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Fusidic acid is an effective treatment against *Toxoplasma gondii* and *Listeria monocytogenes in vitro* but not in mice

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Abstract

Fusidic acid is a bacteriostatic antibiotic that inhibits the growth of bacteria by preventing the release of translation elongation factor G (EF-G) from the ribosome. The Apicomplexan parasite *Toxoplasma gondii* has an orthologue of bacterial EF-G that can complement bacteria and is necessary for parasite virulence. Fusidic acid has been shown to be effective in tissue culture against the related pathogen *Plasmodium falciparum*, and current drug treatments against *T. gondii* are limited. We therefore investigated the therapeutic value of fusidic acid for *T. gondii* and found that the drug was effective in tissue culture but not in a mouse model of infection. To determine whether this trend would occur in another intracellular pathogen that elicits a Th1 type immune response, we tested the efficacy of fusidic acid for the bacterium *Listeria monocytogenes*. Similarly to its effects on *T. gondii*, fusidic acid inhibits the growth of *L. monocytogenes in vitro* but not in mice. These findings highlight the necessity of *in vivo* follow-up studies to validate *in vitro* drug investigations.

Keywords

Fusidic acid; elongation factor G; *Toxoplasma gondii* and *Listeria monocytogenes*

Introduction

Fusidic acid is a bacteriostatic antibiotic used to treat Gram-positive bacteria, most notably skin infections as well as bone and joint infections caused by *Staphylococcus aureus* (Collignon and Turnidge 1999). Fusidic acid has been in clinical use since as early as 1962 in 23 countries including Canada; however, it is not approved for use in the United States (Kraus and Burnstead 2011). Fusidic acid inhibits protein translation by preventing the release of translation elongation factor G (EF-G) from the ribosome (Kinoshita et al. 1968). Eukaryotic pathogens from the phylum Apicomplexa contain an orthologue of bacterial EF-G, and a bacterial strain with an EF-G mutation can be complemented with the *Toxoplasma gondii* EF-G (TgEFG) (Payne et al. 2011). Translation occurs in three distinct locations in most Apicomplexan parasites, the cytosol, the mitochondria and a primitive chloroplast-like structure called the apicoplast. Each compartment requires its own derivative of EF-G; therefore, three are encoded in the *T. gondii* genome. TgEFG is localized in the apicoplast,

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and its precise regulation is essential only during animal infection (Payne et al. 2011). Approximately one third of the world's human population is infected with *T. gondii*. While infection of immunocompetent people is generally asymptomatic, infection of immunocompromised hosts and fetuses can have dire consequences, including death. A limited number of treatments like sulfonamides exist for the fast-replicating form of *T. gondii* that characterizes acute infection, and there are no treatments for the encysted, chronic form of the parasite (Montoya and Liesenfeld 2004). Drug discovery is therefore an important area of *T. gondii* research. Fusidic acid was previously shown to be effective in tissue culture against the causative agent of malaria, *Plasmodium falciparum* (Black et al. 1985). Thus fusidic acid might be an effective new therapeutic for *T. gondii*, so we tested its efficacy against *T. gondii* in both tissue culture and in mice.

Materials and methods

Effects of fusidic acid on *T. gondii* in vitro

Parasites of the type II Prugniaud (Pru) strain were grown in confluent monolayers of human foreskin fibroblasts (HFFs) at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutathione and 1% penicillin-streptomycin and buffered with 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES). To test the efficacy of fusidic acid against *T. gondii* in vitro, HFFs in 24 well plates were pretreated for one hour with various concentrations (0–100 µg/ml) of fusidic acid sodium salt and then infected with 100 *T. gondii* tachyzoites force lysed from HFFs in 25 cm² tissue culture flasks. After seven days of undisturbed parasite growth, and then the number of lytic plaques in each well were counted. The percentage of plaques formed in the presence of fusidic acid compared to no drug was determined, and a half maximal inhibitory concentration (IC₅₀) was calculated for fusidic acid.

Effects of fusidic acid on *Listeria monocytogenes* in vitro

The minimum inhibitory concentration (MIC) of fusidic acid sodium salt against the EGD strain of *L. monocytogenes* was determined in broth culture in both culture tubes and a 96 well plate. Fusidic acid sodium salt was dissolved in brain heart infusion broth (BHI), filter sterilized (0.22 µm), and dilutions (100, 75, 50, 40, 30, 20, 10, 5, 3, 1.5, 1 and 0.5 µg/ml) were transferred to culture tubes (2 ml/tube) and a 96 well plate (200 µl/well). *L. monocytogenes* was grown in BHI at 37°C with shaking to an OD₆₀₀ of 0.5, and then added to the culture tubes and 96 well plate to a final concentration of 5×10⁵ colony forming units (CFUs)/ml. Cultures were grown overnight at 37°C with shaking, and OD₆₀₀ readings were taken. MIC was determined as the lowest concentration with no detectable growth. *L. monocytogenes* was grown in BHI with no drug for a negative control and in BHI with streptomycin or erythromycin (100 mg/ml) for positive controls.

T. gondii infection and fusidic acid treatment of mice

Female BALB/c mice (8–10 week old, NCI) were infected by intraperitoneal (i.p.) injection with 5×10³ or 5×10⁴ tissue culture grown tachyzoites engineered to express firefly luciferase (Pru HPT::Luc) (Tobin and Knoll 2012). Beginning eight hours post infection, mice were treated every eight hours with fusidic acid sodium salt in saline (20 mg/kg, providing a total

of 60 mg/kg/day) or an equivalent volume of saline, administered subcutaneously (s.c.). As a positive treatment control, a separate group of *T. gondii* infected mice were fed Uniprim chow (Harlan Teklad) that contains sulfadiazine and trimethoprim (SDZ/TMP), known combination therapy for toxoplasmosis, beginning eight hours post infection and continuing through the duration of the experiment. Mice were monitored daily for health parameters, including activity and weight changes. At five days post *T. gondii* infection, mice were anesthetized with isoflurane, given an intravenous (i.v.) injection of luciferin (3 mg), and then imaged on an IVIS Imaging System (PerkinElmer) to assess parasite burdens.

***L. monocytogenes* infection and fusidic acid treatment of mice**

Female BALB/c mice (8–10 week old, NCI) were i.p. injected with approximately 2×10^3 CFU/animal of *L. monocytogenes* EGD. Beginning eight hours post infection, mice were treated daily with fusidic acid sodium salt in saline (60 mg/kg/day) or an equivalent volume of saline, administered s.c. every eight hours. Mice were monitored daily for health parameters, including activity and weight changes. At three days post infection, mice were euthanized and spleens and livers were removed, weighed, and homogenized in 1 ml 1X PBS in glass Tenbroeck style homogenizers (Bellco Glass Inc.). Ten-fold serial dilutions of the organ homogenates were plated on blood agar plates (TSA/Trypticase Soy Agar with 5% sheep blood, Fisher Scientific) to determine bacterial burdens.

Analyses of host cytokine responses

Five days post *T. gondii* infection or three days post *L. monocytogenes* infection, blood was collected from mice by tail bleed or cardiac puncture and clotted at room temperature for 30 minutes followed by 4°C for 30 minutes. Serum was isolated by centrifugation at $13,000 \times g$ for 20 minutes at 4°C. Levels of interleukin (IL)-12p70, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , monocyte chemoattractant protein (MCP)-1, IL-10 or IL-6 were measured in the serum using a BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences).

Statement of animal use

Animals were housed under conventional, specific-pathogen-free conditions and were treated in compliance with guidelines set by the Institutional Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health (IACUC), according to an IACUC approved protocol. The University of Wisconsin is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care.

Results and discussion

To test fusidic acid's effects on *T. gondii* in cell culture, we pretreated HFFs in 24-well plates for one hour with various concentrations of fusidic acid sodium salt (0–100 $\mu\text{g/ml}$) and then infected the cells with *T. gondii*. After seven days, lytic plaques were counted and the percentage of plaques formed in the presence of fusidic acid compared to no drug was calculated. Fusidic acid treatment decreased the number of *T. gondii* plaques in a dose-dependent manner, with a half maximal inhibitory concentration (IC_{50}) of $\sim 4 \mu\text{g/ml}$ (Fig. 1). Fusidic acid inhibited a *T. gondii* mutant with a dramatic reduction in TgEFG levels (Payne

et al. 2011), highlighting that either TgEFG levels are in excess of that required for tissue culture growth or that TgEFG is not the only target of fusidic acid. Overall, these results show that fusidic acid is effective against *T. gondii* in tissue culture, and suggest that it might be a useful treatment against *T. gondii* during animal infection.

Fusidic acid treatment in animals is challenging due to its reported toxicity and poor pharmacokinetics (PK). Inoculation of fusidic acid i.p. induces a granulomatous reaction, s.c. inoculation results in an inflammatory reaction (Nicoletti et al. 1995), intramuscular inoculation can cause paralysis (Rowe et al. 1992), and oral administration in rodents fails to achieve appreciable serum levels of the drug due to its poor absorption in rodents (Findon et al. 1991). PK analysis determined that fusidic acid has a short half-life, thus fusidic acid is typically administered in three daily doses in animals and humans (Findon et al. 1991; Turnidge 1999). To examine the potential therapeutic value of fusidic acid for *T. gondii*, mice were infected with 5×10^4 *T. gondii* parasites, treated with three daily doses of fusidic acid sodium salt either i.p. or s.c., and monitored for health parameters, including activity and weight changes. We found that i.p. administration of fusidic acid at doses of 30 or 90 mg/kg/day, which are well below the manufacturer's reported lethal dose 50% (LD₅₀) of 170 mg/kg/day for this route of inoculation, was not well tolerated by the mice and treatments were suspended after two days (data not shown). Fusidic acid was therefore administered s.c. in all subsequent experiments. Despite the manufacturer's reported LD₅₀ of 313 mg/kg/day following s.c. inoculation, toxicity was observed at a dose of 180 mg/kg/day (data not shown), so a lower dose of 60 mg/kg/day (i.e. 20 mg/kg every 8 hours) was used in all experiments. Even though 60 mg/kg/day was generally well tolerated, some mice developed eczematous skin at the site of inoculation. Animal health and survival were not enhanced by 60 mg/kg/day of fusidic acid as compared to saline (Fig. S1 and Fig. 2a). This result was also observed when mice were infected with the lower dose of 5×10^3 parasites (data not shown). Unexpectedly, *T. gondii* infected mice treated with fusidic acid consistently succumbed to infection earlier than saline treated mice, although this trend did not reach statistical significance. As a positive treatment control, a separate group of *T. gondii* infected mice were fed SDZ/TMP chow, known combination therapy for toxoplasmosis. As expected, SDZ/TMP treatment decreased weight loss and increased survival (Fig. S1 and Fig. 2a).

To further explore the ineffectiveness of fusidic acid against *T. gondii*, we examined both parasite burdens and the host immune response at five days post infection. To assess parasite burdens, mice were anesthetized with isofluorane, given an i.v. injection of luciferin (3 mg), and then imaged on an IVIS Imaging System (PerkinElmer). As expected, the SDZ/TMP treated mice had reduced parasite burdens compared to saline treated mice; however, there was no difference in the parasite burden between fusidic acid and saline treated animals (Figs. 2B–C). While there was no decrease in parasite burden to indicate that fusidic acid effectively treats *T. gondii* infection, there also was no increase in parasite burden that would explain the tendency for fusidic acid treated mice to succumb to infection early. *T. gondii* is an obligate intracellular parasite that elicits a strong Th1-type immune response (Denkers and Gazzinelli 1998). As fusidic acid was previously seen to inhibit a Th1-type immune response (Bellahsene and Forsgren 1980), we analyzed Th1-type cytokine levels

from *T. gondii* infected mice at day 5 post infection using cytometric bead arrays (CBA, Becton Dickinson). We observed no differences in serum levels of IL-12, TNF- α , IFN- γ , MCP-1, IL-10 or IL-6 between the saline and fusidic acid treated mice (Fig. S2). Thus the failure of fusidic acid to inhibit *T. gondii* *in vivo* was unlikely due to immune suppression by fusidic acid.

Our data suggest that fusidic acid can inhibit the growth of *T. gondii* in tissue culture but not in animals. To determine whether this trend would occur for a Gram-positive bacterium that also elicits a Th1 response, we tested fusidic acid against *L. monocytogenes*. A wide range of susceptibility to fusidic acid has been reported for *L. monocytogenes*, depending on the strain used, MIC 0.5–32 $\mu\text{g/ml}$ (Osaili et al. 2012; Troxler et al. 2000). Clinical and Laboratory Standards Institute interpretive criteria or breakpoints have not been established for fusidic acid against *L. monocytogenes*. We found the MIC of fusidic acid sodium salt against the EGD strain of *L. monocytogenes* to be $\sim 2 \mu\text{g/ml}$ in broth culture. To examine the efficacy of fusidic acid against *L. monocytogenes* in animals, we infected mice with *L. monocytogenes* EGD and treated them with three daily s.c. doses of fusidic acid sodium salt in saline (60 mg/kg/day). While fusidic acid treatment did not improve the overall health of the *L. monocytogenes* infected animals, it did not consistently decrease their survival as was observed in the *T. gondii* infected mice (data not shown and Fig. 3a). At three days post infection, fusidic acid treatment did not alter bacterial burden in the spleen or liver, the two main sites of *L. monocytogenes* replication (Fig. 3b). Quantification of IL-12, TNF- α , IFN- γ , MCP-1, IL-10 and IL-6 levels at three days post infection revealed no differences in the cytokine response between saline and fusidic acid treated mice (data not shown). Therefore, similar to *T. gondii*, fusidic acid is effective against *L. monocytogenes* *in vitro* but not in animals.

These results highlight the necessity of *in vivo* studies to validate the results of tissue culture drug explorations. The fact that fusidic acid was effective against both *T. gondii* and *L. monocytogenes* *in vitro* but not *in vivo* suggests that the minimum inhibitory concentration was not obtained *in vivo* with the delivery route and amount of fusidic acid used. Drug toxicity prevented higher dosing and alternative routes of inoculation, ruling out fusidic acid as an effective therapeutic option for these pathogens *in vivo*. Our findings with the *T. gondii* TgEFG mutant in tissue culture (Fig. 1) show that fusidic acid may be inhibiting mitochondrial translation as well as apicoplast EF-G in Apicomplexan parasites. Inhibition of mitochondrial translation in animals could be causing toxicity, which may explain the slightly decreased survival of *T. gondii* infected mice treated with fusidic acid (Fig. 2a). Future derivatives of fusidic acid may more specifically target bacterial and apicoplast translation, and thus they would be broad-spectrum antibiotics with little to no toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Fusidic acid inhibits the growth of *T. gondii* in tissue culture

Wild-type *T. gondii* (black squares) and a TgEFG mutant (gray triangles) were grown in the presence of various concentrations of fusidic acid. The percent (%) no drug plaques was calculated as a number of plaques formed with fusidic acid divided by the number of plaques formed with no drug added. Each data point is plotted as the mean $\pm$ standard deviation of three independent experiments. There was no statistically significant difference in the growth inhibition of wild-type *T. gondii* and TgEFG mutant parasites with fusidic acid.

Fig. 2. Fusidic acid does not effectively treat *T. gondii* in mice

a) Percent survival of *T. gondii* infected animals treated with SDZ/TMP (red), fusidic acid (blue) or saline (black). Shown is a representative of four independent experiments with 3–6 mice per group per experiment. The difference between mice treated with fusidic acid versus saline was not significant, but the difference between SDZ/TMP versus saline was significant, $p=0.03$. b) IVIS imaging five days post *T. gondii* infection of mice treated with saline (left three), fusidic acid (middle three) or SDZ/TMP (right three). Shown is a representative experiment of four independent experiments with the mice imaged ventrally. For all images, the signal intensity is represented by radiance, which is defined as photons/second/cm²/steradian (p/s/cm²/sr). The color scale ranges from purple at 1×10^5 p/s/cm²/sr

to red at 1.3×10^6 p/s/cm²/sr. c) Quantification of total flux in photons/second (p/s) for the IVIS images from (b), and each data point is the mean $\pm$ standard deviation. There was no statistically significant difference between mice treated with fusidic acid versus saline, but for SDZ/TMP versus saline, p=0.03.

Fig. 3. Fusidic acid is not an effective treatment for *L. monocytogenes* in mice

a) Percent survival of *L. monocytogenes* infected animals treated with fusidic acid (gray) or saline (black). Shown is a representative of three independent experiments with 6–12 mice per group per experiment. b) At three days post *L. monocytogenes* infection, the number of colony forming units per gram of spleen or liver (CFU/g) from fusidic acid (gray) or saline (black) treated mice were enumerated. Shown is a representative of three independent experiments with 6–12 mice per group per experiment, and each data point is the mean $\pm$ standard deviation. There was no statistically significant difference between mice treated with fusidic acid versus saline.