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Evaluation of Epigallocatechin Gallate and Related Plant Polyphenols as Inhibitors of the FabG and FabI Reductases of Bacterial Type II Fatty Acid Synthase*

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Running Title: Plant Polyphenol Antibacterials

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ABSTRACT

Epigallocatechin gallate (EGCG) is the major component of green tea extracts and possesses antibacterial, antiviral and antitumor activity. Our study focused on validating the inhibition of the bacterial type II fatty acid synthesis system as a mechanism for the antibacterial effects of EGCG and related plant polyphenols. EGCG and the related tea catechins, potently inhibited both the FabG and FabI reductase steps in the fatty acid elongation cycle with IC\textsubscript{50}s between 5 and 15 µM. The presence of the galloyl moiety was essential for activity, and EGCG was a competitive inhibitor of FabI and a mixed-type inhibitor of FabG demonstrating that EGCG interfered with cofactor binding in both enzymes. EGCG inhibited acetate incorporation into fatty acids \textit{in vivo}, although it was much less potent than thiolactomycin, a validated fatty acid synthesis inhibitor, and overexpression of FabG, FabI or both did not confer resistance. A panel of other plant polyphenols was screened for FabG/FabI inhibition and antibacterial activity. Most of these inhibited both reductase steps, possessed antibacterial activity, and inhibited cellular fatty acid synthesis. The ability of the plant secondary metabolites to interfere with the activity of multiple NAD(P)-dependent cellular processes must be taken into account when assessing the specificity of their effects.
INTRODUCTION

Plants are renowned for elaborating compounds of medicinal interest and there is a continuing debate over the clinical value and safety of herbal remedies (1). Botanical extracts include a large variety of low molecular weight secondary metabolites derived from isoprenoid, phenylpropanoid or fatty acid/polyketide pathways. The rich diversity of these compounds is thought to arise from an evolutionary process driven by the selection for the acquisition of resistance to microbiological attack (2). Plants and their natural enemies co-evolve (3), so it is also expected that bacterial defenses have arisen to combat these agents. The majority of plant pathogens are gram-negative bacteria, and consistent with the co-evolutionary hypothesis, gram-positive bacteria are generally more susceptible to the plant secondary metabolites than gram-negative bacteria (4,5). For example, most gram-negative bacteria are refractory to plant secondary metabolites when tested in standard susceptibility tests producing MIC$^1$ values in the range of 0.1 to 1 mg/ml. A primary underlying cause for the resistance of gram-negative bacteria to a wide range of plant toxins is the existence of efflux pumps that prevent the intracellular accumulation of polyphenols (4,6). Genetic elimination of these pumps can increase the efficacy of the plant metabolites by 1-2 orders of magnitude (6).

Green tea, and the individual compounds purified from tea extracts, are among the best known plant polyphenols and possess numerous biological activities including antimicrobial activity against a variety of organisms (7). The main components of a cup of green tea (200 ml) are the well-characterized catechins consisting of (–)-epigallocatechin gallate (EGCG) (140 mg), (–)-epigallocatechin (EGC) (65 mg), (–)-epicatechin gallate (ECG) (28 mg), and (–)-epicatechin (EC) (17 mg) (8). Avid tea drinkers consume several cups a day, and although the potency of the catechins is low, the large quantities of the polyphenols that are ingested make it reasonable to think that they have potential for antibacterial activity. An example is the correlation between the in vivo and in vitro susceptibility of Helicobacter pylori to green tea (9,10).

There is an enormous literature on the biological and biochemical activities of green tea extracts and isolated compounds, but a unifying hypothesis that accounts for their biological
activities has not emerged, and the underlying biochemical targets remain unidentified (8,11,12). For example, EGCG and related polyphenols inhibit the fugal and mammalian type I fatty acid synthase system, most likely by interacting with their reductase and perhaps condensation subdomains (13-16). Indeed, the inhibition of fatty acid synthesis is consistent with many biological effects of EGCG, and it is tempting to conclude that fatty acid synthesis is an important target for tea catechins and plant secondary metabolites. Bacterial fatty acid synthase consists of multiple individual enzymes, each encoded by a separate gene (17,18), in contrast to the mammalian fatty acid synthase, which is a homodimer of single multifunctional polypeptide derived from a single gene (19). The difference between the bacterial and mammalian synthases has been exploited to establish fatty acid synthesis as a target for antibacterial drug discovery (20,21). There are several naturally-produced antibiotics, such as cerulenin (22), thiolactomycin (TLM) (23) and CT2108A (24), and synthetic molecules, like isoniazid (25) and triclosan (26,27), that specifically target this pathway. Some of these, like cerulenin (28), inhibit both the type I and type II fatty acid synthases, while others, like thiolactomycin (23), are selective for the bacterial type II system and are potentially useful therapeutics (29).

The goals of this study were to determine if the tea catechins and related plant polyphenols were inhibitors of bacterial type II fatty acid synthase and to determine if this inhibition was related to their antibacterial properties. We find that EGCG is a potent inhibitor of both the β-ketoacyl-[acyl carrier protein] (ACP) reductase (FabG) and the trans-2-enoyl-ACP reductase (FabI) components in the bacterial type II fatty acid synthase system, a property that is common to a broad range of plant polyphenols. However, the inhibition of fatty acid synthesis was not the sole reason for the antibacterial activity of the tested compounds in the E. coli model system.
EXPERIMENTAL PROCEDURES

Materials—Amersham supplied [1-14C]acetyl-CoA (60 µCi/µmole), [1-14C]acetate (54 µCi/µmole) and [2-14C]malonyl-CoA (52 µCi/µmole); Sigma supplied acetoacetyl-CoA, malonyl-CoA, ACP, NADPH, NADH and chloramphenicol. The trans-2-octenoyl-N-acetylcysteamine was the generous gift of Rocco Gogliotti and John Domagal a (Parke-Davis Pharmaceutical Research). His-tagged FabB, FabH, FabG and FabI from E. coli were purified as described previously (30,31). All other reagents were of the highest available grade.

Green tea extract compounds, including EGCG, EGC, ECG, EC, (−)-galocatechin gallate, (−)-gallocatechin, (−)-catechin gallate and (+)-catechin, propyl gallate, gallic acid, butein, isoliquiritigenin, resveratrol, piceatannol, fisetin, queretin, rhein and plumbagin were purchased from Sigma. Fustin, taxifolin, 2,2′,4′-trihydroxychalcone and 7,3′,4′-trihydroxyisoflavone were purchased from Indofine Chemicals Co. The compounds were dissolved in DMSO at 10 mM.

Bacterial strains—Strain ANS1 (metB1 relA1 spoT1 gyrA216 tolC::Tn10 λ− λR F−) cultured in tryptone broth (1% tryptone) was used to determine the minimal inhibitory concentrations (32). Chemically defined M9 medium (33) was used in the 14C-acetate labeling experiments described below. ANS1 was constructed by P1-mediated transduction of the tolC::Tn10 element in strain EP1581 into strain UB1005 followed by selection for tetracycline resistance. The TolC-dependent type I secretion and multidrug efflux systems are defective in ASN1 (32). Plasmid expressing fabG or fabI was constructed by moving the XbaI-BamHI fragment of the His-tag version of either the fabG or fabI gene(34) in pET15-b into pBluescript II KS(+), respectively. To select for the presence of both reductases, the pBluescript plasmid carrying the enoyl-ACP reductase gene from Clostridium acetobutylicum was modified to replace the AmpR gene with and KanR cassette (35). The His-tagged versions were used to confirm expression using an anti-tag antibody.

β-Ketoacyl-ACP reductase (FabG) assay—The disappearance of NADPH, the cofactor for FabG reaction was measured spectrophotometrically at 340 nm as described previously (36). The reaction mixture contained 0.5 mM acetoacetyl-CoA, 0.2 mM NADPH, 4 µg of FabG protein, 0.1 M
sodium phosphate buffer, pH 7.4 in a final volume of 300 µl. Test compounds were added to the reaction mix at the concentrations indicated in the text and figure legends. The reaction was initiated by the addition of acetoacetyl-CoA. Decrease in the absorbance at 340 nm was recorded for 2 minutes. The initial rate was used to calculate the enzymatic activity. Reactions with DMSO solvent alone were used as controls.

Enoyl-ACP reductase (FabI) assay—Reduction of the trans-2-octenoyl-N-acetylcysteamine substrate analog was measured spectrophotometrically by following the utilization of NADH at 340 nm at 25°C as described previously (30). Briefly, a standard reaction contained 0.1 M sodium phosphate, pH 7.5, 100 µM trans-2-octenoyl-N-acetylcysteamine, 200 µM NADH, and 12 µg of FabI in a final volume of 300 µl. Decrease in absorbance at 340 nm was measured at 25°C for the linear period of the assay (usually the first 1-2 min). Test compounds were added in the assay to the indicated concentrations as described in the text and figure legends. Equal volume of the solvent was used for the untreated control.

β-Ketoacyl-ACP synthase I (FabB) and III (FabH) assay—The coupled assay of FabH, as described previously (34), contained 25 µM ACP, 1 mM β-mercaptoethanol, 65 µM malonyl-CoA, 45 µM [1-14C]acetyl-CoA (specific activity 60 µCi/µmole), 1 µg purified FabD, 0.1 M sodium phosphate buffer, pH 7.0, and 20 ng FabH protein in a final volume of 40 µl. The FabD protein was present to generate the malonyl-ACP substrate for the reaction. The ACP, β-mercaptoethanol and buffer were pre-incubated at 37°C for 30 minutes to ensure the complete reduction of ACP. The reaction was initiated by the addition of FabH. After incubation at 37°C for 15 minutes, 35 µl of reaction mixture was removed and dispensed onto a paper filter disc (Whatman No. 3MM filter paper). The disc was washed successively with ice cold 10%, 5%, and 1% trichloroacetic acid with 20 minutes for each wash and 20 ml of wash solution per disc. The filter discs were dried and counted for 14C-isotope in 3 ml of scintillation fluid.

The condensation assay for FabB was the same as described previously (37). Briefly, FabB assays contained 45 µM myristoyl-ACP, 50 µM [2-14C]malonyl-CoA (specific activity, 52
µCi/µmole), 100 µM ACP, 1 µg FabD and 25 ng FabB in a final volume of 20 µl. ACP was reduced by 0.3 mM DTT before the other reaction components were added. The reaction was initiated by the addition of the enzyme. After incubation at 37°C for 15 min, the reaction was stopped by adding 0.4 ml of the reducing reagent containing 0.1 M K₂HPO₄, 0.4 M KCl, 30% tetrahydrofuran and 5 mg/ml NaBH₄. The reaction contents were vigorously mixed after the addition of the reducing reagent and incubated 37°C for 40 minutes before being extracted into 0.4 ml toluene. The ¹⁴C-isotope in the upper phase was quantitated by scintillation counting. IC₅₀s were determined in a 10-point assay at a series of concentrations. A line was drawn between the points and the IC₅₀ was the interpolated concentration that gave 50% inhibition (see Fig. 1).

**Determination of the MIC**—The MICs of the test compounds against *E. coli* strain ANSI were determined by a broth microdilution method. ANSI was grown to mid-log phase in 1% tryptone broth and then diluted 30,000-fold in the same medium. A 10 µl aliquot of the diluted cell suspension (3,000 to 5,000 colony forming units) was used to inoculate each well of a 96-well plate (U-bottom with low evaporation lid) containing 100 µl of tryptone broth with the indicated concentration of inhibitors. The plate was incubated at 37°C for 20 hours before being read with a Fusion™ Universal Microplate Analyzer (Packard, Canada) at 600 nm. The absorbance was normalized to the solvent treated control which was considered as 100%.

**[1-¹⁴C]Acetate labeling**—Strain ANSI was grown to mid-log phase in M9 minimal medium supplemented with 0.1% casamino acids, 0.4% glycerol and 0.0005% thiamin. A one ml aliquot of cells was treated with the antimicrobials for 10 minutes at indicated concentrations as described in the figure legends. An equal volume of the solvent DMSO was added to the untreated control. Cells were then labeled with 10 µCi of [1-¹⁴C]acetate for 1 hour before being harvested by centrifugation. The cell pellets were washed with PBS and resuspended in 100 µl of M9 medium. The total cellular lipids were extracted and the incorporated ¹⁴C-isotope in lipids in the chloroform phase was quantitated by scintillation counting. Results reflect duplicate experiments.
RESULTS

Inhibition of FabG and FabI by tea catechins—The enzymes of the type II fatty acid synthase were assayed for inhibition by EGCG, the major catechin of green tea (Fig. 1A). The elongation condensing enzyme (FabB) was refractory to EGCG inhibition, whereas FabH, the initiating condensing enzyme was inhibited by EGCG with an IC$_{50}$ of 40 µM. The most potently inhibited enzyme was FabG, the NADPH-dependent ketoreductase in the pathway which exhibited an IC$_{50}$ of 5 µM. FabI, the NADH-dependent enoyl reductase was inhibited by EGCG with an IC$_{50}$ of 15 µM. FabG was used as a model enzyme to determine the inhibitory potency of the other significant green tea catechins (Fig. 1B). EGCG was the most potent, but the other catechins, ECG, GCG and CG, all exhibited activity against FabG with IC$_{50}$s ranging from 5-15 µM. Similarly, the four compounds exhibited activity against FabI with IC$_{50}$s between 5 and 15 µM (Table 1). Also, we found that the gallate substitution was critical for the inhibitory activity of the compounds (Table 1). Removal of the galloyl moiety from any of the tea catechins resulted in complete loss of inhibitory activity in vitro. The galloyl group itself has no significant inhibitory activity as evidenced from the lack of FabG and FabI inhibition by propyl gallate and gallic acid (Table 1). These data establish that green tea catechins have significant inhibitory activity against the reductase enzymes of the bacterial type II fatty acid synthase. Furthermore, the structure activity relationship shows that the portion of the EGCG molecule required for inhibition is defined by the boxed area in Scheme 1.

Scheme 1. The portion of the EGCG molecule that is required for its inhibitory activity against FabG and FabI as determined from the data in Fig. 1 and Table 1 is enclosed by the box. This structure is rotated 90° clockwise and stripped of the irrelevant substituents to reveal a putative active nucleus composed of two polyhydroxyphenol rings joined by an ester linkage to gallic acid.
Mechanism for tea catechin inhibition of FabG and FabI—The kinetic mechanism for the inhibition of FabG and FabI was determined using EGCG as the model compound (Fig. 2). Both FabG (38) and FabI (39) have compulsory ordered mechanisms with the nucleotide cofactors as the leading substrates. This knowledge allowed us to design a kinetic analysis that would distinguish between the three possible outcomes: EGCG could bind to the free enzyme, the enzyme-substrate complex, or both to prevent catalysis. In the first case, the inhibition pattern with respect to the cofactor would be competitive; in the second, the inhibition pattern would be non-competitive; and in the third case, mixed-type inhibition would be observed (40). The inhibition of FabG by EGCG was mixed with respect to NADPH (Fig. 2A). Thus, EGCG binds to both the free enzyme to prevent the binding of the nucleotide cofactor and also to the FabG-NADPH complex to prevent the binding of the substrate. In contrast, EGCG was a competitive inhibitor of FabI with respect to NADH (Fig. 2B), meaning that EGCG interferes with activity by binding to the free enzyme and preventing the binding of NADH. These data illustrate that EGCG inhibits both enzymes by association with the nucleotide cofactor binding site, and with FabG, EGCG has the additional property of binding to the enzyme-cofactor complex.

Antimicrobial effect of EGCG—As reported previously, EGCG has moderate antibacterial activity (5,7). Most gram-negative bacteria are resistant to plant polyphenols, therefore we used our E. coli strain ANS1 (tolC) to eliminate the activity of a major class of multidrug efflux pumps (6,32). Such tolC mutants are used routinely as a platform to investigate the mechanism of drug action (41). In strain ANS1, EGCG had an MIC of 500 µM (Fig. 3A), although the parent wild-type strain UB1005 had the same MIC in this case. To evaluate whether the antibacterial properties of the catechins could be attributed to their effects on fatty acid synthesis, we first determined if the compounds blocked the incorporation of acetate into membrane fatty acids (Fig. 3B). EGCG indeed attenuated fatty acid synthesis in vivo compared to the untreated control cells and cells treated with chloramphenicol, a protein synthesis inhibitor. However, the effect of EGCG was not nearly as great as the effect of TLM, an established inhibitor of fatty acid synthesis at the elongation condensing enzyme step (42,43). These data demonstrate that EGCG inhibits fatty
acid synthesis in vivo, albeit it is not as potent when present at double its MIC compared to a bone fide inhibitor added to the cells at the same relative concentration, suggesting that fatty acid synthesis may not be the sole pathway inhibited by EGCG.

Overexpression of individual genes and the isolation of resistant mutants are powerful genetic tools for target validation in vivo. For example, FabB was unequivocally established as the critical in vivo target for TLM by demonstrating that overexpression of FabB increased the resistance to TLM and that a point mutation conferring resistance to the drug was localized to the fabB gene (32,42). Similarly, FabI was validated as the triclosan target through analyzing the effects of FabI overexpression and the isolation of resistant mutants in the fabI gene (26,27). Unfortunately, there are no known specific inhibitors against FabG to corroborate the function of our FabG expression plasmid, but the construct increases FabG expression, and like the FabB and FabI constructs with identical promoter elements, is anticipated to shift the dose-response curve for an antibacterial that selectively targets FabG. Therefore, we examined the effect of the overexpression of FabG, FabI, or both on the MIC for EGCG in strain ANS1 (Fig. 3A). Unlike other drugs that primarily target lipid synthesis, none of these plasmids increased the resistance of strain ANS1 to EGCG. We also attempted to raise EGCG-resistant mutants using the techniques described previously to isolate mutants specifically resistant to TLM (32) or triclosan (27). However, we were unable to obtain colonies resistant to 1 mM EGCG. These data do not support fatty acid synthesis as a primary target for EGCG’s action and the inability to isolate specific mutants is consistent with the existence of multiple targets in vivo.

**FabG/FabI inhibition by other plant natural products**—In order to determine if the inhibition of FabG and FabI was a property of other natural products with the biphenyl core structure diagramed in Scheme 1, we tested a panel of plant polyphenols (Table 2). The MIC values were obtained using strain ANS1 to eliminate the effects of efflux pumps, and the MICs for a wild-type and tolC isogenic pair were reported previously (6). Some of the natural products, like coumestrol, rhein and plumbagin, had potent antibacterial activity, but were not significant inhibitors of either the FabG or FabI reductases. However, in general all of the tested natural products with the
biphenyl chalcone nucleus inhibited both enzymes. Resveratrol, piceatannol, fustin, taxifolin and 7,3',4'-trihydroxyisoflavone were good inhibitors for FabG and FabI, but their MIC values were high, indicating only weak activity against any target in vivo.

The remaining polyphenols with comparable reasonable MIC values (Table 2) were further evaluated for their effects on fatty acid synthesis at 4-times their MICs. The inhibition of [14C]acetate incorporation by beuten, 3HC, fisetin and quercetin was between 20 and 50% of treated with DMSO (Table 2). The highest inhibition was observed with isoliquiritigenin, which reduced the [14C]acetate incorporation by about 75%. The FabG/FabI inhibitor with the lowest MIC against strain ANS1, 3HC, was selected for more detailed investigation to determine if its antibacterial action could be linked to blocking fatty acid synthesis (Fig. 4). FabH was not inhibited by 3HC. FabB, FabG and FabI were all inhibited by 3HC with IC50s of 100, 25 and 40 µM, respectively (Fig. 4A). Thus, 3HC was less potent than EGCG in vitro. The MIC for 3HC was 6.25 µM, and the MIC was not shifted by the overexpression of either FabG or FabI (Fig 4B). Finally, acetate incorporation studies showed that fatty acid synthesis was inhibited only at concentrations significantly higher than the MIC for the compound (Fig. 4C). Although like most of the other plant polyphenols, 3HC inhibited the reductase steps in fatty acid synthesis in vitro, these data establish that the most effective antibacterial examined in the screen did not primarily inhibit cell growth by blocking the type II fatty acid synthesis. Likewise, we examined the inhibition of fatty acid synthesis in vivo using four other analogs with MICs below 75 µM (Table 2). Although all of the compounds inhibited acetate incorporation into cellular fatty acids, none were as potent as the TLM control. Thus, these polyphenols reduced fatty acid synthesis, but the extent of inhibition was not consistent with fatty acid synthesis as the primary target for their antibacterial activity.
DISCUSSION

The emergence of multidrug resistance in pathogenic bacteria is a global problem that calls for the development of new antibiotics with unique cellular targets (44). Consequently, the differences between bacterial and mammalian fatty acid biosynthesis are being exploited to develop the type II fatty acid synthase as a target for novel drug discovery (21,45,46). FabG is ubiquitously expressed in all bacteria, is highly conserved across species and is the only known isozyme that catalyzes the essential keto reduction step in the elongation cycle (47). Although there are no known FabG inhibitors (21,45,46), FabG represents an ideal focus for the development of new antibiotics based on the hypothesis that FabG would be vulnerable to inhibitors that interact with its cofactor binding site (48). Our study identifies a small molecule scaffold with potent inhibitory effects against FabG, as well as FabI, an established target in bacterial fatty acid synthesis. Green tea catechins (EGCG) and related plant polyphenolic compounds are selective inhibitors of the FabG and FabI reductase steps of bacterial type II fatty acid synthesis. The galloyl moiety of the catechins is absolutely essential for inhibition. Two hydroxyphenyl rings connected by a 2-3 carbon linker is the basic requirement for inhibition as illustrated in Scheme I. Aspects of this core structure is also found in the hydroxydiphenyl ether class of potent FabI inhibitors (27), except in this case the two hydroxyphenyl rings are connected by a single bridging oxygen. The kinetic analysis indicates EGCG binds to the ligand-free form of both FabG and FabI and blocks NAD(P)H binding. This result does not necessarily mean that EGCG occupies the same pocket as NAD(P)H, so to advance the design of inhibitors based on this scaffold it is important to determine the structure of the EGCG-enzyme binary complex. This structure is particularly significant in light of the high degree of conformational flexibility in FabG (38). The tight binding of triclosan to FabI is due to its ability to lock the reductase in a closed confirmation (31), thus identifying a slow-binding polyphenol derivative with a high-affinity interaction with a specific FabG conformation has the most promise as an antibacterial lead.

The green tea catechins also inhibit the reductase and condensation reactions of the polyfunctional mammalian type I fatty acid synthase (13-15). Thus, same steps are targeted by
EGCG in the mammalian enzyme as the bacterial system, and it is tempting to speculate that the anticancer, proapoptotic and hypolipidemic activities associated with green tea extracts and EGCG arise from the inhibition of fatty acid synthesis (13-15,49-51). However, these data must be considered correlative in the absence of a genetic validation of fatty acid synthase as the principal cellular target for the catechins in animal cells. These experiments are critical to the interpretation of the biochemical data in light of the potentially high number and diversity of EGCG biochemical targets, such as matrix metalloproteinases (52) and the laminin receptor (53), that may also contribute to its anticancer activity.

Treatment of bacterial cells with EGCG results in the inhibition of fatty acid production, implicating this pathway as a target for their antibacterial activity. However, EGCG was not as effective at blocking acetate incorporation as established fatty acid synthase inhibitors when compared at 2 times their MICs. Also, overexpression of either one, or both, of the reductase targets (FabG or FabI) did not confer increased resistance to EGCG. Genetic target validation, such as the acquired resistance to TLM or triclosan by either overexpression or mutations in the target genes fabB (32) or fabI (27), is essential to the confirmation of fatty acid synthesis as a target for EGCG. Our experiments failed to forge a definitive link between the FabG/FabI reductases and the antibacterial effect of EGCG arguing against fatty acid synthesis as the sole antibacterial target for EGCG in vivo. We reached the same conclusions with 3HC, a chalcone secondary metabolite with a low MIC against strain ANS1 and inhibitory activity against FabG and FabI. In a similar study, inhibitors of fungal fatty acid synthesis were identified from plant extracts (16); however, a correlation between the fatty acid inhibitory activity and the antifungal action of the compounds could not be established.

Understanding the potential benefits and risks of tea drinking is complicated not only from the great variety of secondary metabolites present (8), but also from our data demonstrating that a single compound from tea has multiple targets. This finding opens the potential for diverse mechanisms of action depending on the relative importance of the targets in the different experimental systems under investigation. Both FabG and FabI are members of the short-chain...
dehydrogenase/reductase (SDR) superfamily (54,55). This family is composed of a large cohort of proteins that bind nicotinamide nucleotide cofactors using a similar protein fold and catalyze a long list of essential reactions in intermediary metabolism (56,57). Our kinetic results indicate that EGCG interferes with the binding of the nicotinamide adenine dinucleotide cofactor in both enzymes, suggesting that the EGCG scaffold recognizes a common feature in their NAD(P) binding domains. This concept leads to the hypothesis that EGCG and related polyphenol structures may have inhibitory activity against other enzymes in the SDR superfamily. The effects may even be broader if they extend to the nucleotide binding sites in other protein families as well. The ability of EGCG to inhibit FabH and not FabB may be understood based on this hypothesis since FabH uses acetyl-CoA (an adenine nucleotide) as a substrate whereas FabB does not. Not only are NAD(P) cofactors essential in intermediary metabolism, the importance of their signaling functions in controlling cell physiology is becoming increasingly apparent (58). For example, many of the same flavanoids that inhibit FabG/FabI (Table 2) also interact with Sir2, an NAD-binding protein that influences lifespan in yeast (59). These data point to the ability of the plant polyphenols to bind to a variety of enzymes that use NAD(P) and more work needs to be done to provide information on their multiple effects on the cell biology of different systems and the multiple targets presented by the diversity of enzymes that utilize NAD(P). Also, minor changes in the hydroxylation pattern of two ring systems and the distance and composition of the linkers that connect them are likely to have a major impact on the spectrum of enzymes targeted by the polyphenols. Considering the large number of potential protein targets for these compounds this appears to be a daunting task, but nonetheless, plant polyphenol secondary metabolites may eventually prove to be suitable chemical scaffolds for the future development of selective inhibitors of fatty acid synthesis in bacteria and other systems.

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FOOTNOTES

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Abbreviations used are: MIC, minimal inhibitory concentration; EGCG, (–)-epigallocatechin gallate; EGC, (–)-epigallocatechin; ECG, (–)-epicatechin gallate; EC, (–)-epicatechin; IC₅₀, concentration of compound required for 50% inhibition under defined assay conditions; ACP, acyl carrier protein; FabG, β-ketoacyl-ACP reductase; FabI, enoyl-ACP reductase; FabH, β-ketoacyl-ACP synthase III; FabB, β-ketoacyl-ACP synthase I; TLM, thiolactomycin; 3HC, 2,2′,4′-trihydroxychalcone; SDR, short-chain dehydrogenase/reductase; DMSO, dimethylsulfoxide.
REFERENCE LIST


TABLE 1. Inhibitory effects of green extract compounds on the activity of fatty acid synthetic enzymes (FabG and FabI) and bacterial growth.

<table>
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<th>Compound</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>IC<sub>50</sub> and MIC values greater than 100 µM and 200 µM, respectively, were not determined. The IC<sub>50</sub> values were rounded to the nearest 5 µM.
TABLE 2. Inhibitory effects of polyphenol compounds on the activity of fatty acid synthetic enzymes (FabG and FabI) and bacterial growth.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
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<th>MIC (µM)ᵃ</th>
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ᵃIC₅₀ and MIC values greater than 100 µM and 200 µM, respectively, were not determined. The IC₅₀ values were rounded to the nearest 5 µM.
ᵇThe amount of [¹⁴C]acetate incorporated into the cells treated with DMSO was 100%. The compounds were tested at 4 times their MICs.
ᶜNot tested.
FIGURE LEGENDS

FIG. 1. Inhibitory effects of EGCG and its analogs on fatty acid biosynthetic enzymes.

Panel A, EGCG inhibited the enzymatic activities of E. coli FabG (○), FabH (●) and FabI (□) with apparent IC₅₀s of 5 µM, 15 µM and 40 µM, respectively. FabB (■) was refractory to EGCG. Enzyme treated with equal volume of the solvent (DMSO) was taken as the 100% active control. The specific activity of FabB was 0.36 nmol [¹⁴C]malonyl-CoA incorporated per min per µg protein; FabG was 2.6 nmol NADPH oxidized per min per µg; FabH was 0.77 nmol [¹⁴C]acetyl-CoA incorporated per min per µg; and FabI was 0.33 nmol NADH oxidized per min per µg. Panel B, EGCG (○) and its analogs, ECG (△), CG (▲) and GCG (●), potently inhibited E. coli FabG with apparent IC₅₀s between 5 and 15 µM. The assay conditions are described under “Experimental Procedures.” Error bars show standard deviations.

FIG. 2. The mechanism of inhibition of EGCG on E. coli FabG and FabI. Panel A, EGCG is a mixed-type inhibitor of FabG with respect to NADPH. The double reciprocal plot of 1/v versus 1/[NADPH] at different concentrations of EGCG, 6 µM (■), 4 µM (●) and 0 µM (○), intercepted to the left of 1/v axis and above the 1/[NADPH] axis indicating EGCG is a mixed-type inhibitor for NADPH. Panel B, EGCG is a competitive inhibitor of FabI with respect to NADH. The double reciprocal plot of 1/v versus 1/[NADH] at different concentrations of EGCG, 30 µM (■), 20 µM (□), 10 µM (●) and 0 µM (○), intercepted on the 1/v axis indicating EGCG is a competitive inhibitor for NADH. The assays were performed using the conditions described under “Experimental Procedures.” Error bars show standard deviations.

FIG. 3. Inhibitory effects of EGCG on the growth and fatty acid biosynthesis of E. coli.

Panel A, EGCG inhibited the growth of E. coli strain ANS1 with an MIC at 500 µM (○). Addition of constructs expressing fabG (□), fabl (●) or both (△) did not rescue the cells from EGCG. Growth of cultures treated with DMSO was considered as 100%. Panel B, the effects of the antimicrobials on fatty acid biosynthesis were tested by monitoring the [¹⁴C]-acetate incorporation into lipids at 1x (clear bars) and 2x (gray bars) MIC concentrations of the drugs. When the cells were treated with
EGCG at 2x the MIC concentration (1 mM), \(^{14}\text{C}\)-acetate incorporation was inhibited by 50% suggesting that fatty acid biosynthesis was compromised in the presence of EGCG. When at the MIC concentration of EGCG (500 \(\mu\)M) was used, 25% inhibition was observed. TLM, a known inhibitor of fatty acid biosynthesis, exhibited a more profound inhibitory effect on \(^{14}\text{C}\)-acetate incorporation at 5 \(\mu\)M and 10 \(\mu\)M. No inhibition was obtained with chloramphenicol, a known inhibitor of protein synthesis, at 5 \(\mu\)M and 10 \(\mu\)M.

**Fig. 4. Biochemical and biological effects of 2,2',4'-trihydroxychalcone.** Panel A, 2,2',4'-trihydroxychalcone inhibited *E. coli* FabB (○), FabG (□) and FabI (●) activity with apparent IC\(_{50}\)s of 100 \(\mu\)M, 25 \(\mu\)M and 40 \(\mu\)M, respectively. FabH (■) was refractory to this compound. Panel B, 2,2',4'-trihydroxychalcone inhibited *E. coli* growth with a MIC of 6.25 \(\mu\)M. Overexpression of *fabG* (▲) or *fabI* (●) did not make the cells more resistant to the drug compared to control strain ANS1 (○). Panel C, \(^{14}\text{C}\)-acetate incorporation was inhibited when cells were treated with increasing concentrations of 2,2',4'-trihydroxychalcone. TLM (a known fatty acid biosynthesis inhibitor) and chloramphenicol (a known protein synthesis inhibitor) were used as positive and negative controls. The assays were performed using the conditions described under “Experimental Procedures.” Error bars show standard deviations.
FIGURE 1
FIGURE 2

A

$1/\nu (\Delta \text{OD}_{340\text{nm}}/\text{min})^{-1}$

$1/[\text{NADPH}] (\mu\text{M})^{-1}$

B

$1/\nu (\Delta \text{OD}_{340\text{nm}}/\text{min})^{-1}$

$1/[\text{NADH}] (\mu\text{M})^{-1}$
FIGURE 3
FIGURE 4
Evaluation of epigallocatechin gallate and related plant polyphenols as Inhibitors of the FabG and FabI reductases of bacterial type II fatty acid synthase
Yong-Mei Zhang and Charles O. Rock

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