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Flavonoid inhibition of overexpressed human 3β-hydroxysteroid dehydrogenase type II

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Abstract

The inhibitory effects of various flavonoids on human 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase type II (3β-HSD type II), overexpressed in baculovirus, were investigated, and the structure–inhibition relationship was examined. The isoflavone derivatives daidzein, genistein, formononetin and biochanin A inhibited 3β-HSD type II activity at a concentration of 10 μM and of these, genistein was the most potent inhibitor. 6-Hydroxyflavone (6-HF), a synthetic flavone, also strongly inhibited 3β-HSD activity but 5-HF, 7-HF and other natural flavones were less potent. Energy minimization structures of the flavonoids, as produced using MOE software, showed that isoflavones and flavones have an almost flat A–C ring structure, and that flavonoids that acted as inhibitors had similar steric structures to DHEA. Genistein, 6-HF and cyanoketone, which is known as a typical 3β-HSD inhibitor, were found to act as competitive inhibitors with K_i values of 0.12 μM, 0.19 μM and 0.67 nM, respectively. Furthermore, the LUMO (lowest unoccupied molecular orbital (LUMO)) values, as calculated using WinMOPAC (Fujitsu, Japan), of the inhibitors were correlated with the IC_{50} values ($r^2 = 0.84$). From these results, it appears that inhibitory effects of flavonoids are due to the combination of steric structure and electron affinity between the active center of 3β-HSD type II and the flavonoid molecule.

Keywords: 3β-Hydroxysteroid dehydrogenase type II; Phytoestrogen; Flavonoids; Genistein; Enzyme inhibition; Isoflavone

1. Introduction

The isoflavones daidzein and genistein, as well as their glycosides, are particularly abundant in soybeans and soy products, which are a major component of traditional Asian diets. These isoflavones are structurally analogous to estrogen and are known to exhibit estrogenic activity [1]. Epideminological studies have demonstrated marked geographic differences in the incidence of hormone-related cancers, such as breast and prostate cancer [2,3]. Specifically, the incidence of these cancers is significantly lower in Asia when compared with Northern Europe and in the US [4,5]. There are numerous reports suggesting that environmental factors, such as dietary compounds, have a major impact on cancer development and progression, and phytoestrogens, which include soy isoflavones, are thought to be responsible for the protective affects against cancers, such as those of the breast and prostate [6–9]. On the other hand, we reported that several flavonoids, including daidzein and genistein, inhibited steroidogenesis by human adrenocortical H295R cells via the inhibition of cortisol production. We also reported that these isoflavones inhibited 3β-hydroxysteroid dehydrogenase [EC 1.1.1.145] coupled with Δ^5-Δ^4 isomerase [EC 5.3.3.1] (3β-HSD) and cytochrome P450c21 (CYP 21B gene product) [10]. 3β-HSD is a bifunctional, single protein enzyme that catalyzes the oxidation and isomerization of 5-ene-3β-hydroxysteroids (e.g. pregnenolone or dehydroepiandrosterone (DHEA)) to the corresponding 4-ene-3-ketosteroids (e.g. progesterone or androstenedione (AD)). In humans, there are two types of 3β-HSD (types I and II), the genes of which encode deduced proteins of 371 and 372 amino acids, respectively, and have been characterized [11,12]. 3β-HSD type I is exclusively expressed in the placenta and skin, while type II is predominantly expressed in the adrenals, testes and ovaries [12,13]. Consequently, 3β-HSD type II is the key enzyme in the biosynthesis of not only corticoids, but also other steroid hormones, such as androgens, estrogens and progestin. In the present study, we investigate the inhibitory effects of 25 flavonoids (listed in Fig. 1), including 10 plant-derived isoflavones, 11 native and synthetic flavones, 2 flavanones and 2 flavonoid derivatives, on recombinant human 3β-HSD type II expressed in a baculovirus expression system, and

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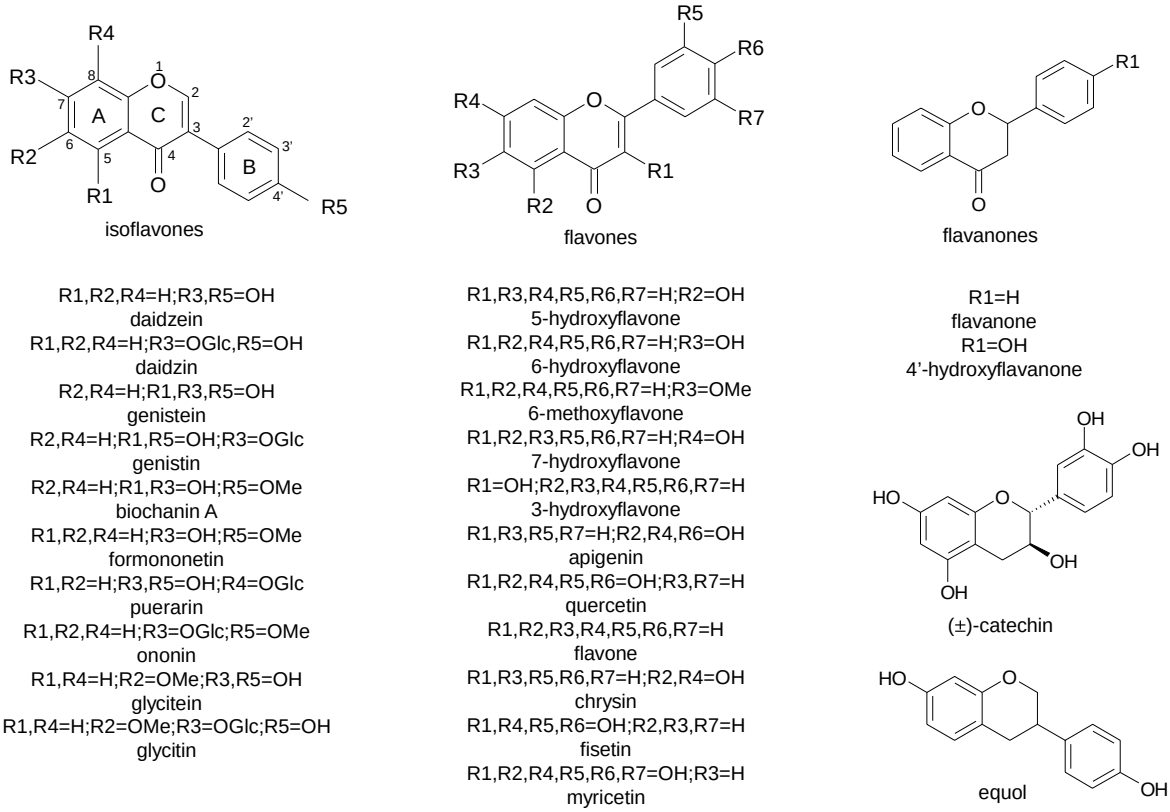

Fig. 1. Flavonoids used in the present study.

compare their effects to those of 2α-cyano-17β-hydroxy-4, 4, 17α-trimethylandrost-5-en-3-one (cyanoketone), which are known to be specific 3β-HSD inhibitors.

2. Materials and methods

2.1. Chemicals

5-Hydroxyflavone (5-HF), 6-hydroxyflavone (6-HF), 7-hydroxyflavone (7-HF), 6-methoxyflavone (6-MF), apigenin, quercetin, formonetin, chrysin, myricetin, ononin, equol, 4′-hydroxyflavanone (4′-HFN), glycitin, puerarin were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Daidzin, genistein, daidzein, genistin, flavone, 3-hydroxyflavone (3-HF), flavanone, fisetin were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Biochanin A, glycitein, (±)-catechin, dehydroepiandrosterone (DHEA), androstenedione were obtained from Sigma–Aldrich, K.K., Japan (Tokyo, Japan). cDNA library, human adrenal gland from Takara Bio Inc. (Shiga, Japan), and Cyanoketone from Sterling-Winthrop (Rensselaer, NY) were used. Radio-labeled [4-^{14}C] dehydroepiandrosterone (0.74 MBq/(ml/(ethanol)), produced by NEN Life Science Products Inc., was obtained from Daiichi Pure Chemicals (Tokyo, Japan).

All other reagents were of the best commercially available grade.

2.2. Sf9 cell culture

Sf9 insect cells were cultured in Sf900 II SFM (Invitrogen Corporation, Carisbad, CA) containing the antibiotics penicillin (50 IU/ml) and streptomycin (50 μg/ml) using adherent monolayer cell culture or suspension cell culture at 28 ± 0.5 °C. For suspension cell culture, we used a 250 ml spinner vessel and a 150 ml working volume of suspension at a constant stirring rate of 120 rpm and maintaining cell density between 3×10^5 and 2×10^6 cells/ml.

2.3. Expression of human 3β-HSD type II

Human recombinant 3β-HSD type II was prepared by overexpression in Sf9 insect cells following baculovirus infection using Bac-to-Bac baculovirus expression systems (Invitrogen, Carlsbad, CA). Briefly, in order to prepare the bacmid construct for expression in insect cells, human 3β-HSD type II cDNA was amplified by PCR using a forward primer (5′-AGAATTCAAATGGGCTGGAGCTGCCTT-3′) and a reverse primer (5′-GGACTGCAGATCACTGAGTCTTGGACTTCA-3′). PCR product was digested with *Eco*RI and *Pst*I restriction enzymes and subcloned into an

*Eco*RI–*Pst*I-digested pFastBac1 donor plasmid to give the donor construct. Max efficiency DH10Bac competent cells having the bacmid and helper plasmid were transformed with the donor construct. *Escherichia coli* colonies in which the transposition of 3β-HSD cDNA into the bacmid occurred were verified by color selection and the recombinant bacmids were isolated after overnight culture. Recombinant bacmids ware transfected into Sf9 cells using CellFECTIN reagent. After culturing for 3 days at 27 °C, culture media were kept as baculovirus stock. Virus stocks were used for further infection of Sf9 cells in order to increase the titer. After infection of Sf9 cells, 3β-HSD assays were performed using Sf9 cell lysate in order to confirm overexpression.

2.4. Preparation of membrane fraction and subcellular fraction of Sf9 cells

Baculovirus-infected Sf9 cells were washed twice with phosphate buffered saline (PBS) and frozen at −80 °C. Cells were homogenized with Physcotron® (Microtec Co., Chiba, Japan) in 20 mM KPB (pH 7.4)–0.25 M sucrose–0.5 mM EDTA (pH 7.4) for 15 s six times in the presence of proteinase inhibitors (complete mini; Roche). The total membrane fraction was prepared by centrifugation of the homogenate at 105,000 × *g* for 1 h, and the pellet was re-suspended in the same buffer, while the supernatant was used as the cytosol fraction. To determine the 3β-HSD type II distribution, part of the total membrane fraction was re-centrifuged. Unbroken cells and the nuclear fraction were separated at 600 × *g* for 10 min, mitochondria were pelleted from the supernatant by centrifugation at 9000 × *g* for 15 min, and microsomes were pelleted according to the method of Kamath and Rubin [14]. Microsomes were resuspended in the same buffer containing 20% glycerol. The total membrane and subcellular fractions were stored at −80 °C until use in enzyme assay.

2.5. 3β-HSD (type II) assay

3β-HSD II activity was measured by incubation with Sf9 cell extract in 0.2 ml of 100 mM potassium phosphate buffer (KPB, pH 7.4) containing 0.5 mM NAD^+, sample solution and [4-^{14}C] DHEA (500 Bq/1 nmol/2 μl ethanol) at 37 °C. After incubation, steroids were extracted with 0.6 ml of ethyl acetate/2,2,4-trimethylpentane (1/1 (v/v)). After concentration by evaporation, metabolite steroids were separated by TLC (Kieselgel 60F254, Merck, Darmstadt, Germany) using benzene/acetone (8/1 (v/v)), and were detected by autoradiography (Scientific Imaging Film, Biomax MR, Eastman Kodak, Rochester, NY). The amount of androstenedione metabolite from DHEA were quantitated indirectly as the integrated optical density (IOD) of the autoradiography film or directly as the radioactivity of relevant TLC bands in ACS II liquid scintillation fluid (Amersham Pharmacia Biotech, Tokyo, Japan) using a Tri-Carb 1600 TR liquid scintillation analyzer (Packard Japan Co., Tokyo, Japan). 3β-HSD activity was expressed as pmol product/min/mg protein.

2.6. Statistical analysis

Statistical analysis of data was performed using Student's *t*-test. Statistical significance was set at the $P < 0.05$ level.

3. Results

3.1. Expression of 3β-HSD (type II) in insect Sf9 cells and subcellular distribution

3β-HSD activity, which was demonstrated by conversion of DHEA to AD, was investigated using the homogenate prepared from recombinant human 3β-HSD type II baculovirus-transfected Sf9 cells. 3β-HSD activity was confirmed in the homogenate of baculovirus-transfected Sf9 cells when compared with control Sf9 cells (Fig. 2). Specific activity of 3β-HSD type II in Sf9 cell homogenate was found to be 3.6 pmol/min/mg protein. In addition, specific activity of the pellet after centrifugation at 105,000 × *g* for 1 h (resulting in removal of cytosol from homogenate) was found to be 18.3 pmol/min/mg protein. Subcellular distribution of 3β-HSD activity in Sf9 cells was as follows: nuclear fraction, 42.4% of total activity; mitochondria, 39.6%; microsomes, 15.7%; and cytosol, 2.3%. Specific activities observed in these subcellular fractions were almost uniform, except in the cytosol where it was extremely low (data not shown), and thus the pellet at 105,000 × *g* (total membrane fraction) was used as the enzyme source for all other experiments.

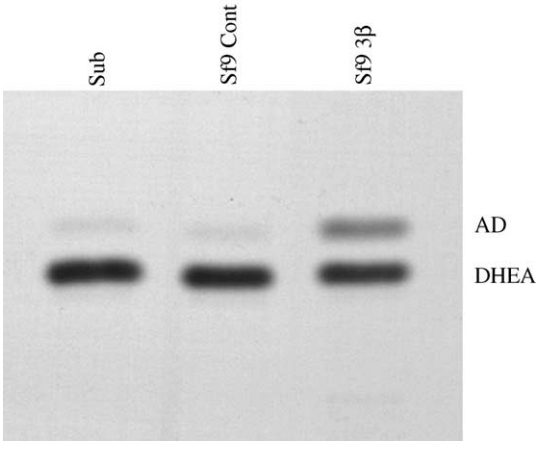

Fig. 2. Detection of 3β-HSD activity in Sf9 cell. [4-^{14}C] DHEA was incubated in the presence of NAD^+ without (Sub) or with homogenate (0.58 mg protein) prepared from control Sf9 (Sf9 Cont) or vaculovirus-transfected Sf9 cells (Sf9 3β) at 37 °C for 120 min. Conversion of DHEA to AD was assayed by thin layer chromatography.

3.2. Effect of various flavonoids on human 3β-HSD type II activity

Using the total membrane fraction of transfected Sf9 cells, the activity of 3β-HSD type II in the presence of various flavonoids (10 μM) was investigated with a total 25 flavonoids. Strong inhibitory effects were detected with the isoflavone derivatives of daidzein and genistein, and these were comparable to the effects obtained with 5 nM cyanoketone, which is a known 3β-HSD inhibitor. The isoflavones formononetin and biochanin A, also exhibited weak inhibitory effects, but no inhibitory effects were observed with isoflavone glycoside molecules. Interestingly, inhibitory effects were seen with 6-HF, a synthetic flavone, and this effect was almost the same as that of genistein, but no inhibitory effects were seen with either 5- or 7-HF. 3-HF, 6-methoxyflavone, chrysin, apigenin, quercetin and myricetin, which are all flavone derivatives, showed comparatively weak but significant inhibitory effects, whereas the effect was undetectable with flavanone derivatives (Fig. 3). Among these flavonoids, eight effective compounds

Table 1
Inhibition of 3-HSD type II activity by flavonoids

Compound	IC_{50} (M)	Relative potency	LUMO (au)
Genistein	2.58×10^{-7}	100	−0.44
6-Hydroxyflavone	5.58×10^{-7}	46	−0.15
Biochanin A	9.44×10^{-7}	27	−0.43
Form ononetin	3.18×10^{-6}	8.1	−0.45
Daidzein	3.28×10^{-6}	7.9	−0.46
Myricetin	1.08×10^{-5}	2.4	−0.094
Q uercetin	8.35×10^{-5}	0.3	−0.039
Apigenin	1.26×10^{-4}	0.2	−0.006
Cyanoketone	7.60×10^{-9}	3400	−9.99

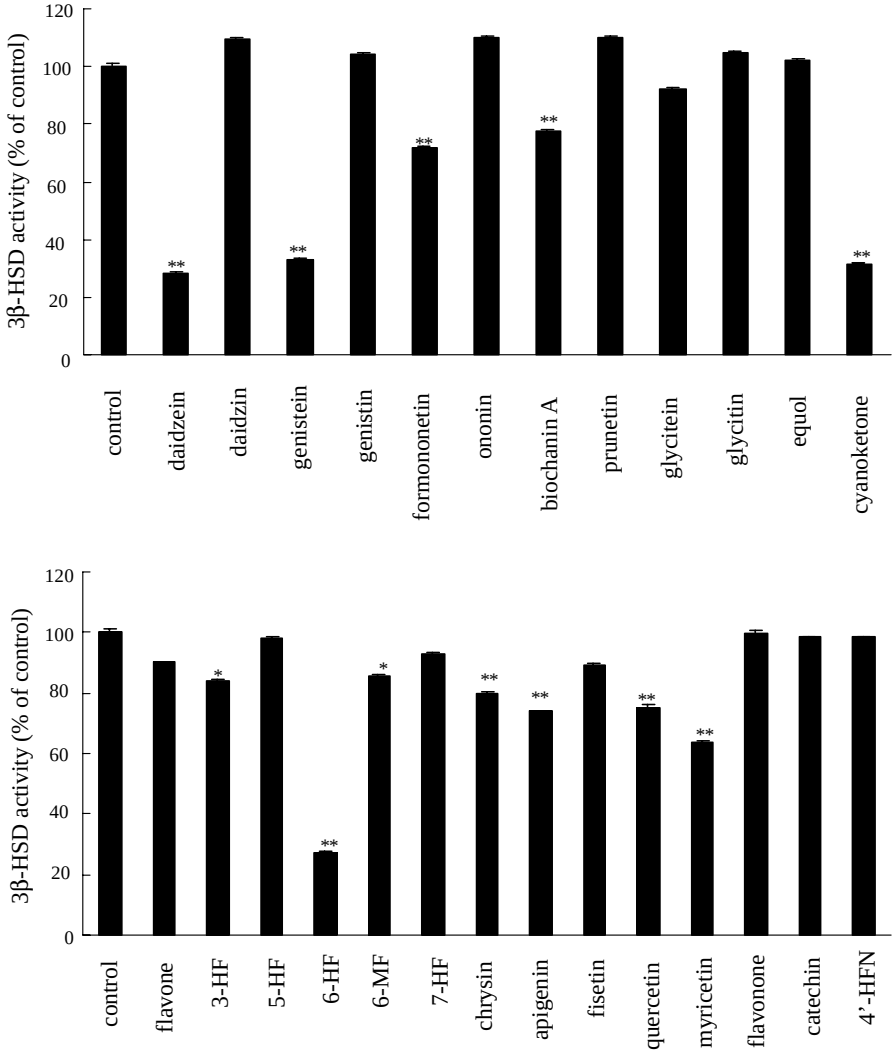

Fig. 3. Effect of various flavonoids on human 3β-HSD type II activity. Total membrane fraction prepared from Sf9 cells transfected with the recombinant vaculovirus carrying human 3β-HSD type II was incubated with [4-^{14}C] DHEA and NAD^+ in the presence of 10 μM of the flavonoids listed or 5 nM cyanoketone for 90 min. 3β-HSD type II activity was expressed as % of control ±S.E.M obtained from 3 to 10 independent experiments. Control used the above conditions but substituted ethanol for the inhibitors and the specific activity was 18.3 pmol/min/mg. * and ** indicate statistical differences when compared to the control ($P < 0.05$ and $P < 0.01$, respectively).

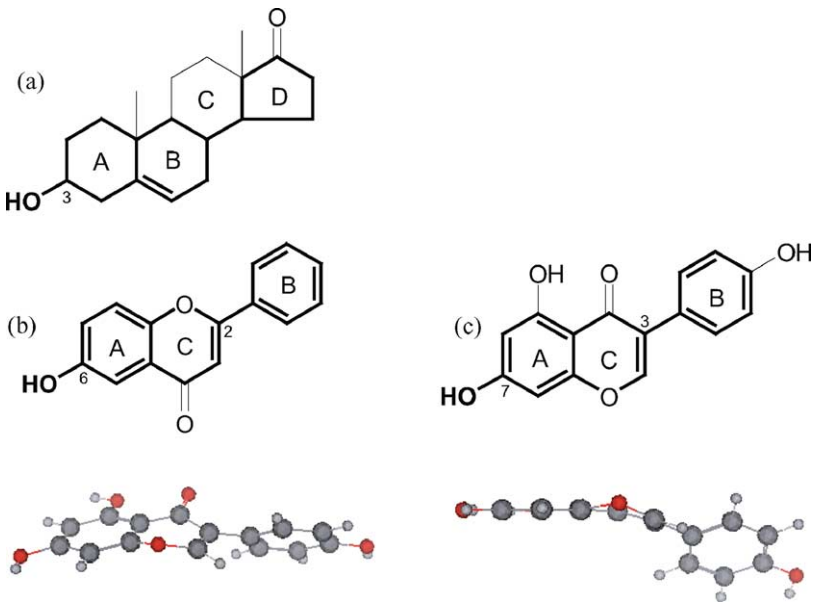

Fig. 4. Molecular conformations of DHEA (a), 6-HF (b) and genistein (c and d). Structural similarities estimated are indicating as thick line and energy minimization structure of genistein were constructed by MOE.

were selected and their IC_{50} values compared with that of cyanoketone (Table 1). The inhibitory potency relative to that of genistein, which was arbitrarily assigned a value of 100, are also shown in Table 1. Cyanoketone substantially inhibited 3β-HSD activity, with a potency over 30 times higher than that of genistein. Genistein and biochanin A had relatively strong inhibition among isoflavone derivatives. Although daidzein was recognized as a strong inhibitor in Fig. 3, its relative potency calculated from the IC_{50} value was only 8% of genistein and thus daidzein is though to act as inhibitor only when present at high concentrations. 6-HF also strongly inhibited 3β-HSD activity (50% of genistein) but was twice as potent as biochanin A, and these three flavonoids (genistein, 6-HF and biochanin A) exhibited IC_{50} values in the order of 10^{-7} M, whereas those of myricetin, quercetin and apigenin, and flavone derivatives were in the order of 10^{-5} to 10^{-4} M. Next, the eight substances along with cyanoketone were subjected to analysis of energy minimization structure using MOE computer software (Chemical Computing Group Inc.; info@chemcomp.com), and the structural specificity of 3β-HSD type II inhibition was determined. This analysis revealed that isoflavones and flavones have an almost flat structure in the A–C ring, and that flavonoids have hydroxy groups at the six and/or seven positions of the A-ring and a B-ring positioned diagonally from the hydroxy group. The structural similarities between these compounds and DHEA appear to be responsible for the inhibitory effects on 3β-HSD activity (Fig. 4).

Based on energy-minimized structures, the lowest unoccupied molecular orbital (LUMO), the highest occupied molecular orbital (HOMO) and the log*P* of flavonoid samples and cyanoketone were calculated using the computer software WinMOPAC (Fujitsu, Japan) (HOMO and log*P* data not shown). LUMO values of these substances were distributed between the minimum range at −9.985 (au) for cyanoketone and the maximum range at −0.0063 (au) for apigenin (Table 1). LUMO values of these inhibitors were comparatively well correlated to their IC_{50} values (r^2 = 0.84) (Fig. 5), whereas HOMO and log*P* values did not show any correlation to IC_{50} (data not shown).

3.3. Kinetic analysis

Lineweaver–Burk plots were used to investigate type of inhibition and these were compared between three substances shown to be comparatively strong inhibitors of human 3β-HSD type II activity; genistein (isoflavone), 6-HF (flavone) and cyanoketone (steroid). For 3β-HSD type II, the K_m value of DHEA was estimated to be 1.75 μM.

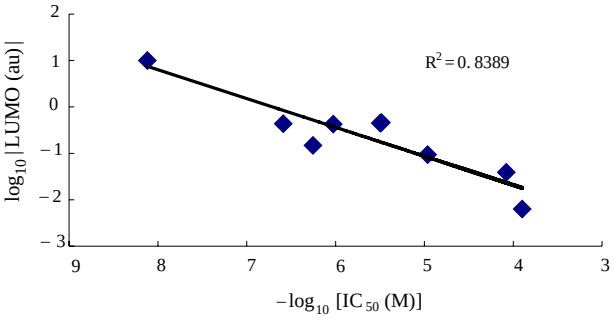

Fig. 5. Correlation of IC_{50} and LUMO. IC_{50} values were calculated from human 3β-HSD type II activity using the total membrane fraction of transfected Sf9 cells in the presence of various concentrations of flavonoid or cyanoketone. Results shown are from at least two independent experiments carried out in triplicate. LUMO values were calculated by WinMOPAC software.

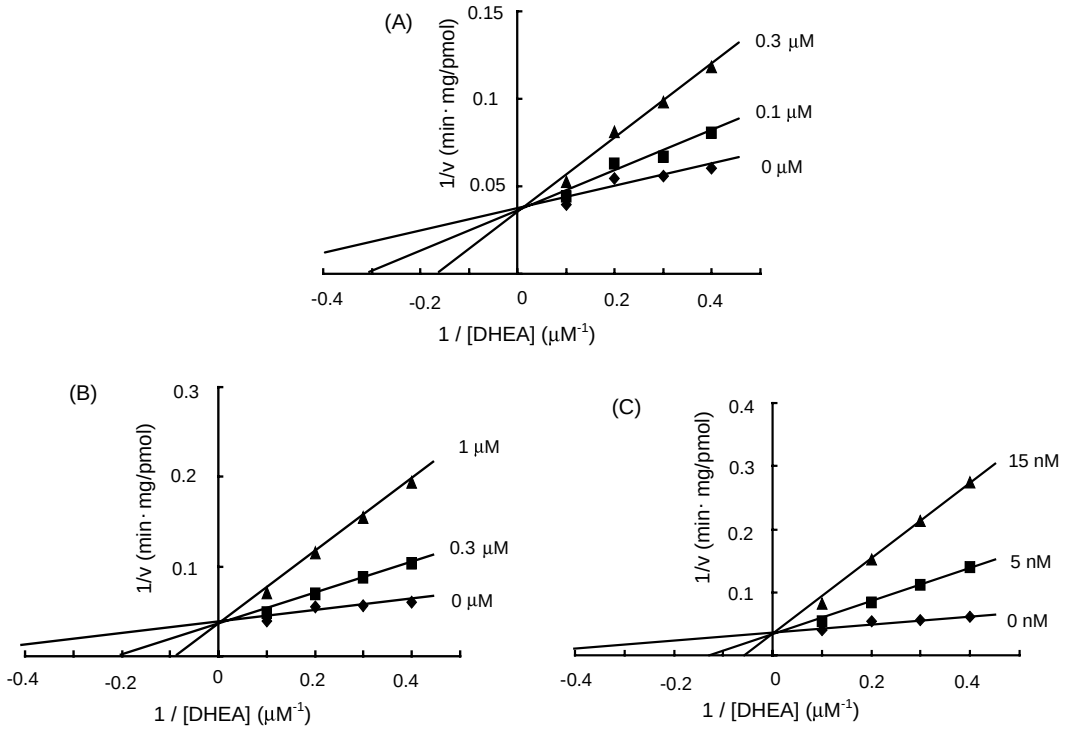

Fig. 6. Kinetic analysis of inhibition by genistein (A), 6-HF (B) and cyanoketone (C). Each point represents the mean of two independent experiments conducted in duplicate.

Genistein, 6-HF and cyanoketone all behaved as typical competitive inhibitors (Fig. 6), and had inhibition constants (K_i), as estimated by Dixon plot, of 0.12 μM, 0.19 μM and 0.67 nM, respectively.

4. Discussion

Isoflavones are known to be phytoestrogens [1], and the traditional Asian diet of soy-derived foods is rich in the isoflavones daidzein and genistein as well as their glycosides (daidzin and genistein) [15–17]. Several recent epidemiological studies have reported that, when compared to Northern European or American individuals, Japanese and other individuals of Asian ethnicity, who tend to have higher daily intakes of soy products, showed a significantly lower risk of hormone dependent cancers, such as prostate cancer in men and/or breast cancer in women [4,5]. In addition, several reports have found that soybean isoflavones have a major protective impact on breast or prostate cancers in humans [6–9], while rodent studies also support the hypothesis that soy-isoflavone diets exert a protective effect against prostate cancer-cell growth [18–22]. Although genistein is a well-known tyrosine kinase inhibitor [23], the mechanisms of suppression of cancer cell growth remain uncertain. Based on these studies, it appears that the continuous intake of soy isoflavones exerts some beneficial effects, such as cancer risk reduction. However, there are also reports that excess administration of phytoestrogens in rodents lead to irregularities in steroidogenesis in vivo [24,25], and maternal vegetarian diets during pregnancy are associated with hypospadias in humans [26].

Our previous report suggested that daidzein and genistein could significantly reduce cortisol production without estrogen receptor mediation in human adrenocortical H295R cells, and that daidzein acts as a potent inhibitor of 3β-HSD and a comparatively weak inhibitor of P450c21 [10]. Daidzein, genistein, biochanin A and formononetin potently inhibit the gamma-isozymes of mammalian alcohol dehydrogenase that catalyze the oxidation of 3β-hydroxysteroids [27] and Wong et al. [28] found that these isoflavones inhibit the 3β-HSD activity of bovine adrenal short-chain alcohol dehydrogenase. Furthermore, in a study using human placental microsomes, it was shown that isoflavonoids, as well as other compounds that possess a phenolic B ring in the 3 position of the pyran ring, preferentially inhibit 3β-HSD and/or 17β-HSD activity [29]. Therefore, investigation of the inhibitory mechanisms of isoflavones on human 3β-HSD is considered to be very important.

3β-HSD catalyzes the conversion of pregnenolone to progesterone and of DHEA to androstenedione, and is thus a very important membrane-associated enzyme involved in steroid hormone biosynthesis [30]. In humans, there are two isozymes, types I and II. Type II is predominantly expressed in the adrenals and gonads [12], while type I is expressed in the placenta, skin and other peripheral tissues [13]. Both types I [31,32] and II human isozymes [33] have successfully been expressed in a baculovirus system. Although the

structure of native enzyme has not yet been elucidated, the type I isozyme has been crystallized [34] using soluble enzyme, which is modified by removal of the membrane-associated regions [35]. In the present study, 3β-HSD type II was overexpressed using a baculovirus expression system and was not distributed in the soluble fraction, but was broadly detected in all membrane-associate fractions of Sf9 cells. Therefore, we used the total membrane fraction as the enzyme source to investigate the relationship between structure and inhibition using various flavonoids and human 3β-HSD type II.

Human 3β-HSD type II activity was strongly inhibited by isoflavone derivatives, and the common structural feature of these inhibitors was the hydroxy group at the C7 position. However, although glycitein has a hydroxy group at C7, no inhibitory effect was seen, and this is probably due to steric interference of the methoxy group at C6. In addition, the glycoside forms of the isoflavones showed no inhibitory effects, and this may also be due to steric interference by the sugar(s). It is believed that daidzin and genistin are not able to be directly absorbed by the intestinal wall and must first undergo catabolism in enteric bacteria. Soy isoflavones mainly exist in the glycoside form and all of these substances are absorbable when they are in the aglycone form [36]. Consequently, daidzin and genistin would exist as daidzein and genistein, respectively, in human body and thus it is possible that they inhibit 3β-HSD type II activity.

The inhibitory effects on 3β-HSD were not limited to isoflavones but were also seen with flavone derivatives. The most effective chemical among the flavone derivatives was the synthetic flavone 6-HF, which exhibited a comparable degree of inhibition as genistein and biochanin A, whereas 5-HF and 7-HF had no inhibitory effect. This clearly suggests that the position of the hydroxy group is a critical factor for inhibition of human 3β-HSD type II activity. From the energy-minimized structure obtained using MOE software, flavone and isoflavone derivatives have an almost flat A–C ring structure, and thus flavones may orient themselves in such a way as to be nearly analogous with isoflavones (Fig. 4). 6-HF may have been recognized as 7-hydroxy-isoflavone, and exhibited similar inhibitory effects on 3β-HSD as genistein or biochanin A. Therefore, the intensity of inhibitory effects of flavonoids on 3β-HSD type II are related to the steric conformation between the B-ring and the hydroxy group at the C6 or C7 position on the A-ring, which is similar to the structural relationship between the 3β-hydroxy group and C-D ring deflection against the A-B ring in steroid substrates, such as DHEA or progesterone. This was also the case for apigenin, quercetin, myricetin and chrysin, all of which are natural flavones having a hydroxy group at the C7 position, and all of which were able to inhibit 3β-HSD type II activity, but to an extremely small degree.

3β-HSD belongs to the short-chain alcohol dehydrogenase-reductase (SDR) family [11]. The catalytic site, Ser···Tyr-X-X-X-Lys, is well conserved in all SDR-family enzymes [37], and the reaction mechanisms are also well understood. First, the NAD^+ cofactor binds to the enzyme and deprotonates the tyrosine residue at the catalytic site. This functions as the base catalyst and may be responsible for deprotonation of the hydroxy group and incorporation of the oxidative cofactor in the dehydrogenase reaction [38]. In the present study, LUMO values calculated from energy-minimized structure of individual flavonoid molecules and IC_{50} values were correlated. Generally, differentiation between HOMO and LUMO reflects the intensity of electron affinity, and lower differentiation suggests higher electron affinity. We believe that strong flavonoid inhibitors might have a high affinity for the electron supplied by the deprotonated tyrosine residue at the catalytic site. If this hypothesis is correct, each inhibitor would be acting as a competitive inhibitor with the substrate, and we thus investigated and compared the type of inhibition observed with cyanoketone, genistein and 6-HF. These three substances were found to behave as typical competitive inhibitors. Therefore, the degree of 3β-HSD inhibition by each flavonoid is related to not only their steric conformation but also the combined effects of electron affinity between the flavonoid molecule and the catalytic site of the enzyme.

Of the 25 flavonoids we investigated in this study, the soy isoflavone genistein inhibited 3β-HSD type II activity most effectively and is thought to exert an affect on steroidogenesis. However, the mechanism of this inhibition will remain uncertain until the 3D structure of human 3β-HSD type II is elucidated.

Acknowledgements

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References

[1] K.D.R. Setchel, Naturally occurring non-steroidal estrogens of dietary origin, in: J. McLachlan (Ed.), Estrogens in the Environment, Elsevier, New York, 1985, pp. 69–85.
[2] J.A. Thomas, Diet, micronutrients, and the prostate gland, Nutr. Rev. 57 (1999) 95–103.
[3] B. Armstrong, R. Doll, Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices, Int. J. Cancer 15 (1975) 617–631.
[4] W.D. Flander, Prostate cancer epidemiology, Prostate 5 (1984) 621–629.
[5] H. Shimizu, R.K. Ross, L. Bernstein, R. Yatani, B.E. Henderson, T.M. Mack, Cancers of the prostate and breast among Japanese and white immigrants in Los Angels country, Br. J. Cancer 63 (1991) 963–966.
[6] M.J. Messina, V. Persky, K.D. Setchell, S. Barnes, Soy intake and cancer risk: a review of the in vitro and in vivo data, Nutr. Cancer 21 (1994) 113–131.
[7] L.J. Lu, K.E. Anderson, J.J. Grady, M. Nagamani, Effects of soya consumption for one month on steroid hormones in premenopausal women: implications for breast cancer risk reduction, Cancer Epidemiol. Biomarkers Prev. 5 (1996) 63–70.

[8] L.J. Lu, K.E. Anderson, J.J. Grady, F. Kohen, M. Nagamani, Decreased ovarian hormones during a soya diet: implications for breast cancer prevention, Cancer Res. 60 (2000) 4112–4121.
[9] N.B. Kumar, A. Cantor, K. Allen, D. Riccardi, C.E. Cox, The specific role of isoflavones on estrogen metabolism in premenopausal women, Cancer 94 (2002) 1166–1174.
[10] S. Ohno, S. Shinoda, S. Toyoshima, H. Nakazawa, T. Makino, S. Nakajin, Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells, J. Steroid Biochem. Mol. Biol. 80 (2002) 355–363.
[11] V. Luu-The, Y. Lachance, C. Labrie, G. Leblanc, J.L. Thomas, R.C. Strickler, F. Labrie, Full length cDNA structure and deduced amino acid sequence of human 3β-hydroxy-5-ene steroid dehydrogenase, Mol. Endocrinol. 3 (1989) 1310–1312.
[12] E. Rhéaume, Y. Lachance, H.F. Zhao, N. Breton, M. Dumont, Y. de Launoit, C. Trudel, V. Luu-The, J. Simard, F. Labrie, Structure and expression of a new complementary DNA encoding the almost exclusive 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase in human adrenals and gonads, Mol. Endocrinol. 5 (1991) 1147–1157.
[13] F. Labrie, J. Simard, V. Luu-The, G. Pelletier, A. Bélanger, Y. Lachance, H.F. Zhao, C. Labrie, N. Breton, Y. de Launoit, M. Dumont, E. Dupont, E. Rhéaume, C. Martel, J. Couët, C. Trudel, Structure and tissue-specific expression of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase genes in human and rat classical and peripheral steroidogenic tissues, J. Steroid Biochem. Mol. Biol. 41 (1992) 421–435.
[14] S.A. Kamath, E. Rubin, Interaction of calcium with microsomes: a modified method for the rapid isolation of rat liver microsomes, Biochem. Biophys. Res. Commun. 49 (1972) 52–59.
[15] H. Wang, P.A. Murphy, Isoflavone content in commercial soybean foods, J. Agric. Food Chem. 42 (1994) 1666–1673.
[16] H. Wang, P.A. Murphy, Isoflavone composition of variety, crop year, and location, J. Agric. Food Chem. 42 (1994) 1674–1677.
[17] Y. Nakamura, S. Tsuji, Y. Tonogai, Determination of the levels of isoflavonoids in soybeans and soy-derived foods and estimation of isoflavonoids in the Japanese daily intake, J. AOAC Int. 83 (2000) 635–650.
[18] A. Bylund, J.X. Zhang, A. Bergh, J.E. Damber, A. Widmark, A. Johansson, H. Adlercreutz, P. Aman, M.J. Shepherd, G. Hallmans, Rye bran and soy protein delay growth and increase apoptosis of human LNCaP prostate adenocarcinoma in nude mice, Prostate 42 (2000) 304–314.
[19] Y.H. Choi, W.H. Lee, K.Y. Park, L. Zhang, p53-Independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells, Jpn. J. Cancer Res. 91 (2000) 164–173.
[20] A. Dalu, J.F. Haskell, L. Coward, C.A. Lamartiniere, Genistein, a component of soy, inhibits the expression of the EGF and ErbB2/Neu receptors in the rat dorsolateral prostate, Prostate 37 (1998) 36–43.
[21] M. Landstrom, J.X. Zhang, G. Hallmans, P. Aman, A. Bergh, J.E. Damber, W. Mazur, K. Wahala, H. Adlercreutz, Inhibitory effects of soy and rye diets on the development of Dunning R3327 prostate adenocarcinoma in rats, Prostate 36 (1998) 151–161.
[22] J.X. Zhang, G. Hallmans, M. Landstrom, A. Bergh, J.E. Damber, P. Aman, H. Adlercreutz, Soy and rye diets inhibit the development of Dunning R3327 prostatic adenocarcinoma in rats, Cancer Lett. 114 (1997) 313–314.
[23] T. Akiyama, J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, Y. Fukami, Genistein, a specific inhibitor of tyrosine-specific protein kinases, J. Biol. Chem. 262 (1987) 5592–5595.
[24] K.S. Weber, K.D. Setchell, D.M. Stocco, E.D. Lephart, Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5alpha-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague–Dawley rats, J. Endocrinol. 170 (2001) 591–599.
[25] S. Ohno, Y. Nakajima, K. Inoue, H. Nakazawa, S. Nakajin, Genistein administration decreases serum corticosterone and testosterone levels in rats, Life Sci. 74 (2003) 733–742.
[26] K. North, J. Golding, A maternal vegetarian diet in pregnancy is associated with hypospadias. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood, BJU Int. 85 (2000) 107–113.
[27] W.M. Keung, Biochemical studies of a new class of alcohol dehydrogenase inhibitors from Radix puerariae, Alcohol Clin. Exp. Res. 17 (1993) 1254–1260.
[28] C.K. Wong, W.M. Keung, Bovine adrenal 3(-hydroxysteroid dehydrogenase (E.C. 1.1.1. 145)/5-ene-4-ene isomerase [E.C. 5.3.3.1]: characterization and its inhibition by isoflavones, J. Steroid Biochem. Mol. Biol. 71 (1999) 191–202.
[29] J.C. Le Bail, Y. Champavier, A.J. Chulia, G. Habrioux, Effects of phytoestrogens on aromatase, 3β and 17β-hydroxysteroid dehydrogenase activities and human breast cancer cells, Life Sci. 66 (2000) 1281–1291.
[30] J.L. Thomas, R.P. Myers, R.C. Strickler, Human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5-4-ene-isomerase: purification from mitochondria and kinetic profiles, biophysical characterization of the purified mitochondrial and microsomal enzymes, J. Steroid Biochem. 33 (1989) 209–217.
[31] J.L. Thomas, B.W. Evans, G. Blanco, R.W. Mercer, J.I. Mason, S. Adler, W.E. Nash, K.E. Isenberg, R.C. Strickler, Site-directed mutagenesis identifies amino acid residues associated with the dehydrogenase and isomerase activities of human type I (placental) 3β-hydroxysteroid dehydrogenase/isomerase, J. Steroid Biochem. Mol. Biol. 66 (1998) 327–334.
[32] W.E. Nash, R.W. Mercer, G. Blanco, R.C. Strickler, J.I. Mason, J.L. Thomas, Over-expression of human type I (placental) 3β-hydroxy-5-ene-steroid dehydrogenase/isomerase in insect cells infected with recombinant baculovirus, J. Steroid Biochem. Mol. Biol. 50 (1994) 235–240.
[33] J.L. Thomas, J.I. Mason, S. Brandt, B.R. Spencer Jr., W. Norris, Structure/function relationships responsible for the kinetic differences between human type 1 and type 2 3beta-hydroxysteroid dehydrogenase and for the catalysis of the type 1 activity, J. Biol. Chem. 277 (8) (2002) 42795–42801.
[34] J.L. Thomas, J.I. Mason, G. Blanco, M.L. Veisaga, The engineered, cytosolic form of human type I 3β-hydroxysteroid dehydrogenase/isomerase: purification, characterization and crystallization, J. Mol. Endocrinol. 27 (2001) 77–83.
[35] J.L. Thomas, B.W. Evans, G. Blanco, J.I. Mason, R.C. Strickler, Creation of a fully active, cytosolic form of human type I 3β-hydroxysteroid dehydrogenase/isomerase by the deletion of a membrane-spanning domain, J. Mol. Endocrinol. 23 (1999) 231–239.
[36] H. Adlercreutz, K. Hockerstedt, C. Bannwart, S. Bloigu, E. Hamalainen, T. Fotsis, A. Ollus, Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG), J. Steroid Biochem. 27 (1987) 1135–1144.
[37] H. Jornvall, B. Persson, M. Krook, S. Atrian, R. Gonzalez-Duarte, J. Jeffery, D. Ghosh, Short-chain dehydrogenases/reductases (SDR), Biochemistry 34 (1995) 6003–6013.
[38] T. Tanabe, N. Tanaka, K. Uchikawa, T. Kabashima, K. Ito, T. Nonaka, Y. Mitsui, M. Tsuru, T. Yoshimoto, Roles of the Ser146, Tyr159, and Lys163 residues in the catalytic action of 7α-hydroxysteroid dehydrogenase from *Escherichia coli*, J. Biochem. Tokyo 124 (1998) 634–641.