

## Serine Protease Inhibitors Block Invasion of Host Cells by *Toxoplasma gondii*

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**We investigated the effect of protease inhibitors on the asexual development of the protozoan parasite *Toxoplasma gondii*. Among the inhibitors tested only two irreversible serine protease inhibitors, 3,4-dichloroisocoumarin and 4-(2-aminoethyl)-benzenesulfonyl fluoride, clearly prevented invasion of the host cells by specifically affecting parasite targets in a dose-dependent manner, with 50% inhibitory concentrations between 1 and 5 and 50 and 100  $\mu\text{M}$ , respectively. Neither compound significantly affected parasite morphology, basic metabolism, or gliding motility within the range of the experimental conditions in which inhibition of invasion was demonstrated. No partial invasion was observed, meaning that inhibition occurred at an early stage of the interaction. These results suggest that at least one serine protease of the parasite is involved in the invasive process of *T. gondii*.**

*Toxoplasma gondii* is a protozoan parasite responsible for toxoplasmosis, a disease affecting humans and other warm-blooded animals. This parasite is highly pathogenic during fetal development or upon immunosuppression (e.g., by AIDS or immunosuppressive treatments). *T. gondii* is an obligate intracellular parasite that can invade a wide range of cells, and a key step in the infection is host cell invasion, which is a prerequisite to parasite multiplication. Toxoplasmosis can be cured in various ways, but existing treatments can lead to deleterious secondary effects, and new therapeutic means are being investigated (13). Proteases have been shown to be of critical importance in development and host cell invasion for other apicomplexan parasites (e.g., *Plasmodium falciparum*, *Eimeria* spp., and *Cryptosporidium parvum*) and are therefore considered as possible new therapeutic targets (1, 10–12, 19). Despite the highly conserved invasion process used by all apicomplexan parasites, little has been reported on the possible involvement of proteases in invasion by *T. gondii*. The purpose of this study was to assay a series of protease inhibitors for their effects on *T. gondii* tachyzoite development to assess the potential targeting of such enzymes in this parasite.

### MATERIALS AND METHODS

**Reagents.** All reagents were obtained from Sigma (St. Quentin, France). Aprotinin, E64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) solutions were prepared in sterile water. Pepstatin 3,4-dichloroisocoumarin (3,4-DCI), phenylmethane sulfonyl fluoride (PMSF), and di-isopropyl fluorophosphate (DIFP) were dissolved in dimethyl sulfoxide. All stock solutions were stored at  $-20^{\circ}\text{C}$  until they were used. 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was solubilized in sterile RPMI 1640 medium (without phenol red) supplemented with 5% fetal calf serum (FCS) and stored at  $4^{\circ}\text{C}$  until it was used. Poly-L-lysine was resuspended in sterile water and stored at  $-20^{\circ}\text{C}$ . Chlorophenol red- $\beta$ -D-galactopyranoside was obtained from Boehringer Mannheim.

**Host cell culture and parasite propagation.** Vero cells (African green monkey kidney cells) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% FCS (Boehringer), 2 mM glutamine, and penicillin (1 U/ml)-streptomycin (1  $\mu\text{g}/\text{ml}$ ) at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. The *T. gondii* RH strain was used throughout this study, together with a stable *lacZ*-transfected RH strain expressing a cytoplasmic  $\beta$ -galactosidase (provided by D. Soldati, Heidelberg, Germany). The *lacZ* transfectant was used for parasite growth assays or

viability assays. The parasites were propagated either in Swiss mice or in Vero cells. When developed in Vero cells, extracellular parasites were recovered, passed twice through a 27-gauge needle, washed twice by 10 min of centrifugation at  $1,000 \times g$  in DMEM-5% FCS, and resuspended in the same medium. Mouse-derived parasites were recovered by peritoneal wash, passed twice through a 27-gauge needle, washed twice by  $1,000 \times g$  centrifugation for 10 min in DMEM-5% FCS, and then resuspended in the same medium before the invasion assay or in DMEM without FCS for the motility assay.

**Protease inhibitor assays.** Inhibitors or control solutions (with DMSO or water) were prepared in DMEM-5% FCS and added to parasite suspensions (vol/vol) 1 h before infection of Vero cells. This preincubation was performed at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere with a  $2 \times 10^6/\text{ml}$  final parasite dilution in both the growth and invasion assays. A final dilution of  $25 \times 10^6$  tachyzoites/ml was used for the MTT assay.

**Growth assay.** Vero cell monolayers grown on 96-well plates were infected with  $10^4$  preincubated parasites either in the presence of the inhibitor or after two washes and resuspension in DMEM-5% FCS. Invasion was allowed for 1 h at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  after 15 min of parasite sedimentation on ice. The wells were then washed three times with medium and incubated for an additional 24 h. The  $\beta$ -galactosidase activity was measured, as previously described (23), by lysis of infected cells followed by chlorophenol red- $\beta$ -D-galactopyranoside assay. The absorbance was read at 540 nm with a microplate reader.

**Invasion assay.** Pretreated tachyzoites ( $4 \times 10^5$ ) were added to confluent Vero cell monolayers grown on glass slides in 2-cm<sup>2</sup> wells. The parasite were permitted to sediment for 15 min on ice, and then invasion was allowed at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 1 h. The wells were then washed twice in phosphate-buffered saline, and the cells were fixed in methanol. The slides were stained with eosin-methylene blue and mounted in Xam (BDH). Quantitation was performed by counting the total number of intracellular parasites present in 100 fields at  $\times 100$  magnification.

**Viability assay.** Parasites or Vero cell monolayers were treated in RPMI 1640 medium (without phenol red) supplemented with 5% FCS, glutamine (2 mM), and antibiotics. Confluent cells or  $2.5 \times 10^6$  tachyzoites per well in a 96-well plate were then incubated for 1 h at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere, and MTT (final concentration, 0.5 mg/ml) was then added for 1 h. Solubilization of formazan formed by metabolic reduction of MTT was obtained by using lysis buffer (2) (10% sodium dodecyl sulfate-45% dimethyl formamide, adjusted to pH 4.5 with glacial acetic acid). The absorbance was read at 540 nm with a microplate reader.

**Motility assay.** Lab-Tek slides (Nunc) were coated with poly-L-lysine (1 mg/ml) for 10 min at room temperature. The slides were then washed three times in water and dried for 1 h. Inhibitors or control solutions were added to freshly isolated parasites resuspended in DMEM without FCS, and  $6 \times 10^5$  parasites were added on poly-L-lysine-coated slides for either 30 min or 1 h at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  atmosphere. The slides were then fixed with 4% formaldehyde for 30 min at room temperature. SAG1 trails left on the slides by moving parasites were detected by incubation with anti-SAG1 monoclonal antibody (5) and stained with fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G antibodies. The slides were mounted in Mowiol, and gliding was quantified by measuring trail lengths, as described by Dobrowolski et al. (7); parasites associated with trails were counted on 10 fields at  $\times 100$  magnification.

**Statistical analysis.** Statistical analysis was performed with the ANOVA program, and probit analysis representation was done with the Sigma Plot program.

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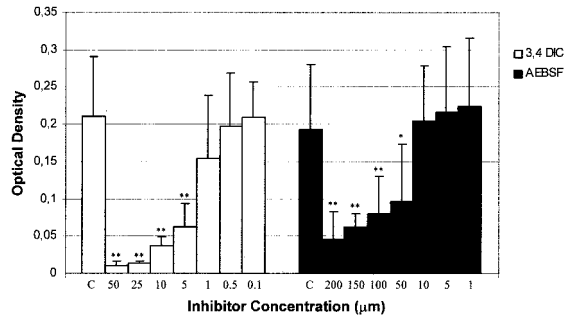


FIG. 1. Inhibition of *T. gondii* growth by various concentrations of 3,4-DCI and AEBSF measured by  $\beta$ -galactosidase assay. Extracellular tachyzoites were first pretreated for 1 h with inhibitors, and then cell infection was permitted for 1 h in the presence of inhibitors. Control values (C) were obtained after treatments with medium supplemented with dimethyl sulfoxide or water for DIC and AEBSF, respectively. Each value is the mean of three experiments done in triplicate + the standard deviation. The asterisks indicate the significant inhibitions compared with the control: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

## RESULTS

**Identification of serine protease inhibitors specifically altering *T. gondii* growth in culture.** A preliminary study of the effect of protease inhibitors on *T. gondii* was performed by using the  $\beta$ -galactosidase assay (growth assay) at 24 h postinfection. This assay has been shown to reliably enumerate viable parasites in a linear fashion based on  $\beta$ -galactosidase production (23) and is an alternative to radioactive assay for the identification of anti-*T. gondii* compounds (16). Under these conditions, the treatment of extracellular tachyzoites with pepstatin and E64 (up to 100  $\mu$ M), which are inhibitors of aspartic and cysteine proteases, respectively, did not alter parasite growth (data not shown) while two irreversible serine protease inhibitors, 3,4-DCI and AEBSF, significantly altered *T. gondii* growth, with 50% inhibitory concentrations ( $IC_{50}$ s) between 1 and 5 and 50 and 100  $\mu$ M, respectively (Fig. 1). When other serine protease inhibitors were tested under similar conditions, no significant inhibitory effect on parasite growth was detected in the same range of concentrations. Among PMSF (up to 1 mM), aprotinin (up to 200  $\mu$ M), and DIFP, only DIFP showed some effect at 200  $\mu$ M, which led to 34% inhibition of  $\beta$ -galactosidase activity compared to the control (data not shown).

The specific effect of 3,4-DCI and AEBSF on parasites was assessed by pretreatment of host cells for 1 h. The cells were then washed before infection with untreated parasites. As shown in Fig. 2, 3,4-DCI and AEBSF showed no significant inhibition of parasite growth under these conditions. When the parasites were treated for 1 h and washed twice before the infection of Vero cells, the measured  $\beta$ -galactosidase activities were strongly reduced compared to those of the untreated control and reached the same inhibitory values as those obtained when infection was performed in the presence of an inhibitor (Fig. 1 and 2).

**3,4-DCI and AEBSF effect on invasion process and intracellular growth of *T. gondii*.** In order to identify the step of parasite growth affected by 3,4-DCI or AEBSF, the inhibitors were tested in an invasion assay. Both inhibitors dramatically decreased the percentage of parasites successfully penetrating cells at concentrations similar to those used for the growth assay (Fig. 3). Using the same inhibitor concentrations, inhibition of invasion and growth were not statistically different, confirming that 3,4-DCI and AEBSF affected the invasion process. Moreover, the correlation coefficients ( $R^2$ ) calculated for the inhibitors (0.976 and 0.996 for 3,4-DCI and AEBSF, re-

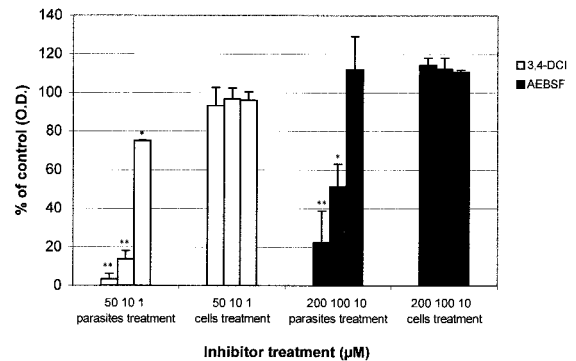


FIG. 2. Effect of 3,4-DCI and AEBSF concentrations on *T. gondii* growth as measured by  $\beta$ -galactosidase assay after a 1-h treatment of host cells or extracellular tachyzoites before infection in the absence of inhibitors. Each value is the mean of three experiments done in triplicate + the standard deviation, expressed as percentages of values (optical density [O.D.]) for control treatment. The asterisks indicate the significant inhibitions compared with the control: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

spectively) strongly support linear dose-dependent effects on the invasion process. The  $IC_{50}$ s calculated from linear plots (probit representation of the relative percentage of inhibition of growth versus the logarithm of the drug concentration) were 2.2  $\mu$ M for 3,4-DCI and 67  $\mu$ M for AEBSF. The efficiency of inhibition of parasite growth was also analyzed with serum-free medium. Under these conditions, the  $IC_{50}$ s decreased to 44  $\mu$ M for AEBSF and 0.2  $\mu$ M for 3,4-DCI. The effects of the inhibitors were also dependent on the duration of pretreatment (either 15 or 60 min). With minimal pretreatment time

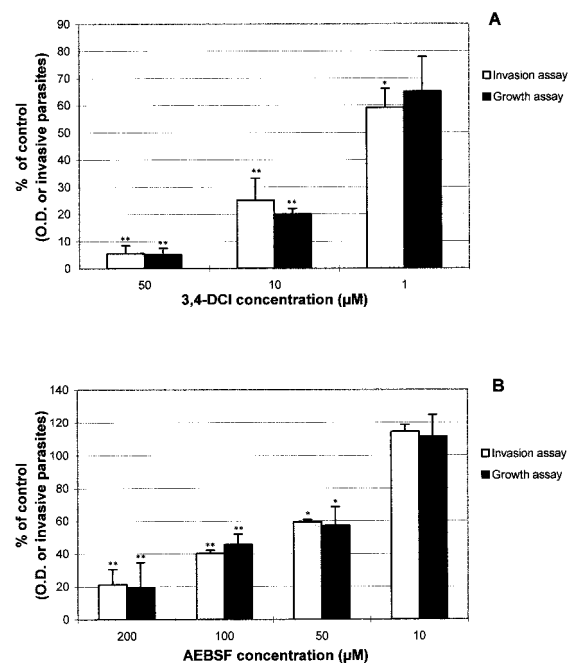


FIG. 3. Comparison of the effect of various concentrations of 3,4-DCI (A) and AEBSF (B) on *T. gondii* growth and host cell invasion. Extracellular tachyzoites were pretreated for 1 h, and cell infection was performed for 1 h. Each value is the mean of three experiments (performed in triplicate) + the standard deviation, expressed as percentages of values (optical density [O.D.]) for control treatment. The asterisks indicate the significant inhibitions compared with the control: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

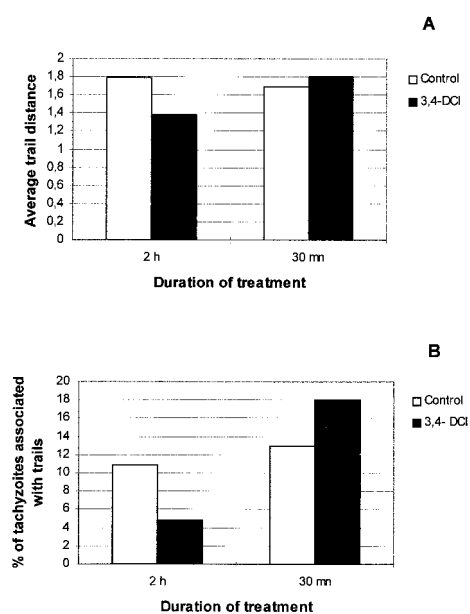


FIG. 4. Time dependency effect of 3,4-DCI on tachyzoite gliding: average trail distance (A) and percentage of tachyzoites associated with trails (B). A gliding assay was performed with the inhibitor either for 30 min with no pretreatment or for 1 h after 1 h of pretreatment (2 h of treatment). The data presented are from a representative experiment with a 3,4-DCI concentration (10  $\mu$ M) leading to 93.4% inhibition of invasion after a 30-min treatment.

(15 min) for parasite sedimentation, the  $IC_{50}$  was above 200  $\mu$ M for AEBSF and between 10 and 25  $\mu$ M for 3,4-DCI (data not shown).

To analyze a possible impairment of intracellular parasite growth, infected cells (taken 4 h after invasion) were treated for 1 h and  $\beta$ -galactosidase activity was measured 24 h later. Under these conditions, AEBSF had no inhibitory effect on parasite growth in the concentration range tested (up to 200  $\mu$ M). In contrast, 3,4-DCI demonstrated significant inhibition of parasite growth (24.7%) at 25  $\mu$ M (data not shown).

**Parasite integrity, viability, and motility.** We then tried to characterize the inhibitory effect of 3,4-DCI and AEBSF on the invasion process. Parasite morphology was unaffected: both propidium iodide assay and electron microscopy analysis confirmed parasite integrity after inhibitor treatments with the highest concentrations tested (data not shown). Parasite viability was then evaluated by a tetrazolium salt reduction assay. Microculture tetrazolium assays are widely applied to quantify cell metabolic activity (2). Therefore, we used such a test to evaluate the possible metabolic toxicity of the protease inhibitors on parasites and host cells. We verified that MTT colorimetric quantitation was a linear function of parasite or cell number (not shown). When host cells were pretreated for 1 h with inhibitors, no significant toxicity was observed with 3,4-DCI up to 50  $\mu$ M or AEBSF up to 200  $\mu$ M. Also, no significant toxicity to parasites was observed with AEBSF in the same range of concentrations. In contrast, a significant difference ( $P < 0.01$ ) was found between tachyzoites treated with over 25  $\mu$ M 3,4-DCI and control tachyzoites (leading to 19.7% inhibition of MTT reduction) (data not shown).

In order to determine whether 3,4-DCI and AEBSF could affect parasite motility, we tested these inhibitors on parasite gliding as described above. Using 3,4-DCI at 10  $\mu$ M, parasite gliding was not affected during a 30-min assay (conditions which in the invasion assay to led 90% inhibition), whereas a

2-h assay (1 h of pretreatment and 1 h of gliding assay) significantly affected parasite motility by decreasing both the average trail distance ( $P < 0.05$ ) and the percentage of parasites associated with trails ( $P < 0.01$ ) (Fig. 4). In contrast, 200  $\mu$ M AEBSF did not affect parasite gliding in a 2-h assay (data not shown).

Microscopic observation of cell monolayers fixed after interaction with treated parasites showed no evidence of abortive invasion (i.e., partial internalization of parasites, such as can be observed by fixation of the monolayer during normal invasion), suggesting that blocking occurs at an early stage of the process.

## DISCUSSION

The invasion process of *T. gondii* is still poorly understood. Myosin-like chain kinase inhibitor (KT5926), myosin ATPase inhibitor (butanedione monoxime), and cytochalasin D, all affecting the motility of the parasite (7, 8), and phospholipase  $A_2$  inhibitors (14, 20, 21, 24) have been described as inhibitors of invasion. Little information concerning the proteases of *T. gondii* has been reported (4, 22), whereas the involvement of such enzymes and their potential targeting have been more thoroughly explored in apicomplexan parasites in the genera *Plasmodium*, *Eimeria*, and *Cryptosporidium* (1, 10–12, 19). In the course of this study we have first confirmed preliminary data previously reported by others (22); treatment of extracellular tachyzoites with pepstatin or E64 (protease inhibitors of aspartic and cysteine proteases, respectively) or aprotinin and PMSF (serine protease inhibitors) did not alter the invasion of host cells by tachyzoites. New data were obtained with two other irreversible serine protease inhibitors, 3,4-DCI and AEBSF, which dramatically decreased penetration by tachyzoites of *T. gondii* without altering their structural integrity. As host cell pretreatment did not affect invasion, these results suggest that a parasite enzyme(s) is the target of these inhibitors.

Harper et al. (15) have described 3,4-DCI as a general protease inhibitor that reacts principally with a broad range of serine proteases. This inhibitor is more potent and reacts more quickly than the two other commonly used serine protease inhibitors, DIFP and PMSF (17). All three of these inhibitors are hydrophobic reagents that are extremely sensitive to hydrolysis. The difference in reactivity among these compounds may explain the discrepancies in efficiency. AEBSF is also a general serine protease inhibitor that is soluble and stable in aqueous solution. In this case, the difference in hydrophilic properties between AEBSF and 3,4-DCI may explain the higher  $IC_{50}$  with AEBSF, if the target(s) of these inhibitors is intracellular. The inefficacy of aprotinin, a macromolecular inhibitor, apart from its narrower range of specificity (it is not a general serine protease inhibitor), could also reflect its inability to access the target(s) affected by 3,4-DCI and AEBSF. An additional piece of evidence that the target is intracellular is that invasion of treated parasites cannot be restored by using supernatant recovered from the interaction between untreated parasites and cells. The likely explanation is that the efficient inhibitors act inside the parasites and that specificity, stability, and accessibility may separately or collectively explain the heterogeneity of effects of the serine protease inhibitors tested.

In order to evaluate the specificity of the effect on invasion versus a possible more general toxic effect of the tested inhibitors, several other assays were performed, including morphological examination, MTT assay, and a gliding assay. MTT assays are colorimetric methods based upon the reduction of tetrazolium salts: most cellular bioreduction of MTT is associated with enzymes of the endoplasmic reticulum and involves

NADH and NADPH pyridine nucleotides (2). Thus, MTT assay reveals the effect of an inhibitor on the basic metabolism of the parasite. No significant effect of 3,4-DCI on this bioreduction was found below 25  $\mu$ M after a 1-h treatment (a condition leading to more than 90% inhibition of invasion), but the effect increased progressively above this value. Therefore, 3,4-DCI can exert a general toxicity on parasites, but the concentrations required exceed those that cause significant reductions in invasion. This toxicity might explain the effect of this reagent on intracellular parasite development. In contrast, AEBSF had no effect on MTT reduction within the range tested. These results lead us to conclude that both 3,4-DCI and AEBSF act on enzyme(s) active during the invasion process. Additionally, 3,4-DCI may exert an effect on another target(s) involved in parasite metabolism at higher doses.

3,4-DCI and AEBSF are essentially protease inhibitors, but both reagents can also inhibit several esterases (6, 17), because the latter enzymes are mechanistically quite similar to serine proteases. Therefore, one could wonder whether these proteinase inhibitors could affect phospholipase A<sub>2</sub> (PLA<sub>2</sub>), which has been suggested to be involved in invasion (14, 20, 21). However, AEBSF minimally inhibits PLA<sub>2</sub> from naja venom and porcine pancreas (6), and treatment of host cells with either 3,4-DCI or AEBSF does not affect infectivity, in contrast to 4-bromophenacyl bromide (a specific PLA<sub>2</sub> inhibitor previously tested [14]). These observations tend to exclude these enzymes as targets of the inhibitors characterized in the present study.

3,4-DCI and AEBSF also block invasion without inhibiting motility (as monitored by the deposition of trails of surface proteins on a substrate [8]), which suggests that the mechanism of gliding is not the target of these inhibitors. However, a prolonged exposure to 3,4-DCI can lead to an inhibitory effect on parasite motility, which might be related to a deleterious effect of this inhibitor on the basic metabolism of the parasite.

Host cell invasion requires parasite motility, followed by the formation of a moving junction with the host cell, exocytosis of secretory organelles involved in the alteration of the host cell membrane, and the development of the parasitophorous vacuole (9). Since we have not found evidence of abortive invasion, our results suggest that an early stage of the process is affected by these serine protease inhibitors, the most likely being the moving junction-exocytosis step. These results can then be compared to other observations of closely related apicomplexan parasites, such as *P. falciparum* and *Plasmodium chabaudi*, in which a serine protease has been reported to be involved in host cell membrane alteration before or during erythrocyte invasion. This serine protease acts by cleaving an external loop of human erythrocyte band 3 and glycophorin A (3, 18). Serine proteases have also been suggested to be involved in host cell invasion by *Eimeria* and *Cryptosporidium* species (1, 10, 12). Therefore, a common mechanism involving serine proteases during invasion by apicomplexan parasites likely exists. This mechanism represents a potential new target for chemotherapy of the diseases caused by these pathogens.

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