Inhibition of green tea and the catechins against 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the key enzyme of the MEP terpenoid biosynthetic pathway

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ABSTRACT: 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) is the first committed enzyme in the MEP terpenoid biosynthetic pathway and also a validated antimicrobial target. Green tea which is rich in polyphenolic components such as the catechins, possesses a plenty of pharmacological activities, in particular an antibacterial effect. To uncover the antibacterial mechanism of green tea and to seek new DXR inhibitors from natural sources, the DXR inhibitory activity of green tea and its main antimicrobial catechins were investigated in this study. The results show that the raw extract of green tea and its ethyl acetate fraction are able to suppress DXR activity explicitly. Further determination of the DXR inhibitory capacity of eight catechin compounds demonstrates that the most active compound is gallocatechin gallate that is able to inhibit around 50% activity of DXR at 25 μ M. Based on these data, the primary structure-activity relationship of the catechins against DXR is discussed. This study would be very helpful to elucidate the antimicrobial mechanism of green tea and the catechins and also would be very useful to direct the rational utilization of them as food additives.

KEYWORDS: Green tea; Catechins; 1-Deoxy-D-xylulose 5-phosphate reductoisomerase; MEP pathway; Inhibition.

Chemical compounds studied in this article

Gallocatechin gallate (PubChem CID: 5276890); Epigallocatechin gallate (PubChem CID: 65064)

Abbreviations

BF: butanol fraction; C: (+)-catechin; CIAP: Calf intestine alkaline phosphatase; CG: catechin gallate; DNPH: 2,4-Dinitrophenylhydrazine; DXP: 1-Deoxy-D-xylulose 5-phosphate; DXR: 1-Deoxy-D-xylulose 5-phosphate reductoisomerase; EAF: ethyl acetate fraction; EC: (–)-epicatechin; ECG: (–)-epicatechin gallate; EGC: (–)-epigallocatechin; EGCG: (–)-epigallocatechingallate; GC: (+)-gallocatechin; GCG: gallocatechin gallate; GTE: green tea extract; MEP: 2-methyl-D-erythritol 4-phosphate; PEF: petroleum ether fraction; WSF: water soluble fraction.

1. Introduction

The use of antibiotics has been playing a critical role for the treatment of infectious diseases. However, the emergence of resistant strains has dramatically decreased the therapeutic effect of various antibiotics year by year. Therefore, seeking for novel antimicrobial agents aiming at new targets is very essential. Terpenoids are one of the biggest families of natural products, playing key metabolic and regulatory roles in all forms of life [1]. Two routes have been found for the biosynthesis of terpenoids: the mevalonate pathway [2] and the MEP pathway [3]. Because the MEP pathway is operative in human pathogens but not in human [3], all the key enzymes of the

pathway are considered good targets to interfere with bacterial growth [4,5].

DXR has been identified as a rate-limiting enzyme of the MEP pathway for terpenoid biosynthesis, which catalyzes the intramolecular rearrangement of DXP (**1**) to MEP (**2**) and provides a perfect target for novel antibacterial screening (Figure 1) [3,6]. Much research has therefore been performed to look for its inhibitors, which have led to the discovery of fosmidomycin (**3**), a known component previously characterized from *Streptomyces lavendulae*. Research has shown the compound is not only a potent DXR inhibitor, but exhibits strong antibacterial effects as well [7]. Clinical studies have indicated that it is somewhat effective in treating malaria caused by *Plasmodium falciparum* [8]. The studies also found some structural analogs of fosmidomycin, such as FR900098 (**4**) which possesses similar DXR inhibitory activity and antimicrobial capacity as **3** [7].

Fig. 1. The first committed step of the MEP terpenoid biosynthetic pathway and its inhibitors.

It has long been aware that higher plants produce enormous secondary metabolites, many of which display excellent pharmacological activities. The plants which are rich in flavonoids and polyphenols often have been reported to have good performance in antibacterial action. Green tea, as the most popular functional drink worldwide, contains quite lots of catechin compounds such as EC (**5**, Figure 2), EGC (**6**), ECG (**7**), EGCG (**8**), C (**9**), GC (**10**), CG (**11**), and GCG (**12**). Research has shown that GTE and its main components, the catechins exhibit potent antimicrobial activity

and also synergistic effect on the activity of the antibiotics like ciprofloxacin [9,10,11,12]. Recent studies in this area are largely concentrated on the evaluation of antimicrobial activities of the catechin compounds against various resistant strains [13,14] and different tissue/organ infections, such as urinary tract infections [15], oral infections [16], and gastroduodenal infections [17]. Meanwhile, some investigations centered on the mechanisms of bactericidal action of green tea [18,19].

In this study, we found that the GTE and its ethyl acetate fraction possess quite strong inhibition against DXR at 0.5 mg/mL (71.5% and 93.4% inhibition, respectively). Further investigation pointed out that the DXR inhibitory activity of the GTE mainly results from GCG (about 50% inhibition at 25 μ M), the major antimicrobial components of green tea. The data obtained in this paper could be very useful for elucidation of the antibacterial mechanism of green tea and also the tea catechins. Herein we would disclose all the experimental details.

Fig. 2. The structures of the catechins.

2. Materials and Methods

2.1. Materials

The fresh green tea was collected at the tea plantations in Qinling Mountains in southeast of Shaanxi Province at April of 2015. It was delivered in ice and then stored at -80°C before use. The

catechins were purchased from Chengdu Biopurify Phytochemicals, Ltd. (Chengdu, China), and the stock solutions of them (10 mg/mL) were prepared in 40% aqueous DMSO just before use. DNPH was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). NADPH was purchased from Gen-View Scientific Inc. (Tallahassee, FL, USA). Fosmidomycin was from Toronto Research Chemicals Inc. (North York, ON, Canada). CIAP was purchased from Takara (Dalian, China). DXP was synthesized according to a procedure previously reported [20]. The preparation of *E. coli* DXR was carried out in accordance with a published method [21]. Deaerated water was prepared by boiling Minipore water for 30 min, cooled to room temperature and then bubbled with a gentle N₂ flow for 6 hours. All the buffers involved in the study were prepared in the deaerated water just before use, unless otherwise stated. HPLC grade methanol and acetonitrile was purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). All other chemicals are of analytical grade.

2.2. Preparation of GTE and its fractionation

The frozen fresh green tea (100 g) was extracted with 600 mL 70% aqueous ethanol (v/v) at room temperature (24 h × 3). The combined extracting solutions were evaporated at 30 °C in vacuo until thick green slurry was produced (about 50 mL). 5 mL of the slurry was then removed, evaporated at 30°C in vacuo to dryness and kept under N₂ atmosphere at -80°C before use. Meanwhile the remaining part was sequentially fractionated with petroleum ether (50 mL × 3), ethyl acetate (50 mL × 3), and butanol (50 mL × 3). The solvents were subsequently removed at 30°C in vacuo and four fractions, namely the PEF, the EAF, the BF, and the WSF were obtained and stored under N₂ atmosphere at -80°C for later analysis.

2.3. HPLC analysis of the GTE and its different fractions

2 mg of the GTE or each of its fractions was dissolved respectively in 1 mL acetonitrile of HPLC grade. After filtrated through 0.22 μ m membrane, the solutions were analyzed on an Agilent 1200 HPLC system and the major components of them were determined by comparison with the HPLC profiles of the commercial catechin compounds. The HPLC conditions: Column, Shim-pack VP-ODS column (250 $\times$ 4.6 mm, 4.6 μ m); Detection wavelength: 280 nm; Mobile phase: acetonitrile-water (containing 1% acetic acid, v/v); gradient: 0 min, 8% acetonitrile; 30 min, 15% acetonitrile; 55 min, 32% acetonitrile; 62 min, 8% acetonitrile; Flow rate: 1.0 mL/min; Injection volume: 40 μ L. Column temperature: 25°C.

2.4. Stability of EGCG and GCG in Tris-HCl buffer (pH 7.4)

Certain amount of EGCG and GCG was dissolved separately in normal 50 mM Tris-HCl buffer (pH 7.4) and in 50 mM Tris-HCl buffer (pH 7.4) prepared in deaerated water (final concentration of each compound was 50 μ M). The solutions were then incubated at 37°C and determined by HPLC every half an hour. The HPLC conditions: Column, Shim-pack VP-ODS column (250 $\times$ 4.6 mm, 4.6 μ m); Mobile phase: methanol-water (containing 1% acetic acid, v/v); Gradient: 0 min, 15% methanol; 12-13 min, 55% methanol; 20-25 min, 15% methanol. Detection wavelength: 280 nm; Flow rate: 0.8 mL/min; Injection volume, 20 μ L; Column temperature: ambient.

2.5. Determination of DXR inhibitory activity of the GTE, its fractions, and the catechins

The inhibitory activity of the GTE, its fractions, and the catechins against DXR was determined using the pre-column derivatization HPLC method (the principle and the process of the protocol see part 1 in the Supplementary Material) established by us with minor modifications [22]. The assay mixture consists of 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.5 mM NADPH, 2%

(W/V) DMSO, and 4 µg/mL DXR in a final volume of 100 µL. The GTE or each of its fractions (final conc. 0.1-0.7 mg/mL) or each of the catechins (final conc. 25-100 µM) was added to the mixture separately before the addition of DXP (final conc. 1 mM) to start the reaction. In a control assay, fosmidomycin (**3**) was used instead of the GTEs or the catechin compounds. The reaction mixture was incubated at 37°C for 30 min before DXR was inactivated by boiling for 3 minutes. Subsequently, 10 µL CIAP buffer (1 M Tris-HCl, 1 M NaCl₂, 50 mM MgCl₂, pH 7.5) and 2U CIAP were added, and the mixture was incubated at 37°C for 120 min. 10 µL DNPH (120 mM in 30% perchloric acid, V/V) and 280 µL methanol were supplemented and mixed well. After incubated at 37°C for another 45 min, the mixture was centrifuged at 6000 rpm for 3 min and the supernatant was used for HPLC analysis on the Agilent system. HPLC conditions: Column, Shim-pack VP-ODS column (250 × 4.6 mm, 4.6 µm); Injection volume, 60 µL; Mobile phase: acetonitrile-water; gradient, 0 min, 40% acetonitrile; 17 min, 80% acetonitrile; 20 min, 40% acetonitrile. Detection wavelength: 360 nm; Flow rate: 0.7 mL/min; Column temperature: ambient.

2.6. Determination of CIAP inhibitory activity of the GTE and its fractions

The HPLC method described above was also used to determine the inhibitory activity of the GTE and its fractions against CIAP. The reaction mixture comprises 10 µL CIAP buffer and 2U CIAP in a final volume of 100 µL. The GTE or each of its fractions (final conc. 1 mg/mL) was added to the mixture separately before the addition of DXP (final conc. 1 mM) to start the reaction. The reaction mixtures were incubated at 37°C for 120 min, and the CIAP was inactivated in boiling water for 3 min. Then, the reaction mixture was derivatized with DNPH and analyzed with the HPLC method described in section 2.5.

3. Results

3.1. Evaluation of the pre-column derivatization HPLC method for its reliability in this study

We have set up a pre-column derivatization HPLC method to determine the steady state kinetic parameters of DXP synthase, another key enzyme of the MEP pathway [22]. In this study, we extended the method to measure the DXR inhibitory activity of the GTE and the catechins. To evaluate the reliability of the method in this experiment, we first chromatographed the GTE and each fraction of it, such as the PEF, the EAF, the BF, and the WSF and also the eight catechin compounds respectively to figure out whether they would interfere with the quantification of DXP. The results show that the presence of these additives would not generate any interference with the determination of DXP (Evaluation process and HPLC profiles see Part 1 and Figures S1 and S2 in the Supplementary Material). Then we used the method to determine the inhibition curve of fosmidomycin against DXR, and the results display that the compound is able to inhibit DXR in a clear dose-dependent manner with an IC_{50} of 267 ± 11 nM (Fig. 3). These data are in good agreement with previously published results [23]. Thus it is obvious that the pre-column derivatization HPLC method is good enough for the measurement of the DXR inhibitory activity of the GTE and the catechins.

Fig. 3. The DXR inhibitory activity of fosmidomycin determined by the pre-column derivatization HPLC method.

3.2. Inhibition of the GTE and its fractions against CIAP

In the DXR inhibition experiment, we use CIAP to dephosphorylate DXP before DNPH derivatization. Thus, whether the GTE and each of its different fractions inhibit this enzyme is very crucial. The experiments indicate that neither the GTE nor each of its fractions possesses suppressing effect on the activity of CIAP at a concentration of 1 mg/mL (Figure 4).

Fig. 4. CIAP inhibitory activity of the GTE, the PEF, the EAF, the BF, and the WSF at a final concentration of 1.0 mg/mL.

3.3. DXR inhibitory activity of the GTE and its different fractions

With the validated HPLC method in hand, we initiated to test the DXR inhibitory activity of the GTE at a final concentration of 0.5 mg/mL. As expected, the results reveal that the extract is truly active in suppressing DXR and it is able to inhibit around 70% of DXR activity at this concentration (Fig. 5A). Encouraged by the results, we further determined the DXR inhibitory effects of the different fractions of the GTE at final concentration of 0.5 mg/mL. From the results depicted in Fig. 5A, we are able to observe that all the fractions display a certain degree of inhibitory activities against DXR, with the EAF exhibiting the highest inhibition (about 93%) and the BF displaying the medium. The other two fractions such as the PEF and the WSF only show DXR inhibition lower than 20%. Moreover, we determined the DXR inhibitory effects of the EAF

and the BF at different concentrations and the results show both fractions can suppress the activity of DXR in a concentration-dependent manner (Fig. 5B).

Fig. 5. A. The DXR inhibitory activity of the control fosmidomycin at 1.0 μ M, the GTE, the PEF, the EAF, the BF, and the WSF at 0.5 mg/mL; B. Inhibition curves of the EAF and the BF against DXR.

3.4. Determination of the main components of the GTE and its different fractions

Having determined that the GTE and its different fractions exhibited distinct inhibition against DXR, we chromatographed these extracts separately on an HPLC system to figure out the difference of their chemical ingredients. Because the catechin compounds have been considered as the main active components of the green tea, [9,10,11,12] we selected 8 catechins and used them as HPLC references. The results (HPLC profiles of the GTE, its different fractions and the reference compounds see Figure S3 in the Supplementary Material) indicate that the extracts such as the PEF and the WSF which exhibit very weak DXR inhibition contain almost no catechin compounds, while the GTE and its EAF and BF which display quite strong DXR inhibition contain several catechins such as C, EGCG, GCG, and ECG. Moreover, for the three active parts, the total amount of the catechins in each sample shows a positive correlation with its DXR inhibitory activity.

3.5. Stability of EGCG and GCG in Tris-HCl buffer (pH 7.4)

It has been reported that the catechins are unstable, especially in basic solutions [27]. Because the DXR assay is carried out usually in Tris-HCl buffer (pH 7.4) at 37°C, we evaluated the stability of the major antimicrobial catechin compounds EGCG and GCG found in green tea under such conditions. The results depicted in Figure 6 show that EGCG and GCG are indeed unstable in the Tris-HCl buffer at almost medium pH without N₂ saturation and EGCG even completely disappears after 2 h incubation at 37°C. Fortunately, in the Tris-HCl buffer with saturated N₂ the two compounds show better stability and they are able to survive after incubated for 1 h at 37°C. Therefore, the DXR assays testing the catechin compounds have to be performed in N₂ saturated buffers.

Fig. 6. Stability of EGCG (50 μ M) and GCG (50 μ M) in 50 mM Tris-HCl buffer (pH 7.4) at 37°C. (■) EGCG in Tris-HCl buffer; (●) EGCG in N₂ saturated Tris-HCl buffer; (♦) GCG in Tris-HCl buffer; (▲) GCG in N₂ saturated Tris-HCl buffer.

3.6. DXR inhibitory activity of the catechin compounds

Based on the DXR inhibitory activity of the GTE and its different fractions, we further selected eight compounds, such as EGCG, GCG, EGC, GC, ECG, CG, C, and EC (their structures were depicted in Figure 2) which represent the major catechin compounds and also the main

antimicrobial principles in green tea to test their DXR inhibitory activity. To ensure the stability of the catechins under DXR assay conditions, the incubations were carried out in 50 mM Tris-HCl buffer (pH 7.4) prepared in deaerated water. The results given in Figure 7 show that at a concentration of 100 μ M, compounds G and EC exhibit undetectable activity against DXR, the other compounds like EGC, GC, ECG, CG, and EGCG only display weak DXR inhibitory activity (from less than 10% to about 30%). The best DXR inhibitor among all the eight compounds is GCG, it can completely suppress the activity of DXR at 100 μ M, and even at a concentration as low as 25 μ M, it still exhibits around 50% DXR inhibition rate.

Fig. 7. The inhibitory activity of the catechins against *E. coli* DXR at different concentrations. The assays were performed in N₂ saturated 50 mM Tris-HCl buffer (pH 7.4) to assure the stability of the catechins.

4. Discussion

Although the antimicrobial property of green tea and its principal component catechins has been discovered for decades, their modes of antimicrobial action are not fully elucidated. So we initiated this study to i) Unravel the antimicrobial mechanism of green tea and the catechin compounds; ii) Look for new DXR inhibitors with totally different structures comparing to fosmydomycin and its analogue. As expected, our experiments show that the GTE is able to inhibit more than 70% of DXR activity at a concentration of 0.5 mg/mL. Further investigation indicates

that the EAF is the most active fraction of the GTE, it is able to suppress more than 90% activity of DXR at the same concentration.

It has been reported that the active ingredients of green tea, for example, the antioxidants and the antimicrobial compounds normally locate in its ethyl acetate soluble part [24,25,26], which are represented by the catechins such as EGCG, GCG, EGC, GC, ECG, CG, and EC et al. Having realized that the most potent DXR inhibition in the GTE is also from its EAF, we predicted that the catechins could be the possible candidates that possess DXR inhibitory activity. The HPLC analyses of the GTE and its different fractions support this prediction. There is almost no catechin compound in the fractions which only show weak DXR inhibition and the contents of the catechins in the fractions which possess strong DXR inhibitory capacity are relatively high. We thus measured the DXR inhibitory capacity of the commercially available catechins. From the literature we knew that the catechins, especially EGCG and GCG are unstable and susceptible to oxidation degradation in the solution, in particular in basic mediums [27]. Therefore we must find a way to overcome the annoying drawback of these compounds before we start to evaluate their inhibition against DXR because the DXR assay is usually carried out under a weak basic condition. It has been reported that EGCG can be stabilized for a few hours in 50 mM phosphate buffer (pH 7.4) saturated with nitrogen [28]. We applied this condition in our experiments and found that the major antimicrobial catechins EGCG and GCG can be stabilized at least for an hour in N₂ saturated 50 mM Tris-HCl buffer (pH 7.4) at 37°C. Under this way, we assayed the DXR inhibitory activity of the eight catechins and the results show that although the catechins share a common flavan-3-ol structural moiety, their DXR inhibitory activities are significantly different. The compounds which do not have a pyrogallol group in their structure, for example EC (**5**) and

C (**9**) show no inhibition against DXR even at a concentration as high as 100 μ M, and the compounds which have one pyrogallolyl group, for example EGC (**6**) and GC (**10**), ECG (**7**) and CG (**11**) start to exhibit weak activity, while the compounds with two pyrogallolyl groups such as EGCG (**8**) and GCG (**12**) display stronger activity. Thus the DXR suppressive ability of the catechins could relate to the number of the pyrogallolyl group in their structure. This observation might suggest the importance of the 5'-OH group in the B ring of the catechins. Similar phenomenon has been observed on the suppressive capacity of the catechins against *Bacillus cereus* [9]. Moreover, it seems that the position of the pyrogallolyl group also affects the DXR inhibition of the catechins, with 3-substitution being better than 2-substitution (for example ECG > EGC and CG > GC). Further analysis of the DXR inhibitory data of these polyphenolic compounds leads us to the conclusion that the isomer holding a 2*S* configuration normally possesses more potent DXR inhibition than its 2*R* epimer (for example GC > EGC, CG > ECG, and GCG > EGCG) and this stereochemical effect becomes much more obvious in the compounds that have two pyrogallolyl groups because the DXR inhibitory capacity of GCG is about 6 times stronger than that of EGCG. Thus the DXR suppressive ability of the catechins might also relate to the orientation of the substituent at position 2 and for EGCG and GCG the chirality of C2 might play a crucial role for their DXR inhibition. It has been reported that GCG displays the strongest effect against *B. cereus* among all the polyphenols tested [9]. Our data have shown that GCG is also the most potent DXR inhibitor among the eight catechins. This would suggest that GCG might be one of the most important antimicrobial agents in green tea. More investigations are necessary to clarify the reason that i) the DXR inhibitory activity of the catechins depends on the number of the pyrogallolyl group they have; ii) the stereochemistry of C3 of EGCG and GCG

influences their DXR inhibitory capacity so much.

In summary, we tested the DXR inhibitory activity of GTE and its different fractions in this study. The results show that the GTE, its EAF, and BF, especially the EAF, possess the main DXR suppressive effect. Further determination indicates that the catechins such as EGCG and GCG which are the principal antimicrobial ingredient of the EAF of the GTE, exhibit medium to strong DXR inhibitory activity. These data would be very helpful to elucidate the antimicrobial mechanism of green tea and the catechins and also would be very useful to direct the rational utilization of them as food additives.

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Graphical Abstract

Inhibition of green tea and the catechins against 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the key enzyme of the MEP terpenoid biosynthetic pathway

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