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Bioorganic & Medicinal Chemistry 16 (2008) 3580–3586
Bioorganic & Medicinal Chemistry

Catechin gallates are NADP$^+$-competitive inhibitors of glucose-6-phosphate dehydrogenase and other enzymes that employ NADP$^+$ as a coenzyme

Eui Seok Shin,[a] Jiyoung Park,[b] Jae-Min Shin,[c,d] Dooho Cho,[d] Si Young Cho,[a] Dong Wook Shin,[a] Mira Ham,[b] Jae Bum Kim[b] and Tae Ryong Lee[a,*]

[a]*R&D Center, AmorePacific Corporation, 314-1 Bora-Dong, Giheung-Gu, Yongin, Gyeonggi 446-729, Republic of Korea*
[b]*School of Biological Sciences, Seoul National University, San 56-1, Sillim-Dong, Kwanak-Gu, Seoul 151-742, Republic of Korea*
[c]*Department of Bioinformatics, Soongsil University, 1-1 Sangdo-Dong, Dongjak-Gu, Seoul 156-743, Republic of Korea*
[d]*SBscience Inc., 3rd Fl. Sungok-Building, 4-1 Sunae-Dong, Bundang-Gu, Gyeonggi 463-825, Republic of Korea*

Received 9 January 2008; revised 1 February 2008; accepted 5 February 2008
Available online 14 February 2008

Abstract—Recent studies have shown that glucose-6-phosphate dehydrogenase (G6PD) is an effectual therapeutic target for metabolic disorders, including obesity and diabetes. In this study, we used in silico and conventional screening approaches to identify putative inhibitors of G6PD and found that gallated catechins (EGCG, GCG, ECG, CG), but not ungallated catechins (ECG, GC, EC, C), were NADP$^+$-competitive inhibitors of G6PD and other enzymes that employ NADP$^+$ as a coenzyme, such as IDH and 6PGD.

1. Introduction

Oxidative stress and associated inflammatory processes are believed to play important roles in the pathogenesis of metabolic syndromes as well as major age-related diseases.[1–3] NADPH is an essential coenzyme for several enzymes that generate oxygen-free radicals, including NADPH oxidase, nitric oxide synthase, and the cytochrome P450 monooxygenases.[4] Thus, a reduction in NADPH production could result in a significant change in cellular oxidative stress. In addition, NADPH is an essential element in lipogenesis[5,6] and contributes to fatty acid and cholesterol synthesis by supplying reducing power. Therefore, NADPH-producing enzymes might be closely associated with oxidative stress, chronic inflammatory signals, and lipid metabolism disorders.

NADPH is produced by reduction of NADP$^+$ in biochemical reactions catalyzed by several enzymes, including malic enzyme (ME), isocitrate dehydrogenase (IDH), and glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), which are the first two enzymes of the pentose phosphate pathway (PPP).[6] Among the four NADPH-producing enzymes, G6PD is the rate-limiting enzyme of PPP, and is highly conserved in most mammalian species.[7] G6PD, which is expressed ubiquitously, is implicated in various cell functions, including cell growth, survival, and redox regulation, and its deficiency causes hemolytic anemia and neonatal jaundice.[8] Interestingly, hormonal or nutritional regulation of G6PD was restricted to liver and adipose tissues.[6] Hepatic G6PD is regulated by nutritional signals, including a high-carbohydrate diet, polyunsaturated fatty acids, and hormonal signals such as insulin, glucagon, thyroid hormone, and glucocorticoids.[6,7] Furthermore, G6PD-deficient patients show a decrease in lipogenic rate and serum lipoprotein concentrations, implying the importance of G6PD in fatty acid synthesis.[9,10] Recent studies have elucidated novel roles of adipose tissue G6PD in the etiology of metabolic disorders. G6PD expression is highly increased in obese subjects including *ob*/*ob*, *db*/*db*, and diet-induced obese mice, and high expression of G6PD in adipocytes is tightly associated with lipid dysregulation, oxidative stress, and the chronic inflammation found in obese or

Keywords: Catechin gallate; Glucose-6-phosphate dehydrogenase; Isocitrate dehydrogenase; 6-Phosphogluconate dehydrogenase; NADP.
* Corresponding author. Tel: +82 31 280 5961; fax: +82 31 899 2595; e-mail: trlee@amorepacific.com

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doi:10.1016/j.bmc.2008.02.030

diabetic subjects.[11,12] These observations indicate that G6PD is a potential therapeutic target for obesity and/or diabetes-related diseases.

Dehydroepiandrosterone (DHEA) is a well-known, uncompetitive inhibitor of G6PD.[13–15] It has anti-oxidative, anti-carcinogenic, anti-obesity, and anti-aging properties.[16,17] Use of DHEA as an anti-obesity drug is hampered by the requirement of high oral dosage and its easy conversion into various active androgens. Thus, it is expected that finding more efficient inhibitors of G6PD could lead to potent therapeutic drugs against obesity and/or diabetes.

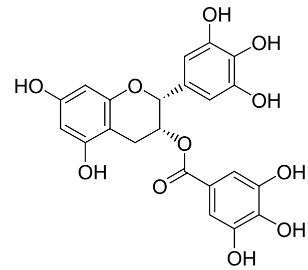
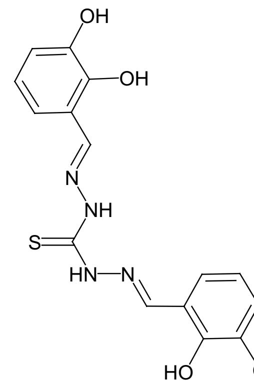
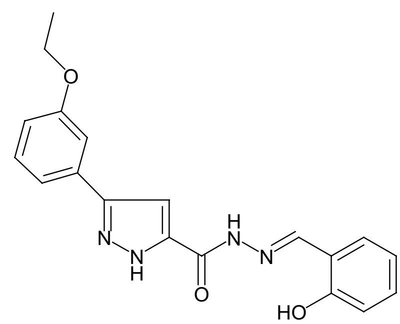
In this study, both in silico and conventional screening approaches targeting the coenzyme ($NADP^+$) and substrate (glucose-6-phosphate, G6P) binding sites of G6PD were performed in an effort to discover candidate G6PD inhibitors, and catechin gallates were identified as potent $NADP^+$-competitive inhibitors of G6PD.

2. Results and discussion

2.1. Identification of EGCG as a potent inhibitor of G6PD through virtual screening approach

From a virtual screening experiment targeting the coenzyme ($NADP^+$) and substrate (G6P) binding sites of G6PD, 250 candidate compounds were selected from a collection of three million commercially available compounds, and purchased from several chemical library distribution companies. These candidate compounds were used in an in vitro G6PD inhibition assay and eight compounds showed more than 50% inhibition at less than 100 μmol/L. Among eight compounds, (−)-epigallocatechin gallate (EGCG) was identified as the most potent inhibitor of G6PD (Table 1 and Fig. 1). EGCG is the most abundant polyphenolic catechin isolated from green tea, which exhibits profound pharmacological activities including anti-oxidant activity, inhibition of cell proliferation, inhibition of ultraviolet B (UVB)-induced inflammatory responses, modulation of cell cycle regulation, anti-cholesterolemic activity, suppression of angiogenesis, and anti-carcinogenic effect.[18–21]

Table 1. IC_{50} values of G6PD inhibitors

Structure	IC_{50} (μmol/L)
	0.25
	56.37
	21.76

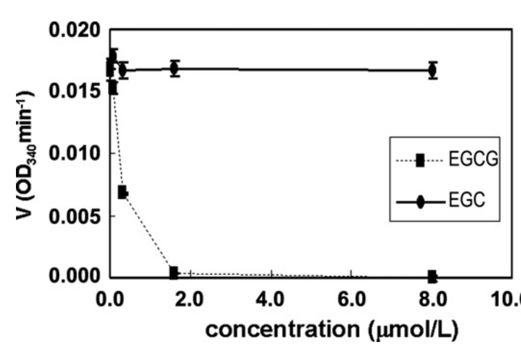
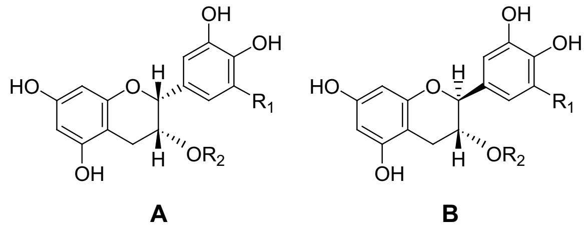


Figure 1. Dose–response curves for EGCG and EGC on the rate of G6PD catalysis. The activity of G6PD was measured in the presence of various concentrations of EGCG (■) and EGC (●).

2.2. Galloyl moiety of catechins is an essential structural feature in the inhibition of G6PD

To elucidate the structure–activity relationship of the inhibitory effects of EGCG on G6PD, the inhibition kinetics of EGCG and other green tea catechins were investigated. Interestingly, the inhibitory activity of green tea catechins on G6PD was restricted to gallated catechins, such as EGCG, (−)-gallocatechin gallate (GCG), (−)-epicatechin gallate (ECG), and (−)-catechin gallate (CG) when compared to ungallated catechins, such as (−)-epigallocatechin (EGC), (−)-gallocatechin (GC), (−)-epicatechin (EC), and (−)-catechin (C) (Fig. 1 and Table 2). All gallated catechins exhibited similar IC_{50} values (0.18–0.25 μmol/L) for the inhibition of G6PD (Table 2). Therefore, the galloyl moiety of catechins is an essential structural feature in the inhibition of G6PD, while the 5′-hydroxyl group on the B ring and the stereochemistry of the 2-position of the catechin skeleton are not necessary for inhibition of G6PD. Several supportive studies have shown that the galloyl moiety has active biological features. Tian and co-workers reported that EGCG is a potent inhibitor of fatty acid

Table 2. G6PD, 6PGD, and IDH IC_{50}values for gallated catechins



Compound	General structure	R1	R2	IC_{50} (μmol/L)		
				G6PD	6PGD	IDH
EC	A	H	H	≫1000	≫1000	≫1000
EGC	A	OH	H	≫1000	≫1000	≫1000
ECG	A	H	3,4,5-Trihydroxybenzoyl	0.18 ± 0.01	1.21 ± 0.13	10.8 ± 1.66
EGCG	A	OH	3,4,5-Trihydroxybenzoyl	0.25 ± 0.02	0.72 ± 0.07	6.44 ± 1.12
CG	B	H	3,4,5-Trihydroxybenzoyl	0.24 ± 0.01	1.28 ± 0.08	6.62 ± 0.63
GCG	B	OH	3,4,5-Trihydroxybenzoyl	0.23 ± 0.02	1.45 ± 0.08	2.72 ± 0.21
GC	B	OH	H	≫1000	≫1000	≫1000
C	B	H	H	≫1000	≫1000	≫1000

synthase (FAS), and that the galloyl moiety is the critical structural feature in the inhibition of the β-ketoacyl reductase activity of FAS via a reversible association with the NADPH-binding site or with an adjacent area of the β-ketoacyl reductase of FAS.[22,23] In addition, EGCG and ECG, but not EGC and EC, are potent inhibitors of glutamate dehydrogenase (GDH) with EC_{50}s in the nanomolar range. EGCG is a non-competitive inhibitor of both GDH substrates (NADH and 2-oxoglutarate), but acts in an allosteric manner.[24]

2.3. Catechin gallates are $NADP^+$-competitive inhibitors of G6PD and other enzymes that employ $NADP^+$ as a coenzyme

To examine the manner in which gallated catechins inhibit G6PD activity, various concentrations of EGCG and CG were added to reactions containing various concentrations of $NADP^+$ and G6P. Both EGCG and CG are competitive inhibitors of $NADP^+$, but are uncompetitive inhibitors of G6P (Fig. 2). Based on the $NADP^+$-competitive inhibition patterns of gallated catechins, it was proposed that gallated catechins could act as general inhibitors of enzymes that employ $NADP^+$ as a coenzyme. Gallated catechins were indeed potent inhibitors of 6PGD and IDH, enzymes which both employ $NADP^+$ as a coenzyme (Table 2).

It is well known that green tea catechins affect the reduction of body weight and prevent obesity-related metabolic disorders such as diabetes, hyperlipidemia, and hypertension in various animal models and in humans.[25,26] Although catechins have been shown to be effective inhibitors of G6PD, it has not been clearly determined whether the therapeutic effects of catechins on metabolic disorders are directly associated with G6PD inhibition. Gallated catechins are not only inhibitors of G6PD, but are also inhibitors of 6PGD and IDH, which use $NADP^+$ as a coenzyme. In addition, gallated catechins are good inhibitors of FAS and GDH, which play important roles in lipid synthesis and insulin secretion, respectively. Green tea catechins are also reported to be inhibitors of pancreatic phospholipase A2 (PLA2), and were found to inhibit the intestinal absorption of lipids in ovariectomized rats.[27] Thus, although gallated catechins are effective inhibitors of G6PD in vitro and show good anti-obesity effects in vivo, the extent of the effects that are directly associated with the inhibition of G6PD by catechins remains unclear.

2.4. Effects of EGCG on endogenous dehydrogenase activity in 3T3-L1 adipocytes lysates

To further investigate the inhibitory effects of EGCG, ECG, GCG, and CG on the production of NADPH by G6PD and 6PGD in adipocytes, measurement of NADPH production was performed using cell lysates from differentiated 3T3-L1 adipocytes. As shown in Figure 3A, EGCG, ECG, GCG, and CG effectively suppressed NADPH production in 3T3-L1 adipocytes with IC_{50} values near 25 μmol/L. However, EGC, EC, GC, and C did not suppress NADPH production in 3T3-L1 adipocytes (Fig. 3B). We also compared the inhibitory effect of EGCG with that of DHEA, a well-known uncompetitive inhibitor of G6PD activity.[13–16] As shown in Figure 3C, both DHEA and EGCG inhibited NADPH production. EGCG inhibited NADPH production in a dose-dependent manner, but DHEA showed only 40% maximum inhibition at concentrations above 100 μmol/L. This difference in inhibition patterns between EGCG and DHEA represents a difference in inhibition mechanisms. Consistent with the observation of differing inhibition mechanisms, EGCG also inhibited 6PGD activity in a dose-dependent manner, while DHEA did not inhibit 6PGD activity (Fig. 3D).

EGCG, ECG, GCG, and CG were significantly better inhibitors of endogenous NADPH production when compared to DHEA. However, considering the very low IC_{50} values (0.18–0.25 μmol/L) in activity assay using purified yeast G6PD, it was somewhat disappoint-

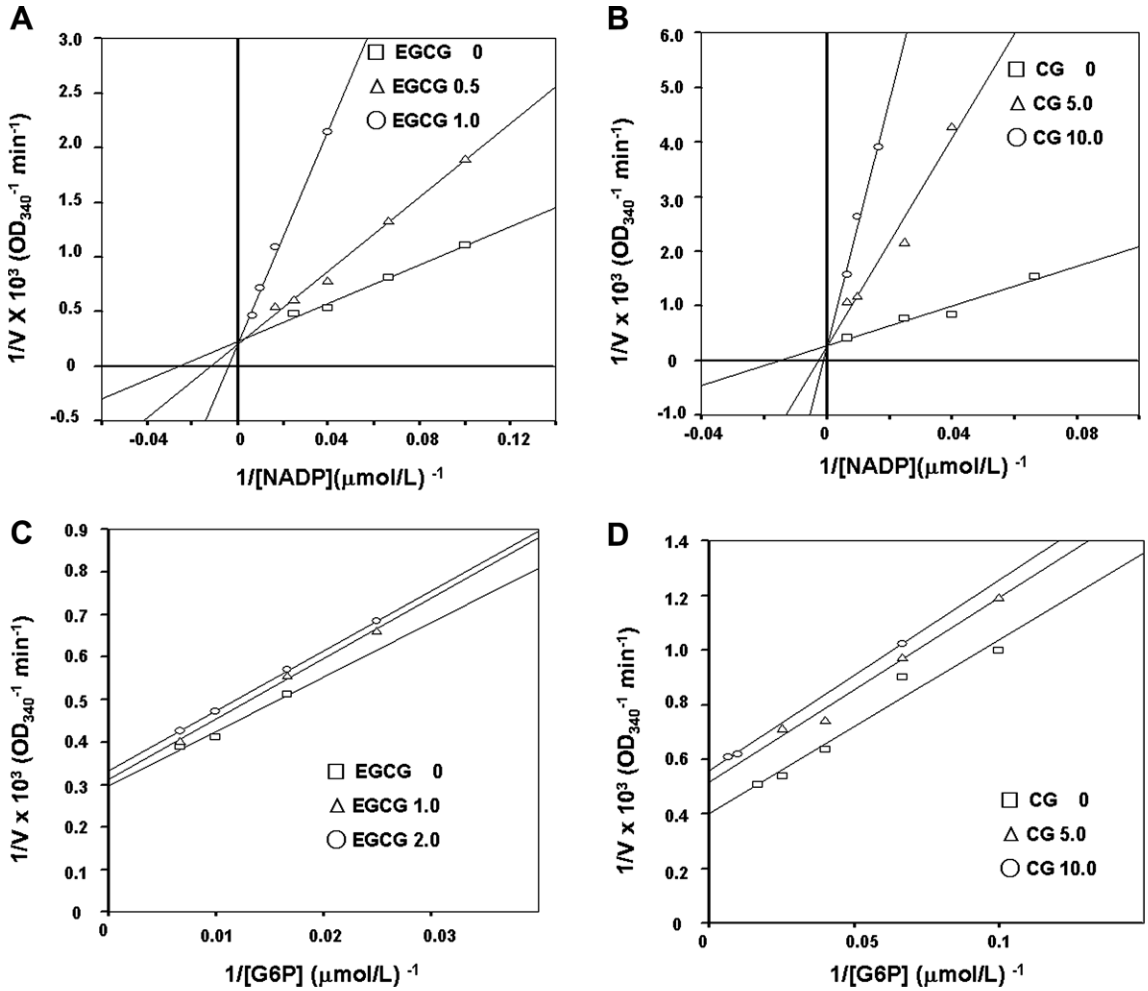


Figure 2. Inhibition kinetics of EGCG and CG with respect to G6P and $NADP^+$. G6PD was analyzed with respect to $NADP^+$ at three concentrations of EGCG and CG. (A) EGCG: 0 μmol/L (□), 0.5 μmol/L (△), and 1.0 μmol/L (○). (B) CG: 0 μmol/L (□), 5.0 μmol/L (△), and 10.0 μmol/L (○). G6PD was analyzed with respect to G6P at three concentrations of EGCG and CG. (C) EGCG: 0 μmol/L (□), 1.0 μmol/L (△), and 2.0 μmol/L (○). (D) CG: 0 μmol/L (□), 5.0 μmol/L (△), and 10.0 μmol/L (○).

ing that catechin gallates showed 100-fold higher IC_{50} values in the NADPH production assay using cell lysates of differentiated 3T3-L1 adipocytes. This difference in IC_{50} values may be partially due to the presence of other proteins, such as IDH, FAS, GDH, and others, which bind with EGCG and consequently reduce the amount of available free EGCG in the 3T3-L1 adipocyte cell lysate. The total plasma concentration after 50 mg EGCG oral intake (estimated amount of EGCG in a cup of green tea) in human is approximately 0.3 μmol/L.[28] Catechin gallate IC_{50} values of approximately 25 μmol/L seem too high to suggest any physiological relevance to known in vivo activities of green tea catechins. However, unlike DHEA, catechin gallates are $NADP^+$-competitive inhibitors of enzymes that employ $NADP^+$ as a coenzyme. Therefore, the in vivo EC_{50} values of catechin gallates will vary depending on the concentration of free $NADP^+$ in the target organ. The concentration of free $NADP^+$ in the target organ is difficult to measure. However, a reasonable estimation can be made based on several previous reports. Total concentration of $NADP^+$ and NADPH in rat liver is approximately 100 μmol/L, and the $NADP^+$/NADPH ratio is about 0.005. A significant portion of the $NADP^+$ and NADPH is bound to protein; in the case of NADH, over 80% of NADH is protein bound. Therefore, the concentration of free $NADP^+$ would be much lower.[29–31] The estimated free $NADP^+$ concentration in a target organ is approximately 0.1 μmol/L, which is more than 1000-fold lower than the concentration of $NADP^+$ used in the in vitro experiment. The high concentration of $NADP^+$ in the in vitro experiment is necessary to obtain a reasonable signal size for measurement. Therefore, EC_{50} values of catechin gallates could be much lower than 0.3 μmol/L. In addition, it was recently reported that multiple treatments with catechins showed synergistic effects.[32] Thus, multiple treatments will further decrease their EC_{50} values and our results may, in fact, be physiologically relevant to known in vivo activities of green tea catechins in humans.

3. Conclusion

In this study, utilizing both in silico and conventional screening approaches, we identified that gallated cate-

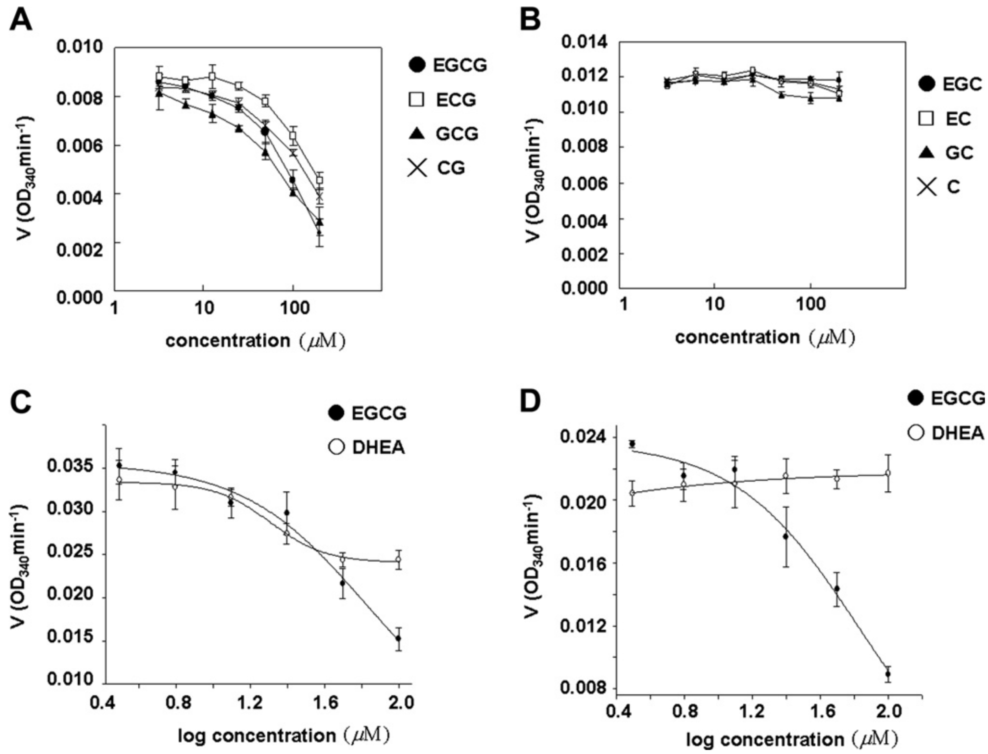


Figure 3. Effects of catechins and DHEA on NADPH production. The production of NADPH by 3T3-L1 cell lysates was measured in the presence of various concentrations of catechins and DHEA. (A) EGCG (●), ECG (□), GCG (▲) and CG (×). (B) EGC (●), EC (□), GC (▲) and C (×). (C) EGCG (●) and DHEA (○). (D) The production of NADPH by 6PGD in 3T3-L1 cell lysates was measured in the presence of various concentrations of EGCG (●) and DHEA (○). Results are represented as means ± SD of three-independent experiments.

chins, but not ungallated catechins, were $NADP^+$-competitive inhibitors of G6PD and other enzymes that employ $NADP^+$ as a coenzyme. Although the extent of the effects that are directly attributable to the inhibition of each enzyme remains unclear, these results along with previous reports concerning the inhibition effects of green tea catechins on FAS, PLA2 and GDH explain how green tea catechins can display such broad in vivo activities against obesity and oxidative stress-related disorders. Catechin gallates showed somewhat high IC_{50} values in the NADPH production assay using cell lysates of differentiated 3T3-L1 adipocytes. However, these activities may still be physiologically relevant to known in vivo activities of green tea catechins in humans, due to $NADP^+$-competitive inhibition of catechin gallates and low in vivo concentrations of free $NADP^+$.

4. Experimental

4.1. Materials

Glucose-6-phosphate, sodium chloride, magnesium chloride, β-NADP, (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), (−)-gallocatechin gallate (GCG), (−)-gallocatechin (GC), (−)-catechin gallate (CG), (−)-catechin (C), Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from bakers yeast, 6-phosphogluconic dehydrogenase from *Saccharomyces cerevisiae* (EC 1.1.1.44), and isocitric dehydrogenase (NADP) (EC 1.1.1.42) from porcine heart were purchased from Sigma–Aldrich.

4.2. Modeling of the binding site of G6PD

Two binary complex structures of the G6PD human deletion mutant of Kotaka et al. with glucose-6-phosphate (G6P, PDB code: 2bhl), and $NADP^+$ (PDB code: 2bh9) were selected for virtual screening.[33] The superimposed structure of the two binary complexes is shown in Fig. S-1. For convenience in structure-based modeling, a combined structure was constructed by copying G6P from the PDB structure 2bhl into the PDB structure 2bh9, in a manner similar to that used by Kotaka et al.[33] (Figure S-2). The software program IDPharmo version 2.0 (Equispharm Inc., Seoul, Korea)[34] was used for virtual screening to search approximately three million commercially available library compounds in a period of eight hours, using a 3GHz, four-CPU Linux PC. IDPharmo is fingerprint-based virtual screening software, and three essential physicochemical features comprise its construction of reliable fingerprints: the hydrogen bond donor, the hydrogen bond acceptor, and the hydrophobic core. After several cycles of pharmacophore generation and refinement, 9 protein–ligand-binding features for the substrate- and coenzyme-binding sites of G6PD were obtained, as shown in Figure S-2. Based on the combination of these 9 ligand-binding features, 12 different pharmacophore models, termed PharmoMaps, were selected. Based on these 12 PharmoMaps, approximately 2000 'virtual hit' molecules were obtained from the three million-compound library in which each compound is pre-compiled in a maximum of 150 different conformations. The scoring methods utilized the combined root mean square deviation (RMSD) scores between the PharmoMaps and docked-molecules, and bump-penalties. In order to reduce the number of virtual hits carried forward further for testing, the 2000 hits were visually inspected for drug-likeness by assessing hydrophobicity, molecule size, and diversity of molecular structure. A total of 250 compounds that could be purchased from different chemical library distribution companies were finally selected.

4.3. Assay of enzyme activity

Enzyme activity was determined using a Spectra MAX 190 (Molecular Devices) by measuring absorbance at 340 nm, at a temperature of 30 °C. The reaction mixture contained 150 mmol/L sodium chloride, 6 mmol/L magnesium chloride, 0.5 mmol/L glucose-6-phosphate, 0.25 mmol/L $NADP^+$, and 0.1 mol/L Tris buffer (pH 7.5) in a total volume of 0.2 mL. An amount of 0.0002 enzyme units was used in each reaction. Glucose-6-phosphate was replaced by 0.5 mmol/L 6-phosphogluconate for the 6PGD assay, or by 0.5 mmol/L isocitric acid for the IDH assay. The reaction mixture was prepared immediately prior to use. The reaction catalyzed the reduction of $NADP^+$ to NADPH, and the rate of the reaction was calculated from the increase in the absorbance at 340 nm. The extinction coefficient of NADPH is $6.02 \times 103\ M^{-1}\ cm^{-1}$. The inhibition effect of the inhibitors was examined by adding each inhibitor to the reaction mixture prior to the initiation of the reaction. The level of inhibition in the presence of the inhibitor was measured by reference to the half-inhibition concentration (IC_{50}). The IC_{50} values of the green tea catechins were determined graphically after linear regression of the inhibitory percentages expressed with the logarithmic concentration of the inhibitors.

4.4. Determination of inhibition mechanism

The mechanism of inhibition by catechin gallates was determined by constructing reciprocal plots of $1/V$ versus 1/[S] for reactions with 1 mmol/L G6P and varying concentrations of $NADP^+$, and with 0.5 mmol/L of $NADP^+$ and varying concentrations of G6P. The plots were assessed by a weighted least-square analysis. The slopes of these reciprocal plots were then plotted against the concentration of the inhibitors (range: 0–2.0 μmol/L for EGCG, 0–10.0 μmol/L for CG) in a weighted analysis.

4.5. Endogenous dehydrogenase enzyme assay

G6PD and 6PGD enzyme activities were determined by measuring the rate of NADPH production as previously described with slight modifications.[11] Because 6PGD also catalyzes a reaction in which NADPH is produced, NADPH production by 6PGD and NADPH produced by total dehydrogenases (G6PD + 6PGD) was measured separately. Fully differentiated 3T3-L1 adipocytes were lysed with NETN buffer (50 mmol/L Tris pH 7.8, 100 mmol/L NaCl, 0.1% NP-40, 1 mmol/L EDTA) and centrifuged at 12,000 rpm at 4 °C for 15 min. Supernatants were used immediately in assays measuring dehydrogenase activity. Each enzyme activity was measured after incubation of the supernatants in reaction buffer with indicated polyphenols and DHEA at 30 °C. For NADPH production assay, the reaction buffer contained 150 mmol/L sodium chloride, 6 mmol/L magnesium chloride, 0.5 mmol/L glucose-6-phosphate, 0.5 mmol/L 6-phosphogluconate, 0.25 mmol/L $NADP^+$, and 0.1 mol/L (pH 7.5) Tris buffer. For 6PGD activity assay, the reaction buffer contained 150 mmol/L sodium chloride, 6 mmol/L magnesium chloride, 0.25 mmol/L 6-phosphogluconate, 0.125 mmol/L $NADP^+$, and 0.1 mol/L (pH 7.5) Tris buffer. In each assay, 1.0 μg of protein was added to the reaction buffer. Protein concentrations were determined using the Bradford method (Bio-Rad), and used to normalize enzyme activity. Inhibitors were dissolved in dimethylsulfoxide and then added to the reaction mixture. The volume of dimethylsulfoxide was less than 0.2% (v/v) of the total reaction volume in order to avoid interference with the enzyme activity. In inhibitor-free control reactions, the same amount of dimethylsulfoxide was added to the reaction mixture.

Acknowledgments

This work was partly supported by the Korea Science and Engineering Foundation (KOSEF) through the National Research Laboratory Program of Korea Institute of Science (No. M10400000359-06J0000-35910), the Molecular and Cellular BioDiscovery Research Program (No. M10748000258-07N4800-25810), and the Technology Evaluation and Planning and Stem Cell Research Center of the 21st Century Frontier Research Program (No. R11-2005-009-01002-0). M. Ham, J. Park, and J. B. Kim are supported by the BK21 Research Fellowship from the Ministry of Education and Human Resource Development.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.02.030.

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