 or EF-2. Preliminary studies using an aspirochlorine-resistant mutant suggest that the mechanism of resistance to aspirochlorine is probably due to a different cytosolic factor. We are currently cloning and sequencing the EF-3 gene from the resistant strain to locate any putative mutation(s) which may confer resistance to aspirochlorine.

Experimental

Materials

Aspirochlorine was isolated from fermentation broths of *A. flavus* strain and identified by mass spectrometry and NMR (Lepetit Research Center). All radiolabelled compounds were purchased from Amersham plc (UK), mycotoxins and all biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of Analar grade.

Cell Free Protein Synthesis

Poly U-directed poly(Phe) synthesis was performed using an *Escherichia coli* S-30 fraction as described previously[16]. Mammalian cell free rabbit reticulocyte protein synthesis system was purchased from Promega (Promega) and used according to the manufacturer's recommendations. S-30 fraction from rat liver, rat liver ribosomes and cytosolic factors, S-100, were prepared as described previously[21]. Fungal cell free protein synthesis systems were prepared from *C. albicans* ATCC 10231[15]. *C. albicans* cells were grown in yeast nitrogen base medium (Difco) in a 50 liter fermenter to mid-log phase and harvested by centrifugation at $10,000 \times g$. The cells were suspended in Tris buffer (10 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine, 10 μg/ml leupeptin) and were disrupted in a grinding mill (Dyno-mill; type KDL, Willy A. Bachofen AG Maschinenfabrik, Basel, CH) employing three cycles of 1 minute with 2-minute cooling intervals (4°C). The cell debris and glass beads were removed by low speed centrifugation and the resulting supernatant was then centrifuged at $30,000 \times g$ for 1 hour to obtain the S-30 fraction. S-30 fraction was used to prepare ribosomes and the S-100 cytosolic factors.

E. coli poly(U)-directed poly(Phe) synthesis was performed as described[16], using S-30. Assays were performed in 100 μl 30 mM Tris/HCl, pH 7.7, 10 mM $MgCl_2$, 80 mM NH_4Cl, 3 mM DTT, 1 mM GTP, 0.8 mM ATP (buffer A) with 80 μg poly(U), 0.6 μg of phenyalanine-specific tRNA from *E. coli*, 17 pmol L-phenylalanine, and 6.87 pmol L-[^{3}H]phenylalanine [specific activity 59 Ci/mmol (1 Ci = 37 GBq)]. Incubation was for 30 minutes at 30°C. Protein synthesis was terminated by adding TCA to a final concentration of 5% (v/v). The samples were heated for 10 minutes at 80°C, cooled on ice and filtered on glass fibre filters using a cell-harvester (LKB).

C. albicans and rat poly(U)-directed poly(Phe) synthesis was performed using S-30 or S-100 and ribosomes[15]. Assays were performed in 100 μl of buffer A with 80 μg poly(U), 0.6 μg of phenylalanine-specific tRNA from *Saccharomyces cerevisiae*, 13.6 pmol L-phenylalanine, and 10 pmol L-[^{3}H]phenylalanine [specific activity 59 Ci/mmol (1 Ci = 37 GBq)] and treated as described above.

Rabbit reticulocyte lysate BMV-directed polypeptide synthesis was performed using the commercially purchased Promega translation system according to the manufacturer's instructions except that a mix of all aminoacids, with the exception of phenylalanine, at a final concentration of 1 nmol and 20 pmol L-[^{3}H]phenylalanine [specific activity 59 Ci/mmol (1 Ci = GBq)]. Incubation was for 60 minutes at 30°C. Protein synthesis was terminated by adding TCA to a final concentration of 5% (w/v). The samples were heated for 10 minutes at 80°C, cooled on ice and filtered on glass fibre filters using a cell-harvester (LKB).

Minimum Inhibitory Concentration (MIC)

The MICs of aspirochlorine were determined against yeasts and bacteria. The MICs were read visually as the lowest concentration of compound which resulted in no discernible growth as previously described[22].

Macromolecular Syntheses in Intact Cells

The effects of aspirochlorine on macromolecular syntheses in whole bacteria and yeasts were investigated as described previously[15]. In brief, macromolecular syntheses in *B. subtilis* G498 were monitored by incorporation of appropriate precursors: for DNA synthesis, [^{3}H]-thymidine (2 μCi/ml) with 2 mg of unlabelled thymidine/liter; for RNA synthesis, [^{3}H]-uridine (1 μCi/ml, 10 mg/liter); for cell wall synthesis, [^{3}H]*N*-acetylglucosamine (2 μCi/ml, 3.5 mg/liter); for protein synthesis, [^{3}H]-tryptophan (1 μCi/ml, 1 mg/liter). Macromolecular syntheses in *C. albicans* ATCC 10231 employed the following precursors: [^{3}H]-adenine for DNA

and RNA (2 μCi/ml, with 0.1 mg of unlabelled adenine/liter for DNA, and 1 μCi/ml 2 mg/liter for RNA); [^{3}H]-feucine for protein synthesis (5 μCi/ml); [^{3}H]-glucose for glucan (4 μCi/ml); [^{3}H]*N*-acetylglucosamine for chitin synthesis (4 μCi/ml, 1.4 mg/liter). The reaction was initiated by the addition of the appropriate precursor and after 5~10 minutes aspirochlorine was added and aliquots were removed at the indicated times. For dose-dependence experiments aspirochlorine was added prior to the initiation of synthesis at final concentrations ranging from 1~1,000 μg/ml. In all experiments, the reactions were stopped by the addition of cold TCA and the amount of incorporated material was determined as previously described[15].

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