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Brief Report

Effects of the green tea catechin (–)-epigallocatechin gallate on *Trypanosoma brucei*

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ABSTRACT

The current pharmacopeia to treat the lethal human and animal diseases caused by the protozoan parasite *Trypanosoma brucei* remains limited. The parasite's ability to undergo antigenic variation represents a considerable barrier to vaccine development, making the identification of new drug targets extremely important. Recent studies have demonstrated that fatty acid synthesis is important for growth and virulence of *Trypanosoma brucei brucei*, suggesting this pathway may have therapeutic potential. The first committed step of fatty acid synthesis is catalyzed by acetyl-CoA carboxylase (ACC), which is a known target of (–)-epigallocatechin-3-gallate (EGCG), an active polyphenol compound found in green tea. EGCG exerts its effects on ACC through activation of AMP-dependent protein kinase, which phosphorylates and inhibits ACC. We found that EGCG inhibited TbACC activity with an EC₅₀ of 37 μM and 55 μM for bloodstream form and procyclic form lysates, respectively. Treatment with 100 μM EGCG induced a 4.7- and 1.7- fold increase in TbACC phosphorylation in bloodstream form and procyclic lysates. EGCG also inhibited the growth of bloodstream and procyclic parasites in culture, with a 48 h EC₅₀ of 33 μM and 27 μM, respectively, which is greater than the EGCG plasma levels typically achievable in humans through oral dosing. Daily intraperitoneal administration of EGCG did not reduce the virulence of an acute mouse model of *T. b. brucei* infection. These data suggest a reduced potential for EGCG to treat *T. brucei* infections, but suggest that EGCG may prove to be useful as a tool to probe ACC regulation.

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1. Introduction

The protozoan parasite *Trypanosoma brucei* (sub-species *gambiense* and *rhodesiense*) is the causative agent of African sleeping sickness, a fatal human disease that ranges across sub-Saharan Africa. In addition to causing substantial morbidity and mortality in humans, a third sub-species, *Trypanosoma brucei brucei* is responsible for causing nagana, a livestock disease that results in wasting and death. Nagana imposes a tremendous economic bur-

den on the region, causing 4.5 billion dollars in economic losses each year (FAO, 2007). Vaccine development is confounded by the parasite's ability to switch its surface coat through antigenic variation (Horn and McCulloch, 2010). Chemotherapeutics are relied upon to battle the disease, yet currently approved drugs cause undesirable side effects, are subject to failure, and can be too expensive for citizens of economically depressed regions (Castillo et al., 2010). To meet this urgent need, investigation of existing compounds are an important avenue to identify potential new drugs that are effective, safe, and economical.

Green tea is amongst the most widely consumed beverages worldwide and is often touted for its wealth of medicinal effects (Moon et al., 2007; Khan and Mukhtar, 2008; Thieleck and Boschmann, 2009; Ahmed, 2010). The best-studied active components of green tea are the catechins, of which (–)-epigallocatechin-3-gallate (EGCG) is one of the most abundant (Lin et al., 2003). In addition to numerous other pathways, EGCG has been demonstrated to inhibit fatty acid synthesis (Wang and Tian, 2001; Brusselmans et al., 2003) through its effect on the regulation of acetyl-CoA carboxylase (ACC) (Huang et al., 2009). ACC catalyzes the first committed step in fatty acid synthesis: the ATP-dependent carboxylation of acetyl-CoA, which provides the two-carbon donor, malonyl-CoA, for fatty acid synthesis (Tong and Harwood, 2006). ACC is negatively regulated by phosphorylation by AMP-activated protein kinase (AMPK), a

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; BF, bloodstream form; DMSO, dimethyl sulfoxide; EGCG, (–)-epigallocatechin gallate; PF, procyclic form; RNAi, RNA interference; SA-HRP, streptavidin conjugated to horseradish peroxidase.

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key regulator of cellular energy metabolism (Barber et al., 2005; Brownsey et al., 2006). EGCG treatment activates AMPK, leading to increased phosphorylation of human ACC, which resulted in its inhibition (Moon et al., 2007; Huang et al., 2009).

In *T. b. brucei*, both the cytoplasmic and mitochondrial fatty acid synthesis pathways are important for growth in culture and virulence in mouse models (Lee et al., 2006, 2007; Stephens et al., 2007; Vigueira and Paul, 2011). We have recently demonstrated that RNA interference (RNAi)-mediated gene knock-down of ACC in *T. b. brucei* reduced fatty acid elongation activity in intact cells and reduced virulence in a mouse model of infection (Vigueira and Paul, 2011). In addition, treatment with thiolactomycin, an inhibitor of fatty acid synthetase, inhibited fatty acid synthesis activity and growth of *T. b. brucei* in culture (Morita et al., 2000). Taken together, these results suggest that fatty acid synthesis has the potential to be an effective drug target in *T. brucei*. Here, we examined the effect of EGCG on *T. b. brucei* and found that physiologically relevant levels of EGCG had no effect on growth in culture and did not reduce virulence in a mouse model of infection, while higher levels of EGCG decreased TbACC activity, likely through an increase in TbACC phosphorylation. These data suggest that although EGCG inhibits *T. b. brucei* ACC activity, EGCG is a questionable candidate for further development as a potential cure for *T. brucei* infection. However, EGCG may be a useful pharmacological tool to investigate the signaling pathways governing phospho-regulation of TbACC.

2. Material and methods

2.1. Materials, cell lines, and media

Most chemicals and reagents were purchased from Thermo Fisher Scientific and Sigma, including EGCG (E4143), which was prepared and frozen in single-use aliquots, either as a 100X stock in dimethyl sulfoxide (DMSO) or in sterile water for use in mice. EGCG stocks were freshly thawed just before use. Minimum essential medium and Iscove's modified Dulbecco's medium, was from Invitrogen. Serum Plus was from JRH Biosciences. [¹⁴C]NaHCO₃ (14.9 mCi mmol⁻¹) was from American Radiolabeled Chemicals. Wild-type (WT) *T. b. brucei* 427 bloodstream form (BF) and tsetse midgut procyclic form (PF) cell lines were a kind gift of Dr. Paul Englund (Johns Hopkins School of Medicine). BFs were grown at 37°C/5% CO₂ in HMI-9 media containing 10% heat-inactivated fetal bovine serum (FBS) and 10% Serum Plus (Hirumi and Hirumi, 1989). PFs were grown at 28°C/5% CO₂ in SDM-79 media containing 10% heat-inactivated FBS and 7.5 µg ml⁻¹ hemin (Brun and Shonenberger, 1979). PF ACC-myc cells are tagged in one genomic locus with a c-terminal fusion to the c-myc epitope and have been described previously (Vigueira and Paul, 2011). BF ACC-myc cells were generated in this study with the same tagging construct used to make the PF ACC-myc cell line. ACC-myc cell lines are maintained in the appropriate growth media supplemented with 2.5 µg ml⁻¹ phleomycin.

2.2. Effect of EGCG on *T. b. brucei* growth

WT BF *T. b. brucei* were diluted into fresh media containing 0.1–1 µM EGCG or 1% DMSO as the solvent control and the cell culture densities measured every 2 days for 6 days by flow cytometry (BD FACScan). Following each cell count, cultures were diluted to maintain logarithmic phase growth, and EGCG/DMSO was added to maintain experimental concentrations. Doubling times were calculated from the growth curves. To determine EC₅₀ for growth, WT BF and PF *T. brucei* were diluted into fresh media containing 5–50 µM

EGCG or 1% DMSO as solvent control and cell culture densities determined after 2 days.

2.3. ACC activity assays

Hypotonic lysates were desalted and TbACC activity was assayed essentially as described (Vigueira and Paul, 2011). Briefly, ACC activity was determined by measuring the incorporation of the [¹⁴C]CO₂ from [¹⁴C]NaHCO₃ into the acid resistant [¹⁴C]malonyl-CoA product, which is quantified by scintillation counting. To test the effect of EGCG on TbACC activity, 5–100 µM EGCG or 1% DMSO only (solvent control) was added to the lysates with or without 1X HALT phosphatase inhibitor cocktail (Thermo Scientific Pierce) just prior to assaying for TbACC activity.

2.4. ACC phosphorylation and blotting

Hypotonic lysates from BF and PF ACC-myc cells were prepared in the presence of 1X HALT phosphatase inhibitor cocktail. Lysates were supplemented with 5 mM ATP, divided, with one aliquot treated with 100 µM EGCG and the other with 1% DMSO as solvent control. Lysates were then incubated at 30 °C for 30 min. under constant mixing (Eppendorf Thermomixer, 500 rpm). ACC-myc was immunoprecipitated from the lysates using the ProFound c-myc IP/Co-IP kit (Thermo Scientific Pierce) according to the manufacturer's instructions and eluted in 150 mM glycine at pH 2.2 followed by immediate neutralization with 9.5 M Tris-HCl, pH 9.0.

To detect phosphorylated ACC-myc, approximately equal quantities of immunoprecipitated ACC-myc (~8.8 × 10⁷ cell equivalents/lane for PF and ~1.5 × 10⁸ cell equivalents/lane for BF) were resolved by SDS–10% PAGE, followed by gel staining with Pro Diamond Q phosphoprotein gel stain (Invitrogen) according to the manufacturer's instructions. Phospho-stained gels were imaged under UV using the Gel Doc XR Imaging System (BioRad). To detect total ACC, identically loaded non-stained gels were transferred to nitrocellulose and probed for ACC as described (Vigueira and Paul, 2011) with streptavidin conjugated to horseradish peroxidase (SA-HRP), which detects the ACC biotin prosthetic group. Densitometric quantitation was performed on gels and appropriately exposed blots (in the linear range of detection) using Image J software (NIH). Phosphorylated ACC-myc was normalized to the total amount of ACC in the ACC-myc immunoprecipitates and lysates.

2.5. Mouse infection

To test the ability of EGCG to clear a *T. b. brucei* infection in mice, we chose a daily dose of 4.13 mg/kg EGCG prepared in sterile H₂O and administered daily by IP injection. This EGCG concentration had been previously determined not to cause mouse liver damage, though actual serum levels of EGCG were not determined (Güida et al., 2007). For the EGCG-treated group, 12 week old Swiss mice were pre-treated with EGCG for 2 days prior to infection and EGCG treatment was continued throughout the course of infection. Both the EGCG-treated (*n* = 5) and untreated controls (*n* = 5) were infected with 1 × 10⁵ BF wild-type 427 parasites as previously described (Bacchi et al., 2009). Tail blood samples were examined by microscopy to confirm successful infection, and time to death (or humane end-point) was determined. All work with animals was carried out in compliance with a protocol approved by the Clemson University Institutional Animal Care and Use Committee.

3. Results and discussion

To investigate whether EGCG could be used to target ACC in *T. b. brucei*, we first tested EGCG for its ability to inhibit TbACC

enzymatic activity in whole cell lysates from two different life cycle stages: the procyclic form (PF) found in the fly midgut, and the mammalian bloodstream form (BF). Because of the known mode of action of EGCG on mammalian ACC phosphorylation, we sought to preserve any potential changes in phosphorylation caused by EGCG by adding HALT, a broad-spectrum phosphatase inhibitor, to the cell lysate. In the presence of HALT, EGCG reduced ACC activity in both BF and PF lysates, with an EC₅₀ of 29 ± 1.3 μM (n = 3) and 55 ± 2.4 μM (n = 3), respectively (Fig. 1A and B). HALT treatment alone had no effect on ACC activity (data not shown). However, in the absence of HALT, we observed no inhibition of TbACC activity in lysates by EGCG. Thus, EGCG treatment inhibited ACC activity only in the presence of phosphatase inhibitors. Although the effect of EGCG on ACC activity in lysates has not been previously measured, our observations are consistent with the reported mode of action of EGCG on ACC by increasing ACC phosphorylation (Collins et al., 2007; Hwang et al., 2007; Moon et al., 2007; Huang et al., 2009; Murase et al., 2009). That no effect was observed in the absence of HALT rules out a more general inhibitory “tannin” effect on the ACC enzyme (Wink, 2008). Thus, we propose that HALT, by inhibiting the endogenous phosphatases in the lysate, preserved an EGCG-driven increase in ACC phosphorylation, resulting in decreased TbACC activity.

To more directly examine the effect of EGCG treatment on TbACC phosphorylation, we used BF and PF cell lines in which the C-terminus of one TbACC allele was fused to the c-myc epitope (TbACC-myc), which allows for expression of TbACC-myc at endog-

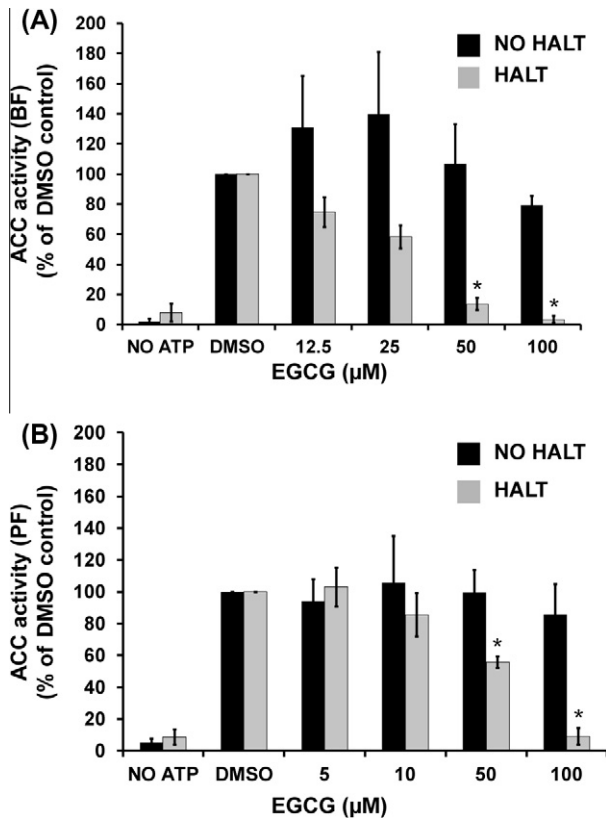


Fig. 1. Inhibition of TbACC activity by EGCG. TbACC activity in lysates of (A) BF and (B) PF trypanosomes was measured in the presence of 5–100 μM EGCG in the absence (black bars) or presence (grey bars) of HALT phosphatase inhibitor cocktail. As a negative control, ATP was omitted from the reaction (No ATP). Values are expressed as a percentage of the DMSO solvent control. DMSO concentrations were maintained at 1% v/v for all conditions. The mean of 3 independent experiments is shown. Error bars indicate ±SEM. The * indicates *P* < 0.01 for the difference between DMSO control and EGCG-treated conditions (student’s *t*-test).

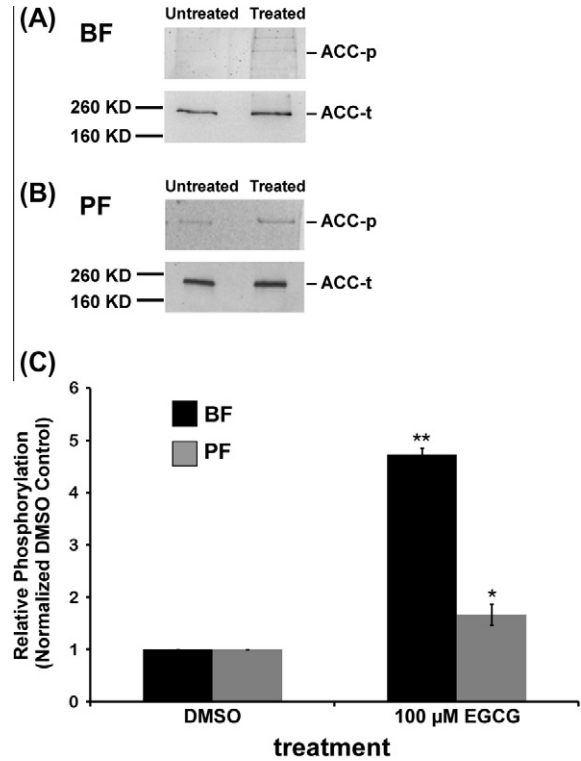


Fig. 2. EGCG induces phosphorylation of TbACC. BF and PF ACC-myc lysates were treated with 100 μM EGCG or DMSO as solvent control in the presence of HALT phosphatase inhibitor cocktail. (A) BF and (B) PF TbACC-myc immunoprecipitates (~5 × 10⁸ cell equivalents/lane) were resolved by SDS-PAGE and stained with Pro Q Diamond phosphoprotein gel stain to detect phosphorylated ACC (ACC-p; upper panels). Identically loaded gels were blotted to nitrocellulose and probed for total ACC with SA-HRP (ACC-t; bottom panels). Representative gels and blots are shown. Unlabeled upper band in panel A is an unknown phosphorylated contaminant. (C) Densitometric quantitation of phosphorylated ACC from phospho-stained gels normalized to total ACC detected by SA-HRP blotting. The mean of 3 independent experiments is shown. Error bars indicate the ±SEM. The * indicates *P* < 0.05 and ** indicates *P* < 0.00001 for the difference between DMSO control and EGCG-treated conditions (student’s *t*-test).

enous levels under the native promoter (Vigueira and Paul, 2011; and this study). BF and PF TbACC-myc lysates were treated with 100 μM EGCG or DMSO as solvent control in the presence of HALT. After EGCG treatment, TbACC-myc immunoprecipitates were resolved by SDS-PAGE and phosphorylated TbACC detected by in-gel phospho-staining with Pro Q Diamond stain (Fig. 2A and B, upper panels, respectively). As a loading control, identically-loaded gels were transferred to nitrocellulose and total ACC detected by blotting with streptavidin-HRP (Fig. 2A and B, lower panels). Densitometry of the phospho-stained gels revealed that EGCG treatment resulted in a statistically significant 4.7 ± 0.12-fold (*P* < 0.00001; *n* = 3) and 1.7 ± 0.20-fold (*P* < 0.05; *n* = 3) increase in phosphorylation of TbACC in BFs and PFs, respectively. This increase in phosphorylation is correlated with the observed inhibition of TbACC activity in BF and PF lysates (Fig. 1A and 1B). It is possible that ACC-myc is inefficiently or aberrantly phosphorylated compared to native ACC, which might mask the true response of ACC to EGCG treatment. However, the facts that immunoprecipitated ACC-myc was also highly active and ACC-myc exhibited the same subcellular fractionation as native ACC (Vigueira and Paul, 2011) argue against this idea. In addition, similar increases in ACC phosphorylation were observed in mammalian cells after treatment with similar concentrations of EGCG (50–400 μM) (Hwang et al., 2007; Moon et al., 2007; Huang et al., 2009; Murase et al., 2009). Taken together, the results shown in Figs. 1 and 2

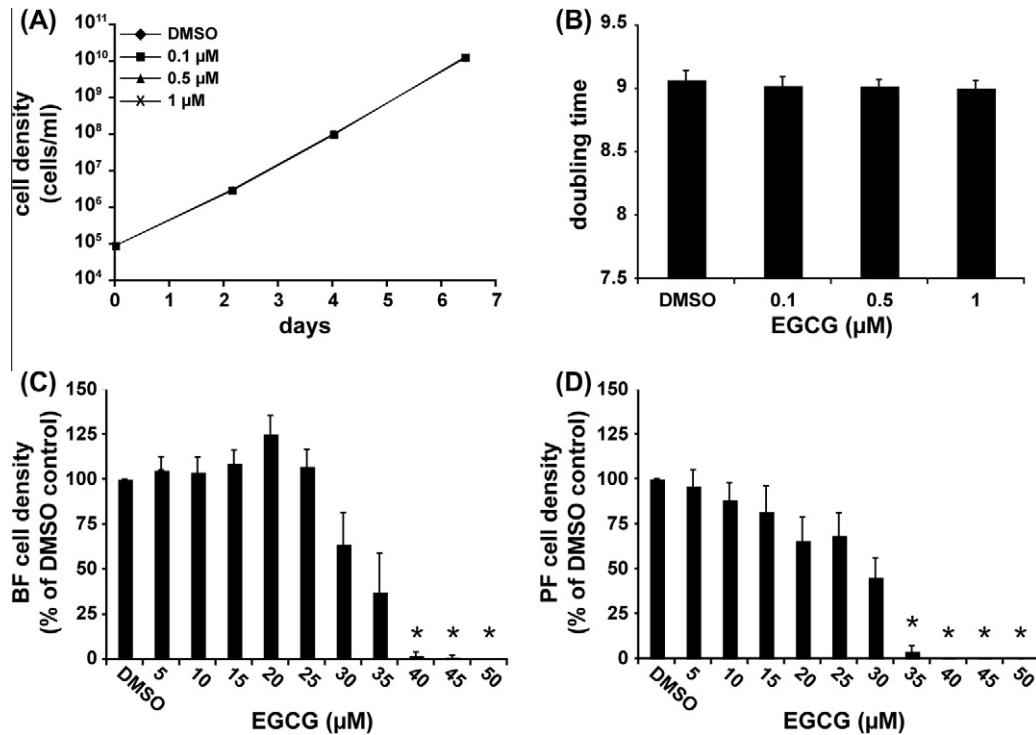


Fig. 3. Effect of EGCG on in vitro growth of *T. brucei*. (A and B) WT BF cells were diluted back to 1×10^6 cells/ml into fresh media containing 0.1–1 μ M EGCG or DMSO as the solvent control and culture cell densities were monitored for 6 days. (A) Representative growth curve showing cumulative culture densities. (B) Mean culture doubling times of 3 independent experiments. Error bars show \pm SEM. (C) WT BF and (D) WT PF cells were back-diluted into fresh media containing 5–50 μ M EGCG or DMSO control. Culture cell densities were determined after 48 h. Values are expressed as a percentage of DMSO control. The mean of 4 independent experiments is shown. Error bars show \pm SEM. The * indicates $P < 0.01$ for the difference between DMSO and EGCG-treated conditions (student's *t*-test).

indicate that ACC is phosphorylated in both BFs and PFs of *T. brucei*, and that EGCG treatment leads to increased TbACC phosphorylation and correspondingly decreased TbACC activity. It is unknown how EGCG treatment leads to phosphorylation and inactivation of TbACC. In mammalian cells, how EGCG activates AMPK is not well understood, but occurs via activation of one or more upstream kinases (e.g. liver kinase B1 (LKB1) and calmodulin-dependent protein kinase kinase (CaMKK)) in a process that may involve the production of reactive oxygen species (ROS) via oxidation of EGCG (Sang et al., 2005; Hou et al., 2005; Hwang et al., 2007; Collins et al., 2007; Moon et al., 2007; Murase et al., 2009). Future work will be needed to determine the identity of the upstream TbACC kinase(s) and the role of ROS in their signaling.

We next examined the effect of EGCG on *T. b. brucei* growth in culture. Orally administered EGCG is rapidly cleared from the body (Ullmann et al., 2003; Chow et al., 2005). In humans, repeated daily oral administration of EGCG can achieve a maximum plasma concentration of $\sim 1 \mu$ M for 5–6 h (Ullmann et al., 2004). A 6 day treatment of BF cells at these physiological EGCG concentrations (0.1–1 μ M) caused no change in growth or doubling time (Fig. 3A and B). Next, we tested higher EGCG concentrations (5–50 μ M), and observed a statistically significant reduction in cell growth over 48 h (Fig. 3C and D), with an EC_{50} of $34 \pm 2.4 \mu$ M and $25 \pm 2.7 \mu$ M for BF and PF parasites, respectively ($P < 0.01$, $n = 4$). These values are consistent with the trypanocidal activity of EGCG (IC_{50} 20.2 μ M at 72 h) previously reported for *T. b. rhodesiense* (Tasdemir et al., 2006). Our previous data showed that TbACC is largely unnecessary in BF parasites cultured *in vitro* and is only required when PF parasites are cultured in low-lipid media (Vigueira and Paul, 2011). Thus, we contend that inhibition of TbACC by EGCG should have little consequence on *T. b. brucei* growth in culture. Consequently, we attribute the reduction in growth observed with EGCG concentrations $> 5 \mu$ M to other previously described target(s) of

EGCG (Ahmed, 2010; Singh et al., 2011), rather than to its inhibition of TbACC.

Finally, we examined the effect of EGCG treatment on the course of *T. b. brucei* infection in mice. Although the EC_{50} values for EGCG for growth in culture are greater than the achievable physiological concentrations in humans, there were three reasons to justify testing EGCG in a mouse model of infection. First, *T. b. brucei* exhibited condition-specific essentiality in the case of both TbACC and enoyl-CoA reductase (EnCAR). PF ACC and EnCAR RNAi cell lines exhibited slowed growth only when exogenous lipids were limited, and a TbACC RNAi line had attenuated virulence in mice (Lee et al., 2006; Vigueira and Paul, 2011). Second, studies in rats showed that intravenous administration of EGCG resulted in greater plasma half-life and in higher maximal plasma concentrations than seen with the oral route of administration (Isbrucker et al., 2006; Misaka et al., 2012). Third, previous studies of EGCG in mice have yielded promising results for treatment of trypanosomiasis. *T. cruzi* mortality in mice and growth in culture was reduced with EGCG treatment (Paveto et al., 2004; Güida et al., 2007). In addition, inflammation caused by *T. b. brucei* infection was reduced and survivorship was extended with oral green tea supplementation (Karori et al., 2008). Taken together, these observations suggest that first, parenteral administration of EGCG might achieve therapeutic plasma concentration of EGCG, and second, the requirement for ACC and thus the parasite's sensitivity to EGCG could be higher *in vivo*.

To test the ability of EGCG to clear a *T. b. brucei* infection in mice, we administered daily IP injections of 4.13 mg/kg EGCG in sterile H₂O two days prior to infection and over the course of the trial. This EGCG concentration had been previously determined not to cause mouse liver damage, though the actual EGCG serum concentration was not determined (Güida et al., 2007). After 2 days of EGCG pre-treatment, Swiss mice were then infected with

1 × 10⁵ BF wild-type 427 parasites as previously described (Bacchi et al., 2009), and time until death (or humane end-point) was determined. In this infection model, EGCG treatment had no effect on infection duration or mouse mortality. Mean time until death was 3.5 days for both treatment and control groups (data not shown). Thus, although EGCG has trypanocidal activity at supra-physiological concentrations, the compound did not attenuate virulence in our acute infection mouse model. EGCG concentrations may not have been sufficiently high in the mouse bloodstream to affect the parasite, however a much lower intraperitoneal dose (0.8 mg/kg/day) led to a significant decrease in parasitemia and increased survivorship of mice infected with *T. cruzi* (Güida et al., 2007). Alternatively, it is possible that the course of infection was too rapid to allow EGCG to exert its effects. If so, a chronic infection model might be better suited to examine the efficacy of EGCG as a possible anti-trypanosomiasis therapy.

In summary, we found that EGCG inhibits TbACC activity in lysates, and this inhibition was correlated with an increase in ACC phosphorylation. This suggests that EGCG may be a useful tool for studying the effects of phosphorylation on TbACC activity. We also demonstrated that EGCG kills both PF and BF parasites in culture. However, EGCG treatment did not affect *T. b. brucei* virulence in a mouse model of acute infection, which reduces its promise as a therapeutic candidate to treat *T. brucei* infection.

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