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Structure–Activity Relationship of Polyphenols That Inhibit Fatty Acid Synthase

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Many flavone derivatives inhibit FAS, and their A and B rings play an important role, but is the C ring necessary for the inhibition of FAS? Here, using nordihydroguaiaretic acid (NDGA), with two phenyl rings connected by a four-carbon chain, as a representative, the structural basis for the inhibition of animal fatty acid synthase (FAS) by polyphenols was investigated. NDGA potently inhibits the overall reaction of FAS (IC_{50} = 9.3 ± 0.1 μM). The kinetic study indicated that NDGA inhibits FAS competitively with respect to acetyl-CoA, noncompetitively with respect to malonyl-CoA, and in a mixed manner with respect to NADPH. The inhibitory mechanism is the same as that of FAS flavonoid inhibitors. This suggests that the C ring of flavonoids is not essential for their FAS inhibitory effect. This conclusion was further confirmed by the results obtained for different polyphenols. A structure-activity relationship study indicated that a biphenyl core exists in all FAS polyphenol inhibitors. Thus, we propose a common model possibly shared by all FAS polyphenol inhibitors. The model includes two almost planar aromatic rings with their respective hydroxyl groups, and a proper ester linkage between the two rings that possibly causes the inhibition of FAS by irreversibly inhibiting the β-ketoacyl reductase domain.

Key words: fatty acid synthase, flavonoid, inhibitor, nordihydroguaiaretic acid, polyphenol.

Abbreviations: Ac-CoA, acetyl-CoA; CG, (–)-catechin gallate; FAS, fatty acid synthase; IC_{50}, the inhibitor concentration required to inhibit the enzyme activity to 50% of that observed in the absence of the inhibitor; Mal-CoA, malonyl-CoA; NDGA, nordihydroguaiaretic acid.

The animal fatty acid synthase (FAS, EC 2.3.1.85) comprises two identical subunits, each containing seven discrete functional domains, *i.e.* the acyltransferase, β-ketoacyl reductase, β-ketoacyl synthase, β-hydroxyacyl dehydratase, enoy reductase, thioesterase and acyl carrier protein domains. FAS synthesizes fatty acids, mainly palmitate, *de novo* from acety-CoA (Ac-CoA), malonyl-CoA (Mal-CoA) and NADPH (*1*, *2*).

FAS is a very significant enzyme in energy metabolism, and is associated with many human diseases (*3*). The inhibition of FAS has been demonstrated to mediate profound weight loss in animals; therefore, FAS is regarded as a potential target for anti-obesity therapies (*3–11*). In addition, recent reports have shown that FAS is emerging as an important therapeutic target for carcinomas of the breast, prostate, endometrium, ovary, and colon (*9*, *12*, *13*).

Potent FAS inhibitors are expected to be applied to the treatment of cancer and obesity. Previously, we reported some natural FAS inhibitors, including flavonoids and tea polyphenols (*14–17*), and these inhibitors have similar molecular skeletons; but is this skeleton necessary for the FAS inhibitory activity? All polyphenol FAS inhibitors have two phenyl rings (A ring and B ring), and differ in the linkage between the two rings. The linkage (C ring) of flavone derivatives produces a conjugate plane with A and B rings, but the A and B rings of tea polyphenols do not lie in the same plane, because the two rings are linked by a twisted C ring. These differences in tridimensional structure possibly result in differences in the FAS inhibitory activity. In addition, the hydroxyl groups in the A and B rings affect the inhibitory activity of the compounds, but the hydroxyl group of the C ring does not (*14*). Therefore, what role does the C ring play in inhibiting FAS? To determine the inhibitory mechanism of animal FAS by polyphenols, different polyphenols were subjected to a kinetic study of FAS inhibition. In addition, based on the structure–activity relationship, the structural effect of polyphenols was analyzed, which revealed a common structural model shared by all FAS polyphenol inhibitors. This model may be helpful for identifying or synthesizing selective inhibitors of animal FAS.

MATERIALS AND METHODS

Materials—Ac-CoA, Mal-CoA, NADPH, coenzyme A, apigenin, baicalein, flavone, genistein, 4′,6,7-trihydroxyisoflavone, daidzein, resveratrol, phloretin, nordihydroguaiaretic acid (NDGA), isoliquirtigenin, morin, (–)-catechin gallate (CG), (–)-epicatechin, (–)-catechin and gallic acid were purchased from Sigma-Aldrich. All other reagents were local products of analytical grade purity.

Preparation of FASs and Substrates—Fowl (duck) FAS was used. The preparation, storage and use of FAS were

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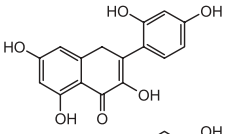
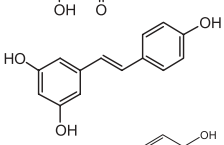
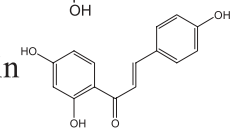
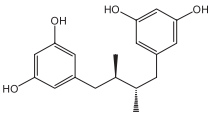
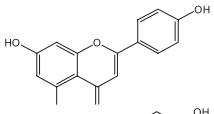
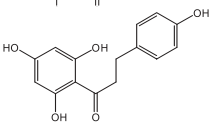
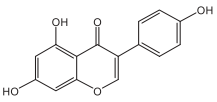
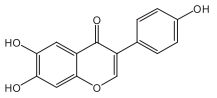
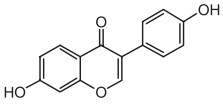
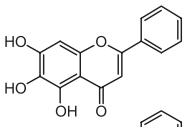
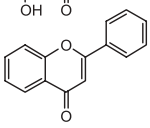
Polyphenols	Structure	IC_{50} (μM)	
		OA	KR
Morin		[a]2.3 ± 0.1	26 ± 2
Resveratrol		8.5 ± 0.2	120 ± 5
Isoliquirtigenin		8.8 ± 0.2	141 ± 6
NDGA		9.3 ± 0.1	427 ± 13
Apigenin		17.6 ± 0.3	[b]>200
Phloretin		26.1 ± 0.9	172 ± 7
Genistein		28.7 ± 1.1	136 ± 10
4',6,7-Trihydroxylisoflavone		29.5 ± 0.9	119 ± 9
Daidzein		73.2 ± 3.4	214 ± 7
Baicalein		[a]111.7 ± 2.3	>1000
Flavone		>1000	>1000

Fig. 1. **Inhibitory effects of polyphenols on FAS.** OA and KR is, respectively, the overall reaction and β-ketoacyl reduction activity of FAS. [a]From Ref. *14*; [b]The assay of FAS activity was influenced by apigenin at high concentration.

performed as described previously (*18*). Briefly, the FAS preparation was homogeneous on polyacrylamide gel electrophoresis in the presence and absence of SDS. The enzyme and substrate concentrations were determined by spectrophotometry with the following extinction coefficients: FAS, 4.83×10^5 M^{-1} cm^{-1} at 279 nm (*19*); Ac-CoA, 1.54×10^4 M^{-1} cm^{-1} at 259 nm, pH 7.0; Mal-CoA, 1.46×10^4 M^{-1} cm^{-1} at 260 nm, pH 6.0; NADPH, 6.02×10^3 M^{-1} cm^{-1} at 340 nm and 1.59×10^4 M^{-1} cm^{-1} at 259 nm, pH 9.0 (*18*, *20*).

Assay of FAS Activity—The overall FAS activity was determined using an Amersham Pharmacia Ultrospec 4300 pro UV-Vis spectrophotometer at 37°C by following the decrease in NADPH at 340 nm. The reaction mixture contained potassium phosphate buffer, 100 mM, pH 7.0; EDTA, 1 mM; dithiolthreitol, 1 mM; Ac-CoA, 3 μM; Mal-CoA, 10 μM; NADPH, 35 μM and FAS, 5 μg in a total volume of 2.0 ml (*18*).

The β-ketoacyl reduction was determined at 37°C by measuring the change of absorption at 340 nm. The reduction reaction mixture (2 ml) contained ethyl acetoacetate, 40 mM; NADPH, 35 μM; EDTA, 1 mM; dithiolthreitol, 1 mM and FAS, 5 μg in 100 mM phosphate buffer, pH 7.0 (*21*, *22*).

Inhibition Studies of Polyphenols—The inhibition by polyphenols was investigated by adding the inhibitors to the reaction system before the addition of FAS. The polyphenols were dissolved in dimethylsulfoxide and then added to the reaction mixture described above. The final concentration of dimethylsulfoxide was under 0.5% (v/v), in

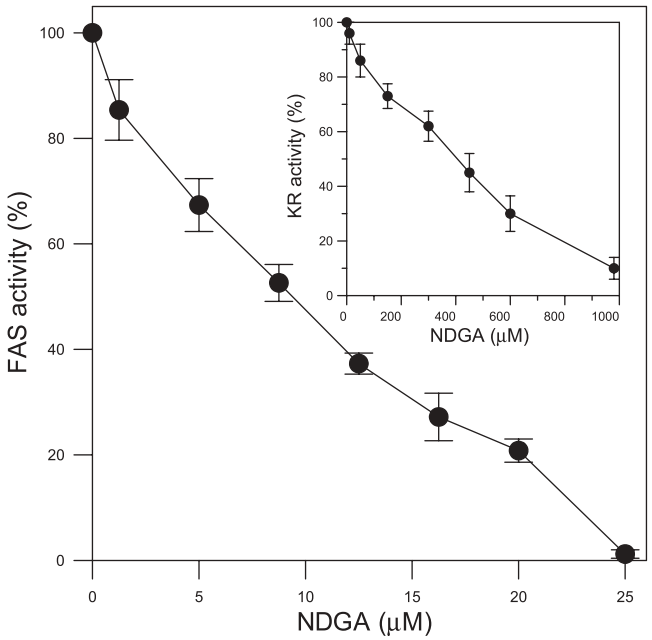


Fig. 2. **Effects of NDGA on FAS activity.** The inhibition of the overall reaction of FAS in the presence of various concentrations of NDGA was measured. The inset shows the inhibition of the β-ketoacyl reduction activity of FAS by different concentrations of NDGA. Error bars show standard deviations. Each point is the mean of 3 experiments.

order to avoid the interference with the FAS activity. The extent of inhibition by the addition of inhibitors was measured by reference to the half inhibition concentration (IC_{50}).

The time course of inactivation was determined by taking aliquots to measure the residual activity at the indicated times after the enzyme solution was mixed with the inhibitor. Time-dependent inhibition is usually a slow-binding inactivation. Vehicles without inhibitor were used as controls. In these experiments, the vehicles did not affect FAS activity over several hours. The apparent first-order rate constant (k_{obs}) can be obtained from the semi-logarithmic plot of the inactivation time course.

Protection Experiments of Inactivation—An inhibitor was added to FAS solutions mixed with one substrate or reversible inhibitors, and then the residual activity of FAS was measured at different time intervals. The same concentrations of the inhibitor and FAS solutions without substrate were used as the control. The apparent first-order rate constants of these reactions were compared to determine the substrate protective effects.

Molecular Modeling Methods—Molecular modeling calculations were carried out using Hyperchem 7.0 for Windows (HYPERCUBE, Inc., Canada). The compounds were constructed and minimized by the molecular mechanics MM+ method to an energy gradient of 0.001 kcal/mol.

RESULTS AND DISCUSSION

We previously reported that some flavone derivatives, morin, luteolin, quercetin, kaempferol, fisetin, baicalein and myricetin, inhibit FAS, and that their inhibitory activities are related to the structures of their A and B rings (*14*). But is the C ring also necessary for the inhibitory effects of polyphenols on FAS? A polyphenol compound containing two phenyl rings linked by a four-carbon chain, NDGA, was used as a representative compound lacking the C ring for further investigation (structure shown in Fig. 1). Figure 2 shows that NDGA inhibits FAS in a concentration-dependent manner and that about 9.3 μM NDGA suppresses the overall FAS reaction activity by 50%. Meanwhile, the effect of NDGA on a partial FAS reaction, β-ketoacyl reduction, was also measured (Fig. 2, inset). Compared with the inhibition of the overall FAS reaction, the inhibition of β-ketoacyl reduction (IC_{50} = 427 ± 13 μM) by NDGA was much weaker. This indicates that the β-ketoacyl reductase domain is not mainly responsible for the inhibition of FAS. In order to characterize the kinetics of the mechanism of FAS inhibition, the effects of increasing the concentration of one substrate, with the other substrate concentrations remaining fixed, on the initial rate was measured with the concentration of NDGA held at a series of fixed values. The results of double-reciprocal plots showed that NDGA inhibits the overall FAS reaction competitively against Ac-CoA (Fig. 3A), noncompetitively against Mal-CoA (Fig. 3B), and in a mixed manner (competitively-noncompetitively) against NADPH (Fig. 3C). NDGA showed no obvious time-dependent inhibition of FAS when FAS solutions (0.9 μM) were mixed with NDGA (250 μM) and aliquots were taken to measure the residual activity (data not shown).

These results are the same as those obtained with flavonoids (*14*), suggesting that the inhibitory mechanism of NDGA is similar to that of flavonoids. The results demonstrated that the C ring is not necessary for the inhibition of FAS by polyphenols. Therefore, other polyphenols compounds lacking the C ring, such as resvertrol, isoquirtigenin and phloretin, and several isoflavonoids whose B rings are linked to the 3-carbon of the C ring, such as 4′,6,7-Trihydroxy-isoflavone, genistein and daidzein, were assayed for FAS inhibition. Also, the effects of some flavone derivatives, morin, apigenin, baicalein and flavone, on FAS were measured to study the structure-activity relationship of these polyphenols compounds. The results are listed in Fig. 1. It was found that, like NDGA, all these polyphenols other than flavone inhibit the overall reaction of FAS, and also show a relatively weak suppression of β-ketoacyl reduction activity of FAS.

Resveratrol, isoquirtigenin, phloretin, apigenin (flavone derivative) and genistein (isoflavone derivative) have the same two phenyl rings with hydroxyl groups, but the linkages between the two rings are different. The two phenyl rings of resveratrol, isoquirtigenin and phloretin are, respectively, connected by two-carbon and three-carbon chains, while the two phenyl rings of apigenin and genistein are associated, respectively, by different C rings. All these compounds show potent FAS inhibitory activity, and moreover, resveratrol and isoquirtigenin are even more potent FAS inhibitors than apigenin and genistein. These inhibitors possess the same inhibitory kinetic characteristics (data not shown), which suggests that they act by the same inhibitory mechanism. The results further confirm the conclusion that the C ring is not indispensable for FAS polyphenol inhibitors. The C ring in flavonoid FAS inhibitors is possibly merely responsible for the

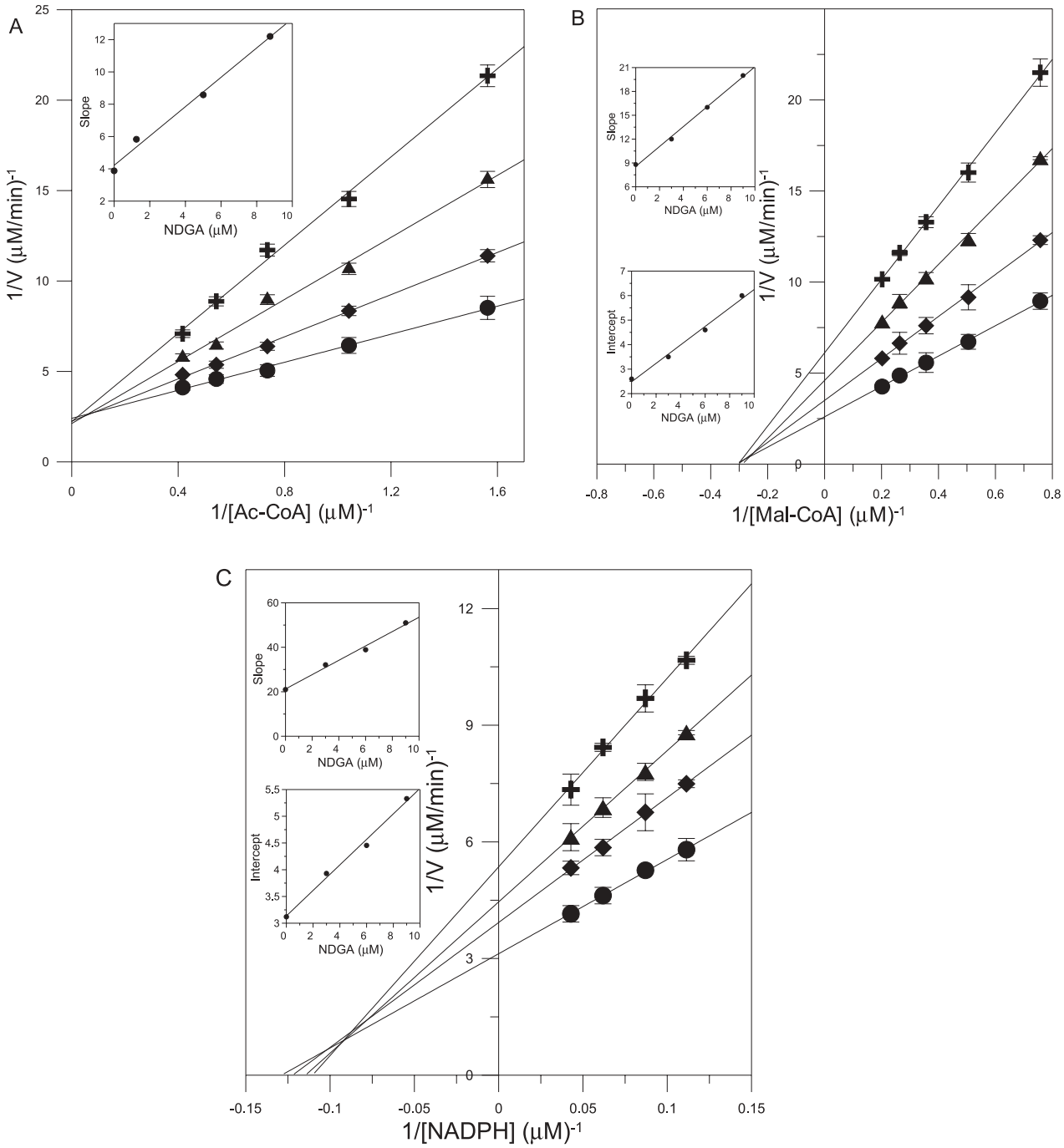


Fig. 3. **Double-reciprocal plots for the inhibition of FAS by NDGA.** The overall reaction of FAS was measured. (A) Ac-CoA was the variable substrate. The concentrations of NDGA were: 0 μM (solid circles), 1.25 μM (solid diamonds), 5 μM (solid triangles), and 8.75 μM (+). The dissociation constant, 4.9 μM, for NDGA binding to free FAS was obtained from the inset plot of the slopes versus NDGA concentration. (B) Mal-CoA was the variable substrate. The concentrations of NDGA were: 0 μM (solid circles), 3.0 μM (solid diamonds), 6.0 μM (solid triangles), and 9.0 μM (+). The dissociation constant for NDGA binding was calculated from the inset plot of the slopes versus NDGA concentration (6.7 μM) or from the plot of the concentration of NDGA versus the intercept (6.9 μM). (C) NADPH was the variable substrate. The concentrations of NDGA were: 0 μM (solid circles), 3.0 μM (solid diamonds), 6.0 μM (solid triangles), and 9.0 μM (+). The dissociation constant, 5.8 μM, for NDGA binding to free FAS was obtained from the inset plot of the slopes versus NDGA concentration, and the dissociation constant, 16.3 μM, for NDGA binding to the FAS-NADPH complex was obtained from the plot of the intercepts against NDGA concentration. Each point is the mean of 2–5 experiments. Error bars show standard deviations.

connection of the A and B rings, and cause the two rings assume some tridimensional structure, while the A and B rings are directly necessary for the FAS inhibitory activity.

In order to analyze further the possible structural basis for the inhibition of FAS by polyphenols, morin and resveratrol were employed as representatives because of their molecular rigidity and potent FAS inhibitory activities.

A common model (Fig. 4) was adapted from morin and resveratrol that is possibly shared by all polyphenol FAS inhibitors. The model consists of two almost planar phenyl rings with their hydroxyl groups, and the distance between the centers of the two rings should be about 0.67 nm. Due to the flexibility of the linkage between the two rings, phloretin, NDGA, and isoliquirtigenin are readily shaped into bioactive conformations. Therefore, they are able to inhibit FAS potently. Additionally, the number of hydroxyl groups in the phenyl rings could affect the FAS inhibitory activity of these polyphenols. For example, daidzein (IC_{50} = 73.2 ± 3.4 μM) with only one hydroxyl group in each ring, and baicalein (IC_{50} = 111.7 ± 2.3 μM), which lacks hydroxyl groups in the B ring, show much higher IC_{50} values than other active polyphenols (IC_{50} < 30 μM) with more hydroxyl groups, whereas flavone without any hydroxyl groups does not inhibit FAS. The failure to inhibit FAS activity is probably due to the fewer hydroxyl groups or no hydroxyl groups in either ring. The optimal pattern, such as morin and resveratrol, for FAS polyphenol inhibitors should probably involve two aromatic rings with hydroxyl groups, with at least one of the rings containing two hydroxyl groups.

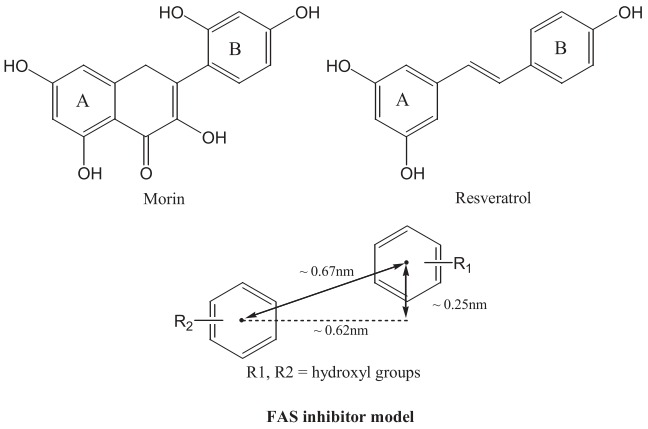


Fig. 4. **The common model of FAS polyphenol inhibitors adapted from morin and resveratrol.** Morin and resveratrol are potent FAS inhibitors and very rigid molecules, and so the relative position of the two aromatic rings changes little when they interacted with FAS. The distances between the two rings of morin and resveratrol are very close. A distance of 0.67 nm between the two rings was calculated from the minimized conformation of morin.

(–)-Catechin, an ungallated catechin (structures shown in Fig. 5), also contains A and B rings with hydroxyl groups, but it does not inhibit FAS. Apparently, the A and B rings of (–)-catechin seem to conform with the inhibitor model, but a molecular modeling analysis indicated that the ligand points of (–)-catechin are incorrectly located. There is a dihedral angle of about 70° between the A and B rings of (–)-catechin, and the hydroxyl groups and center of the B phenyl ring are shifted from the plane of the A ring due to the twisted C ring lacking the double bond and carbonyl oxygen (Fig. 6). Therefore, it is difficult for (–)-catechin to be converted to the active conformation of FAS inhibitors, so that (–)-catechin inhibits FAS only slightly. However, when the 3-hydroxyl group of (–)-catechin is esterified by gallic acid into (–)-catechin gallate (CG), its inhibition of FAS increases dramatically. It has been reported that about 3.4 μM CG blocks 50% of the overall reaction activity of FAS, at the same time, CG only weakly inhibits the β-ketoacyl reductase domain

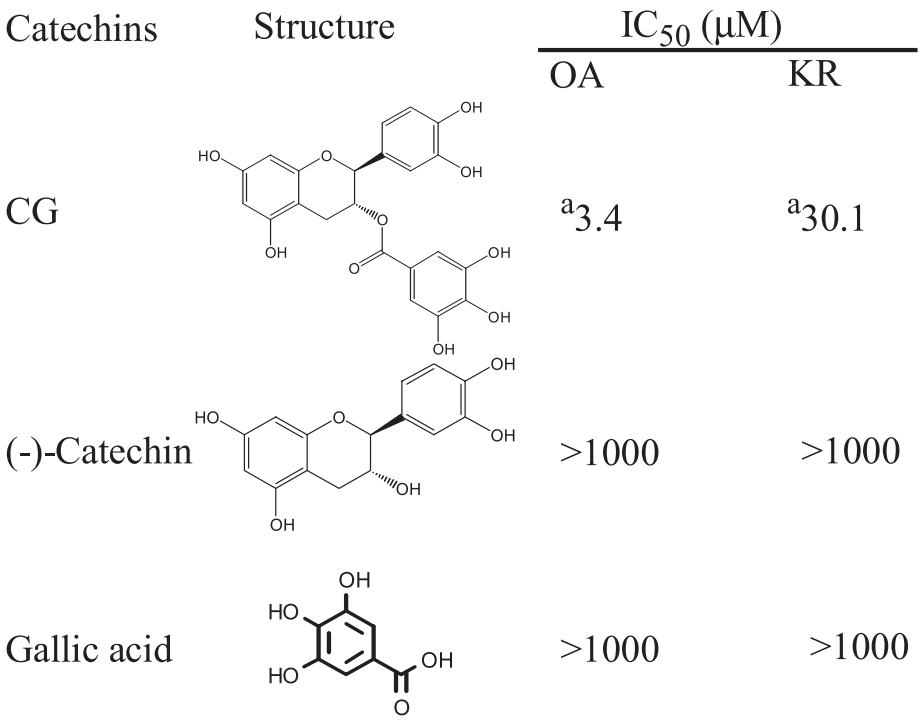


Fig. 5. **Inhibitory effects of catechins on FAS.** OA and KR is, respectively, the overall reaction and β-ketoacyl reduction activity of FAS. aFrom Ref. *23*.

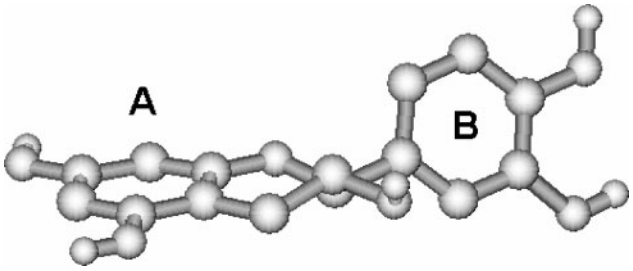


Fig. 6. **The minimized conformation of (–)-catechin.** The twisted linkage between the A and B rings leads to a torsion angle of about 70° between the two rings, which causes the center of one phenyl ring to shift from the plane of the other.

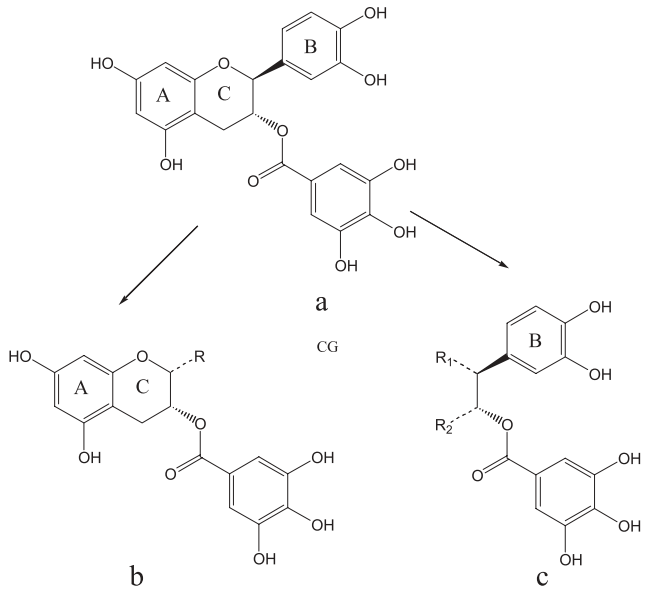


Fig. 7. **The putative portion of CG required for FAS inhibitory activity.** Structure a is CG, and b and c are possible pharmacophores of CG that inhibit FAS. Both pharmacophores are actually biphenyl cores associated through an active aster linkage.

(IC_{50} = 30.1 μM) (Fig. 5). Moreover, CG exhibits competitive inhibition of the overall reaction of FAS against Ac-CoA, noncompetitive inhibition against Mal-CoA, and mixed inhibition against NADPH (*23*). This kinetic mechanism is consistent with that of FAS flavonoid inhibitors. Since neither (–)-catechin nor gallic acid inhibits FAS, the data suggest that the galloyl moiety and the A or B ring of CG probably construct a pharmacophore to inhibit FAS. Considering that the linkage between the galloyl moiety and B ring is more flexible, which allows CG to convert more easily into the active conformation, the section involving the galloyl group and B ring may comprise the main pharmacophore of CG (Fig. 7).

CG has been reported by our laboratory to inactivate FAS irreversibly, and the β-ketoacyl reductase domain was regarded as the immediate target (*15*, *23*). Therefore, we measured the direct slow-binding inhibition of the β-ketoacyl reduction of FAS by CG. It was found that CG can indeed inactivate the β-ketoacyl reduction. With 0.9 μM FAS mixed with CG (1.0 mM), the β-ketoacyl reduction

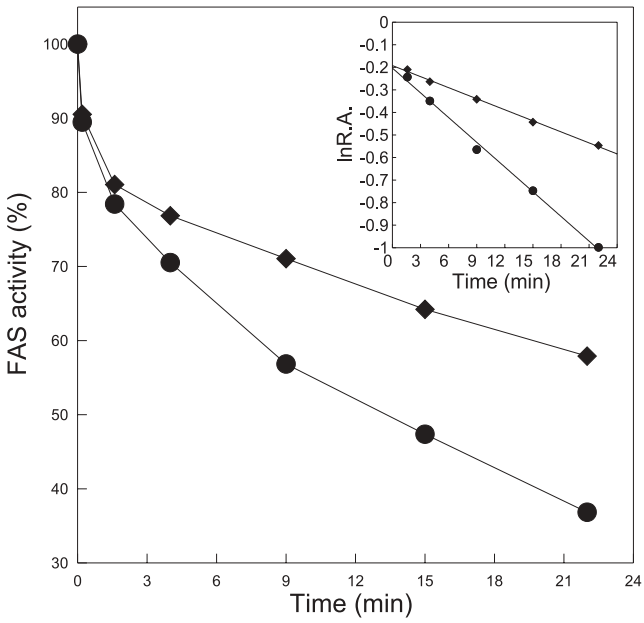
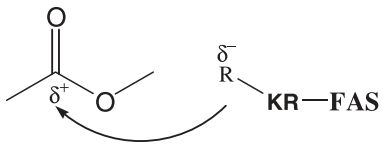


Fig. 8. **Time-dependent inhibition of the β-ketoacyl reduction of FAS by CG.** The β-ketoacyl reduction activities were measured at the indicated times after the addition of 1 mM CG to the FAS (0.9 μM) solutions with (solid diamonds) or without 120 μM NADPH (solid circles). The inset is the semi-logarithm plot of the experimental data. The contribution of the fast-binding reversible inhibition by CG was subtracted. R.A. is the residual activity of the β-ketoacyl reduction. The apparent first-order rate constants were obtained from the linear slopes of the plot.

activity was assayed at the indicated time intervals, and a significant time-dependent inhibition of FAS, including two processes, was observed (Fig. 8). The β-ketoacyl reduction activity declining sharply in the first process that usually results from fast-binding reversible inhibition, and, in the following process, the slow-binding inhibition accounts for the inactivation of β-ketoacyl reduction. The plot of lnR.A versus *t* during the slow-binding inhibition is linear (Fig. 8, inset). Pseudo-first-order rate constants could be obtained from the slopes of this plot. When NADPH was present in the pre-incubation system of FAS and CG, the inactivation rate of the β-ketoacyl reduction appeared to be slow. Fig. 8 shows that 120 μM NADPH reduced the inactivation rate by about 2-fold, from 0.034 min^{-1} to 0.015 min^{-1}, suggesting that CG possibly reacts irreversibly with some residue at the NADPH-binding site to occlude the β-ketoacyl reductase domain of FAS.

The galloyl moiety of green tea catechins is a critical structural feature for both the irreversible and reversible inhibition of FAS (*15*). From the pharmacophoric structures (Fig. 7), it is also found that the galloyl moiety is an indispensable part of the pharmacophore of CG. In the pharmacophore, two aromatic rings with hydroxyl groups are actually associated by an ester linkage, which probably is necessary for the irreversible activity of the inhibitor. Colleagues in our laboratory reported previously that the Mulliken charge of the carbon atom of the ester bond is very important for the irreversible inactivation of FAS, and concluded that the ester bond of the

gallate group probably is nucleophilically attacked by some essential group for the β-ketoacyl reductase of FAS, resulting in the irreversible inactivation of the enzyme (*15*).



In the present study, we have elucidated the inhibitory mechanism and its possible structural basis for some FAS polyphenol inhibitors. A common model shared by all polyphenol FAS inhibitors is proposed that includes several sections: (i) two aromatic rings with their respective hydroxyl groups; (ii) an appropriate linkage that maintains a proper distance of about 0.67 nm between the two rings, and makes the two rings lie closely in a plane in the low-energy conformation of a FAS polyphenol inhibitor; (iii) if there is a proper ester linkage between the two phenyl rings, the compound can simultaneously inactivate irreversibly the β-ketoacyl reduction activity of FAS. Based on this information, more potent and specific FAS inhibitors may be discovered or synthesized for application in the treatment of some diseases associated with FAS, such as cancer and obesity.

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