Structure–activity relationships for inhibition of human 5α-reductases by polyphenols

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Abstract

The enzyme steroid 5α-reductase (EC 1.3.99.5) catalyzes the NADPH-dependent reduction of the double bond of a variety of 3-oxo-Δ^4 steroids including the conversion of testosterone to 5α-dihydrotestosterone. In humans, 5α-reductase activity is critical for certain aspects of male sexual differentiation, and may be involved in the development of benign prostatic hyperplasia, alopecia, hirsutism, and prostate cancer. Certain natural products contain components that are inhibitors of 5α-reductase, such as the green tea catechin (−)-epigallocatechin gallate (EGCG). EGCG shows potent inhibition in cell-free but not in whole-cell assays of 5α-reductase. Replacement of the gallate ester in EGCG with long-chain fatty acids produced potent 5α-reductase inhibitors that were active in both cell-free and whole-cell assay systems. Other flavonoids that were potent inhibitors of the type 1 5α-reductase include myricetin, quercitin, baicalein, and fisetin. Biochanin A, daidzein, genistein, and kaempferol were much better inhibitors of the type 2 than the type 1 isozyme. Several other natural and synthetic polyphenolic compounds were more effective inhibitors of the type 1 than the type 2 isozyme, including alizarin, anthrarobin, gossypol, nordihydroguaiaretic acid, caffeic acid phenethyl ester, and octyl and dodecyl gallates. The presence of a catechol group was characteristic of almost all inhibitors that showed selectivity for the type 1 isozyme of 5α-reductase. Since some of these compounds are consumed as part of the normal diet or in supplements, they have the potential to inhibit 5α-reductase activity, which may be useful for the prevention or treatment of androgen-dependent disorders. However, these compounds also may adversely affect male sexual differentiation. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

The microsomal enzyme steroid 5α-reductase (EC 1.3.99.5) catalyzes the NADPH-dependent reduction of the $\Delta^{4,5}$ double bond of a variety of 3-oxo-Δ^4 steroids [1,2], including the conversion of testosterone to 5α-dihydrotestosterone, a process thought to amplify the androgenic response, perhaps because of the higher affinity of the androgen receptor for 5α-dihydrotestosterone than for testosterone [3]. Two different 5α-reductase isozymes have been characterized in humans, monkeys, rats, and mice [4–7]. The two human isozymes share approximately 50% sequence identity and have different biochemical properties. For example, the type 1 isozyme has a broad basic pH

optimum and low affinity for testosterone ($K_m > 1$ μM), while the type 2 isozyme has an acidic pH optimum and high affinity for testosterone ($K_m < 10$ nM) [8].

Studies of mice with genetically engineered 5α-reductase gene knockouts, as well as investigations of natural 5α-reductase deficiencies in humans, have identified some of the roles this enzyme plays in different biological processes [7]. Female mice deficient in the type 1 5α-reductase have impaired cervical ripening, leading to defective parturition [9,10]. This defect may be due to impaired catabolism of cervical progesterone, which is also a substrate for 5α-reductase. Uterine type 1 5α-reductase is also important for fetal viability, because 5α-reductase activity limits fetal exposure to toxic levels of 17β-estradiol by competing for substrate with aromatase, which catalyzes the synthesis of 17β-estradiol from testosterone [11]. In humans, activity of the type 2 isozyme is critical for differentiation of the prostate and male external genitalia [4,12,13]. Based on studies of individuals with

inherited deficiencies in 5α-reductase activity as well as laboratory studies of affected tissues, 5α-reductase may also have a role in the development of a variety of human disorders including benign prostatic hyperplasia [14], acne [15], alopecia [16,17], and hirsutism [18]. 5α-Reductase also has been proposed to have a role in the development of prostate cancer and possibly may be responsible for differences in prostate cancer mortality among different racial groups [19]. A common missense mutation (V89L) that decreases the activity of the type 2 5α-reductase, is common among Asians, a group that has a lower mortality from prostate cancer compared with African–American men and non-Hispanic whites [20]. Finasteride, a synthetic 5α-reductase inhibitor, is currently used to treat benign prostatic hyperplasia [21] and alopecia [22], and it is also being studied in clinical trials as a chemopreventative for prostate cancer [23].

Diet has an important role in modulating cancer incidence and mortality, and differences in diet may explain geographical differences in prostate cancer mortality [24,25]. Since androgens regulate the growth and function of the normal prostate and prostate cancer [26,27], dietary components capable of altering this growth signaling pathway in the prostate may affect prostate cancer development and progression. We have shown that green tea contains phytochemicals called catechins that are inhibitors of 5α-reductase [28]. It is not known whether green tea catechins modulate androgenic activity *in vivo* in humans, but rats injected with the green tea catechin EGCG have a variety of endocrine changes, including lower serum testosterone concentrations and smaller prostates than the controls [29]. Other natural product inhibitors of 5α-reductase that have been identified include polyunsaturated fatty acids, such as γ-linolenic acid [30]; the macrocyclic ellagitannins, oenothein A and B [31,32]; the flavonoids and lignans, genistein, formononentin, biochanin A, daidzein, coumestrol, equol, and enterolactone [33]; the bisnaphthoquinone, impatienol [34]; and the isoprenylated flavone and stilbene, artocarpin and chlorophorin [35]. Since many of these inhibitors of 5α-reductase are polyphenols, we have investigated the ability of a variety of natural and synthetic polyphenols to inhibit 5α-reductase to determine what structural elements are important for potent inhibition of 5α-reductase by this class of compounds in both the cell-free and whole-cell assay systems.

2. Materials and methods

2.1. Materials

[4-^{14}C]-Testosterone (50–60 mCi/mmol) was a product of Perkin-Elmer Life Sciences. Purified catechins, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and EGCG, were isolated from green tea (*Camellia sinensis*) in our laboratory as described [28].

Other chemicals either were purchased from Sigma or Aldrich or were synthesized in our laboratory as described below. Caffeic acid phenethyl ester was synthesized as described [36]. A variety of semi-synthetic derivatives of the green tea catechin EGC were synthesized by esterifying various aromatic and aliphatic acids to the 3-hydroxy group of EGC (Fig. 1). EGC derivatives were synthesized by acetylating all six hydroxyl groups of EGC using acetic anhydride in pyridine, followed by selective deacetylation in Tris buffer at pH 8.2 to give the 3-monoacetate (**2**). Silylation of the phenolic hydroxyls and subsequent deacetylation afforded pentasilylated epigallocatechin (**4**) [37]. Aryl esters (**5**) of EGC were prepared by transesterification of the 3-hydroxyl with the appropriate methyl esters [38]. Aliphatic esters (**7**) of EGC were prepared by reaction with the requisite acids in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine [39]. Silyl protective groups were then removed from **5** and **7** with triethylamine trihydrofluoride, providing the EGC analogs. All compounds synthesized in our laboratory were purified by silica gel chromatography and were characterized by ^{1}H-NMR. Spectroscopic data were consistent with their structure, e.g. EGC-myristoleate in CD$_3$OD: 6.42 (2H, s, H$_{arom}$), 5.92 (1H, d, $J = 2.28$ Hz, H$_{arom}$), 5.88 (1H, d, $J = 2.28$ Hz, H$_{arom}$), 5.36–5.28 (3H, m, CH=CH, H3), 4.92 (1H, s, H2), 2.82 (2H, t, $J = 7.48$ Hz, CH$_2$CO), 2.00 (4H, m, CH$_2$–C=C–CH$_2$), 1.50–1.0 (14H, m, CH$_2$), 0.90 (3H, t, $J = 7.1$ Hz, CH$_3$) ppm.

2.2. Expression of human 5α-reductase isozymes and preparation of cell extracts

Cell lines expressing the different types of human 5α-reductase were prepared as described previously [28]. In brief, cDNAs for the human types 1 and 2 5α-reductases were isolated from human prostate cDNA libraries and subcloned into the retroviral expression vector pMV7 [40], and high titer stocks of viruses containing the types 1 and 2 5α-reductase cDNAs were generated using the packaging cells BOSC 23 293 [41]. Rat 1A cells [42,43] were infected with retrovirus, and cells containing integrated retrovirus were selected for resistance to G418 [44]. Cells expressing either the type 1 or 2 human 5α-reductase were expanded under G418 selection and, when confluent, were removed from plates by trypsinization. Cells were collected by centrifugation (500 *g* for 10 min at 22°), washed once with culture medium containing 10% fetal bovine serum, quickly frozen on dry ice, and stored at −90° until used in the preparation of a microsomal fraction containing 5α-reductase.

Rat 1A cells were chosen for expression of 5α-reductase based on their low background of 5α-reductase activity (1.8 pmol/min/10^6 cells) compared with a variety of other cell types that were tested. 5α-Reductase activity (type 1 or 2) in cells transduced with recombinant retroviruses containing 5α-reductase cDNA was 28-fold greater (52 pmol/
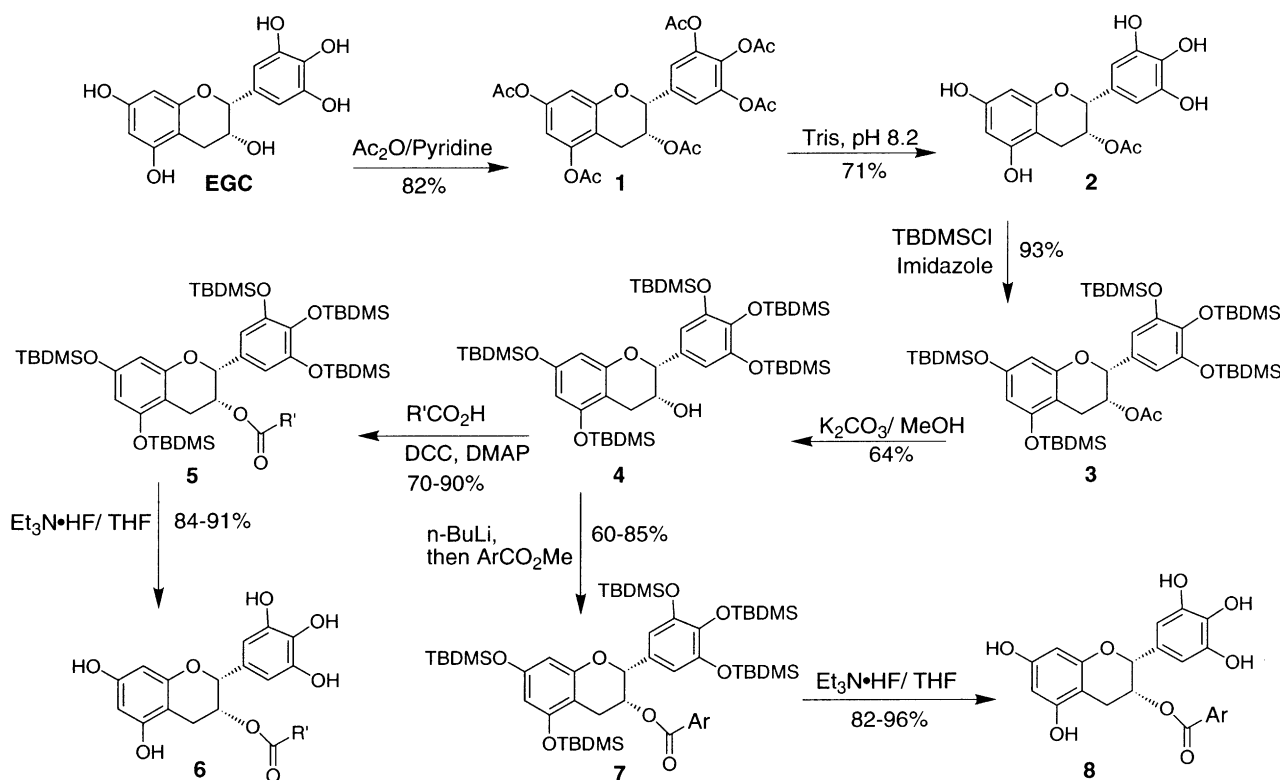
Fig. 1. Synthesis scheme for various aromatic and aliphatic ester derivatives of EGC. Ac: acetate; TBDMS: *tert*-butyldimethylsilane; TBDMSCl: tertiary-butyldimethylsilyl chloride; DCC: dicyclohexylcarbodiimide; DMAP: dimethylpyridine; THF: tetrahydrofuran; R′: aliphatic group; Ar: aromatic group.

min/10^6 cells) than in cells transduced with control virus lacking 5α-reductase cDNA. Since the activity of the recombinant human 5α-reductase activity was so much greater than endogenous rat 5α-reductase, our results should reflect characteristics of the exogenous human and not the endogenous rat enzyme.

For the preparation of microsomes containing 5α-reductase, cells were thawed on ice and then homogenized in 10 vol. of 0.32 M sucrose containing 20 mM potassium phosphate, pH 7, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 1 μg leupeptin/mL, using a Dounce homogenizer and then sonication. The homogenate was centrifuged at 1000 g for 10 min at 4° and the pellet was discarded. The supernatant was centrifuged at 100,000 g for 60 min at 4°, and the resulting pellet (microsomal fraction) was resuspended at 10–20 mg protein/mL in 0.32 M sucrose, 20 mM potassium phosphate, pH 7, 1 mM EDTA, using a Dounce homogenizer. This microsomal fraction was frozen on dry ice and stored at −90° until used in assays of 5α-reductase. We assayed 5α-reductase in various cellular fractions after differential centrifugation of cell extracts. Of the total 5α-reductase activity in cell extracts, 70–80% was present in the microsomal fraction, 10–20% in the nuclear fraction (1000 g pellet), and 2–6% in the cytosolic fraction. 5α-Reductase activity in microsomes isolated from cells transduced with virus containing 5α-reductase cDNA was 31-fold (350 pmol/min per mg of protein; type 1) and 19-fold

(217 pmol/min per mg of protein; type 2) greater than activity (11.5 pmol/min per mg of protein) in microsomes from cells transduced with a control virus.

2.3. Assay of 5α-reductase

The cell-free assay was based on the measurement of 5α-dihydrotestosterone production from testosterone in the presence of microsomes prepared from cells containing either the type 1 or 2 human 5α-reductase. The assay mixture, in a final volume of 0.25 mL, contained 3 μM [4-^{14}C]-testosterone, 0.1 mM NADPH, 100 mM potassium phosphate, pH 7.0. Test compounds were prepared in water or dimethyl sulfoxide, and 2.5 μL of appropriate dilutions was added to a reaction mix prior to the addition of enzyme. Controls contained similar concentrations of solvents. The reaction was started by the addition of 25 μL of microsomes containing 25 μg of protein to 225 μL of an assay mixture at 37°. The mixture was incubated at 37° for 1 hr, and the reaction was stopped by the addition of 0.5 mL of ethyl acetate and mixing for 1 min. After centrifugation (10,000 g for 5 min at 22°), the organic phase was removed, dried under vacuum, dissolved in 25 μL of ethyl acetate and applied to a silica gel 60 TLC plate, which was developed in a solvent system consisting of methylene chloride:ethyl acetate:methanol (85:15:3). Conversion of testosterone to 5α-reduced metabolites was measured by scanning the TLC plate on an

AMBIS radioanalytical scanner or scanning a phosphor screen exposed to the TLC plate on a Storm 860 phosphoimager. 5α-Dihydrotestosterone was the predominant metabolite (>95%), with little or no conversion of testosterone to androstanediols, androstandione, 4-androstenedione, or other metabolites, and recovery of radioactivity was from 90 to 95%.

Inhibition of 5α-reductase activity was also measured in intact cells expressing either human type 1 or 2 5α-reductase. Cells were plated at 50,000 per well in a 24-well plate in 1 mL of Dulbecco's modified Eagle's medium (DMEM) containing 50 U penicillin/mL, 50 μg streptomycin/mL, and 10% fetal bovine serum and then placed in an incubator with 5% CO_2 for 18 hr at 37°. The medium was then changed to 0.5 mL of serum-free DMEM, and 5 μL of test compound in water or dimethyl sulfoxide was added. Cells were incubated for 1 hr at 37° before the addition of [4-^{14}C]-testosterone, at a final concentration of 1.5 μM. Then cells were incubated for 3 hr at 37°, the medium was removed, and radioactive steroids were extracted with ethyl acetate. The amounts of labeled testosterone and 5α-dihydrotestosterone in extracts were determined by TLC and scanning the TLC plate as described above. The concentration of test compound inhibiting the conversion of testosterone to 5α-dihydrotestosterone by 50% (IC_{50}) was determined by interpolation between appropriate

data points. 5α-Dihydrotestosterone was the predominant metabolite (>95%), with little or no conversion of testosterone to androstanediols, androstandione, 4-androstenedione, or other metabolites, and recovery of radioactivity was from 85 to 90%.

2.4. HPLC analysis

The stability of EGCG at 37° in cell culture medium (DMEM) equilibrated with 5% CO_2 (pH 7.4–7.5) or in 100 mM potassium phosphate buffer, pH 7.0, medium or buffers that were used for whole-cell and cell-free assays of 5α-reductase, respectively, was determined by monitoring EGCG concentrations by HPLC using a Novapak (Waters) C18 column (3.9 mm × 150 mm) and isocratic elution at 40° at a flow rate of 1 mL/min with acetonitrile:ethyl acetate:0.05% phosphoric acid in water (12:2:86). EGCG was detected by monitoring the absorbance at 275 nm.

3. Results

The structures of various compounds investigated in this report are shown in Figs. 2 and 3. A comparison of the abilities of the four major green tea catechins, EC, EGC, ECG, and EGCG (Fig. 2), to inhibit the types 1 and 2
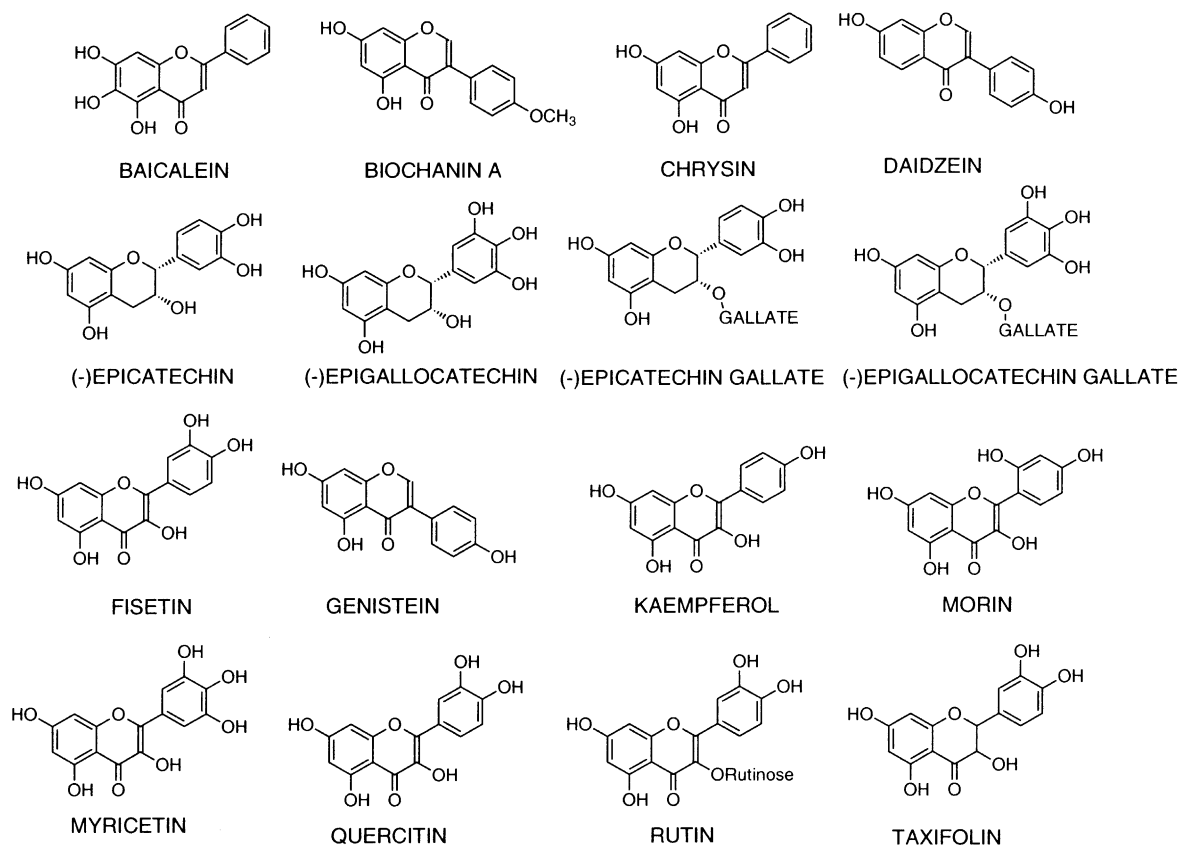

Fig. 2. Structures of flavonoids tested for inhibitory activity against 5α-reductase.
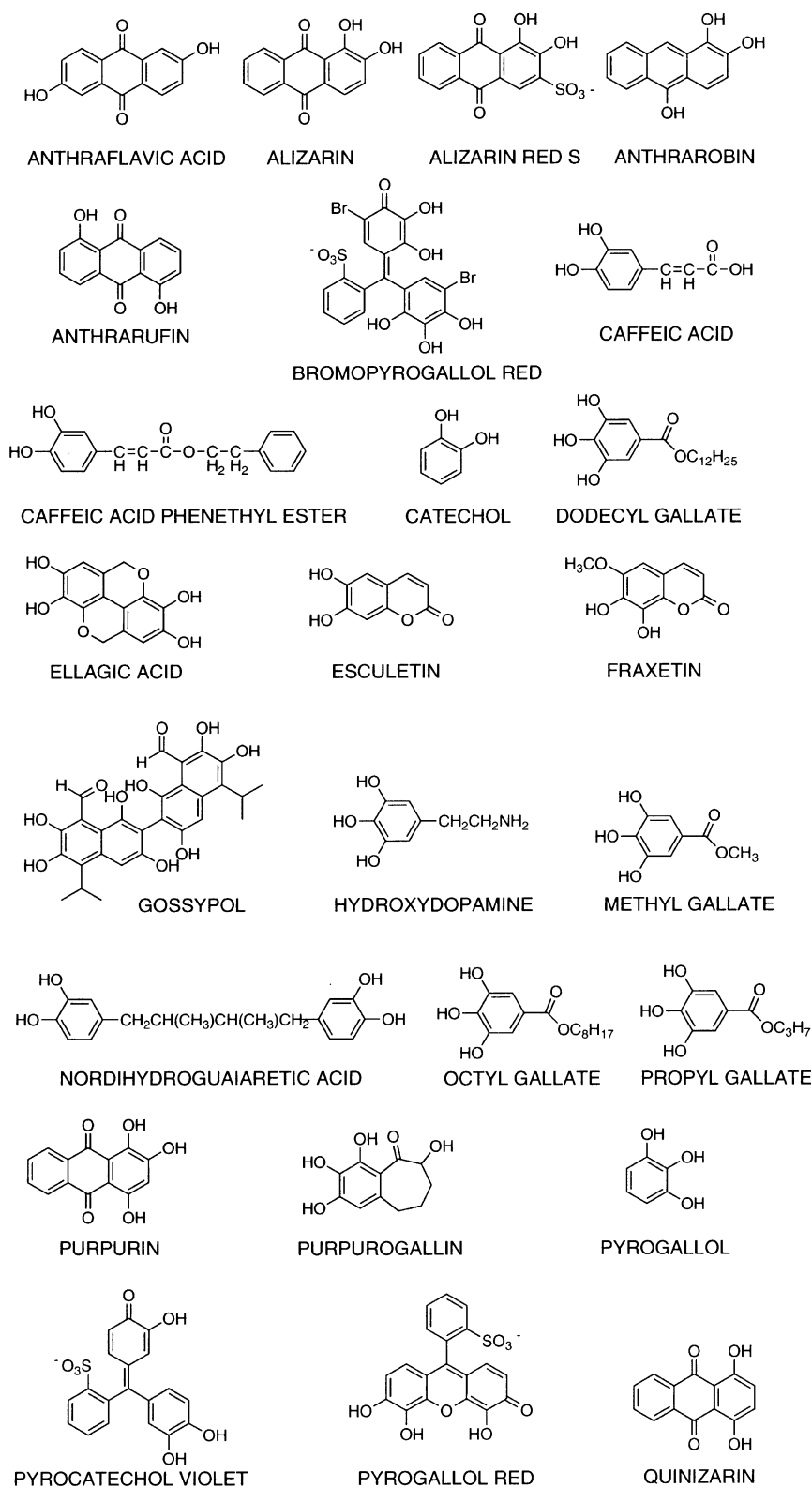
Fig. 3. Structures of polyphenols tested for inhibitory activity against 5α-reductase.

isozymes of 5α-reductase in cell-free and whole-cell assays is presented in Table 1. Using a cell-free assay, ECG and EGCG were better inhibitors of 5α-reductase than were EC and EGC, and the type 1 isozyme was more sensitive to these inhibitors than the type 2 isozyme. Since ECG and EGCG only differ structurally from EC and EGC by the presence of a gallic acid ester on the 3-hydroxyl, the gallate group is important for the enhanced ability of ECG

Table 1
Inhibition of 5α-reductase isozymes by green tea catechins[a]

Catechin	5α-Reductase			
	Cell-free assay IC_{50} (μM)		Whole-cell assay IC_{50} (μM)	
	Type 1	Type 2	Type 1	Type 2
EC	>100 (14)	>100 (4)	>100 (0)	>100 (1)
EGC	>100 (15)	>100 (3)	>100 (15)	>100 (1)
ECG	11 (100)	69 (83)	>100 (0)	>100 (0)
EGCG	15 (99)	74 (74)	>100 (6)	>100 (0)

[a] IC_{50}: concentration (μM) of compound producing 50% inhibition of 5α-reductase activity. Values in parentheses are percent inhibition of 5α-reductase activity in the presence of 100 μM concentration of the indicated compound.

and EGCG to inhibit 5α-reductase. These green tea catechins had little inhibitory activity against 5α-reductase in whole cells. The lack of activity in whole cells may be due to an inability of these catechins to cross the cell membrane or to enzymatic or non-enzymatic changes in the structure of these catechins in assays using whole-cell cultures. The stability of EGCG in culture medium may be responsible, in large part, for the lower activity of EGCG in the cell culture assay, since the half-life of EGCG in culture medium and phosphate buffer used for whole-cell and cell-free 5α-reductase assays was 9.5 ± 0.5 and 74.7 ± 6.4 min (mean ± SEM, $N = 3$), respectively. The stability of EGCG in aqueous solution is highly dependent on pH [45,46], and the difference in pH between culture medium (pH 7.5) and the phosphate buffer for cell-free assays (pH 7.0) may be responsible, in part, for this 8-fold difference in stability. The half-life of EGCG in phosphate buffered saline, pH 7.5, was determined to be 21.2 ± 2.0 min.

Certain flavonoids, including EGCG, produce hydrogen peroxide in aqueous solutions at physiological pH, possibly through a superoxide intermediate [47,48]. To determine if these reactive oxygen species may have some role in inhibition of 5α-reductase by EGCG, we added catalase (25–250 μg/mL) or superoxide dismutase (0.5–5 μg/mL) to assay mixtures containing EGCG. However, addition of these enzymes did not affect inhibition of 5α-reductase type 1 or 2 by 20 or 100 μM EGCG. Therefore, peroxide and superoxide do not appear to be responsible for inhibition of 5α-reductase by EGCG.

We performed a kinetic analysis of the inhibition of type 1 5α-reductase by EGCG using the cell-free assay to determine the mode of inhibition of EGCG [49]. EGCG was a competitive inhibitor of the substrate NADPH and a non-competitive inhibitor of the substrate testosterone based on double-reciprocal plots of the kinetic data (Fig. 4).

To determine what structural features of the gallate group of EGCG were important for inhibitory activity against 5α-reductase, and to determine whether structural changes in or replacement of the gallate group could enhance inhibitory activity in whole cells, a series of
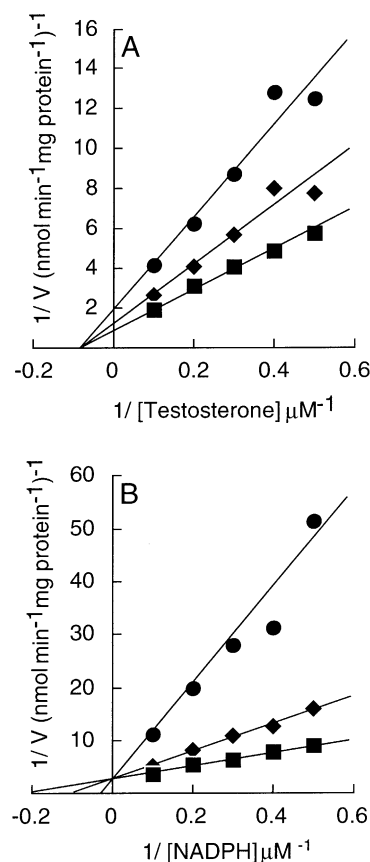

Fig. 4. Kinetic analysis of the inhibition of type 1 5α-reductase by EGCG. Initial reaction velocities (*V*) were determined for different substrate (panel A, testosterone; panel B, NADPH) concentrations and as a function of EGCG concentration: 0 μM (■), 5 μM (◆), and 10 μM (●) in panel A and 0 μM (■), 20 μM (◆), and 30 μM (●) in panel B.

EGC derivatives was synthesized and tested using the cell-free and whole-cell assays (Table 2). Modification of the hydroxyl groups of the gallate ester by methylation or replacement of gallic acid with various aromatic groups without phenolic groups did not improve inhibitory activity in either the cell-free or whole-cell assay. The most significant structural change leading to enhanced activity in the whole-cell assay was introduction of an aliphatic acid ester in place of the gallic acid ester of EGCG. EGC derivatives with long-chain aliphatic acids were better inhibitors than derivatives with short-chain aliphatic acids, and derivatives with aliphatic acids with some degree of unsaturation were better inhibitors than EGC derivatives esterified with saturated aliphatic acids. EGC esterified to either γ-linolenic or myristoleic acid were potent inhibitors of both 5α-reductases in whole cells with IC_{50} values of less than 15 μM. We have reported previously that certain polyunsaturated fatty acids, such as γ-linolenic acid, are inhibitors (IC_{50}: 5–10 μM) of 5α-reductase [30]. Methyl and cholesterol esters of γ-linolenic acid were not potent inhibitors of 5α-reductase ($IC_{50} > 100$ μM) in cell-free and whole-cell assays (data not shown). Therefore, it is likely that the enhanced inhibitory activity of EGC esterified to

Table 2
Inhibition of 5α-reductase by various density of EGC[a]

Group esterified to the 3-hydroxyl of EGC	5α-Reductase			
	Cell-free assay IC_{50} (μM)		Whole-cell assay IC_{50} (μM)	
	Type 1	Type 2	Type 1	Type 2
H (EGC)	62 (61)	>100 (30)	>100 (15)	>100 (1)
Gallate (EGCG)	12 (99)	73 (76)	>100 (11)	>100 (5)
3,4,5-Trimethoxybenzoate	31 (94)	>100 (20)	>100 (12)	>100 (7)
3,5-Dihydroxy-4-methylbenzoate	29 (93)	99 (51)	ND[b]	ND
4-Hydroxy-3,5-dimethoxybenzoate	29 (97)	>100 (21)	43 (83)	62 (72)
Benzoate	48 (85)	>100 (24)	ND	ND
Acetate (C2:0)	>100 (35)	>100 (8)	>100 (8)	>100 (9)
Caproate (C6:0)	47 (90)	>100 (39)	>100 (10)	>100 (0)
Caprylate (C8:0)	30 (98)	78 (74)	58 (89)	72 (83)
Myristate (C14:0)	59 (95)	71 (84)	28 (97)	32 (98)
Myristoleate (C14:1)	20 (99)	76 (96)	7 (99)	8 (98)
Stearate (C18:0)	>100 (31)	>100 (0)	42 (90)	74 (81)
γ-Linolenate (C18:3)	25 (97)	63 (93)	8 (99)	14 (98)

[a] IC_{50}: concentration (μM) of compound producing 50% inhibition of 5α-reductase activity. Values in parentheses are percent inhibition of 5α-reductase activity in the presence of 100 μM concentration of the indicated compound. EGC and EGCG are presented for comparison and are the natural compounds isolated from green tea.

[b] ND: not determined.

γ-linolenic acid is due to the combined functionality of this derivative and not simply due to hydrolysis of the ester bond and release of free γ-linolenic acid. Also, cellular morphology, as determined by light microscopy, was not altered when cells were incubated with EGC derivatives containing γ-linolenic or myristoleic acid esters; therefore, inhibition of 5α-reductase in whole cells was not due to gross changes in cell integrity.

To determine what other structural attributes were important for inhibition of 5α-reductase by polyphenolic compounds, we tested a variety of natural and synthetic polyphenols for their ability to inhibit 5α-reductase isozymes in both the cell-free and whole-cell assays. Several naturally occurring flavonoids with structures related to the tea catechins were tested (Fig. 2, Table 3). Four flavonoids, myricetin, quercitin, baicalein, and fisetin, had marked ($IC_{50} < 100$ μM) activity and were more active against the type 1 than the type 2 isozyme. The number and position of B-ring hydroxyl groups appear to be important for inhibitory activity against the type 1 5α-reductase. The flavonols quercitin, myricetin, and fisetin, with a catechol or pyrogallol configuration in the B-ring (Fig. 2), had greater inhibitory activity against the type 1 isozyme than the flavonols chrysin, kaempferol, and morin that lack hydroxyls in a catechol or pyrogallol configuration (Table 3). A comparison of the structures and inhibitory activities of the flavanols EC and EGC and the flavonols myricetin and quercitin highlights the importance of a 2,3-double bond and a 4-keto group in the C-ring for enhanced inhibitory activity. In contrast to quercitin, rutin, the 3-rutinose glycoside of quercitin, was ineffective against either isozyme ($IC_{50} > 100$ μM). The inactivity of rutin compared with quercitin may be due to the presence of the bulky oligosaccharide rutinose causing steric hinderance or to modification of the 3-hydroxy group. Taxifolin, a flavanone that is structurally similar to quercitin but lacking the 2,3-double bond in the C-ring, was ineffective against either isozyme ($IC_{50} > 100$ μM). Biochanin A, kaempferol, genistein, and daidzein were more effective inhibitors of the type 2 than the type 1 isozyme. With the exception of kaempferol, a flavonol with a single B-ring hydroxyl, these type 2 inhibitors are isoflavones with single hydroxyls on the B-ring. The inhibitory effects of biochanin A, genistein, and daidzein on 5α-reductase have been reported previously [33]. When tested for inhibitory activity in whole cells, most flavonoids showed little or no

Table 3
Inhibition of 5α-reductase isozymes by various natural polyphenols[a]

Polyphenol	5α-Reductase			
	Cell-free assay IC_{50} (μM)		Whole-cell assay IC_{50} (μM)	
	Type 1	Type 2	Type 1	Type 2
Myricetin	23 (96)	>100 (31)	>100 (11)	>100 (11)
Quercitin	23 (96)	>400 (14)	>100 (15)	>100 (29)
Baicalein	29 (79)	99 (51)	>100 (24)	>100 (4)
Fisetin	57 (97)	>100 (4)	>100 (42)	>400 (27)
Biochanin A	100 (50)	17 (74)	64 (64)	5 (93)
Daidzein	>100 (3)	29 (69)	>100 (13)	7 (89)
Kaempferol	>100 (22)	12 (62)	79 (60)	20 (85)
Genistein	>100 (16)	23 (76)	>100 (22)	20 (89)
Morin	>100 (6)	>100 (33)	ND[b]	ND
Taxifolin	>100 (5)	>100 (5)	ND	ND
Chrysin	>100 (2)	>100 (1)	ND	ND
Rutin	>100 (4)	>100 (0)	ND	ND

[a] IC_{50}: concentration (μM) of compound producing 50% inhibition of 5α-reductase activity. Values in parentheses are percent inhibition of 5α-reductase activity in the presence of 100 μM concentration of the indicated compound.

[b] ND: not determined.

Table 4
Inhibition of 5α-reductase isozymes by compounds containing catechols[a]

Catechol	5α-Reductase			
	Cell-free assay IC_{50} (μM)		Whole-cell assay IC_{50} (μM)	
	Type 1	Type 2	Type 1	Type 2
Anthrarobin	4 (99)	50 (97)	6 (91)	>100 (31)
Bromopyrogallol red	7 (98)	84 (58)	ND[b]	ND
Gossypol	7 (99)	21(99)	7 (100)	6 (99)
Pyrogallol red	15 (97)	>100 (27)	ND	ND
Nordihydroguaiaretic acid	19 (99)	50 (80)	19 (99)	22 (99)
Caffeic acid phenethyl ester	25 (97)	>100 (36)	8 (99)	7 (98)
Octyl gallate	27 (99)	58 (90)	7 (99)	18 (94)
Purpurogallin	30 (81)	>100 (31)	ND	ND
Hydroxydopamine	42 (69)	>100 (41)	ND	ND
Dodecyl gallate	43 (88)	>100 (36)	3 (99)	7 (98)
Pyrocatechol violet	48 (85)	>100 (47)	ND	ND
Pyrogallol	70 (60)	>100 (28)	>100 (7)	>100 (15)
Caffeic acid	>100 (13)	>100 (8)	ND	ND
Esculetin	>100 (7)	>100 (13)	ND	ND
Ellagic acid	>100 (7)	>100 (9)	ND	ND
Catechol	>100 (5)	>100 (0)	>100 (9)	>100 (3)
Methyl gallate	>100 (5)	>100 (3)	>100 (0)	>100 (0)
Propyl gallate	>100 (0)	>100 (0)	>100 (5)	>100 (0)
Fraxetin	<100 (2)	>100 (2)	ND	ND

[a] IC_{50}: concentration (μM) of compound producing 50% inhibition of 5α-reductase activity. Values in parentheses are percent inhibition of 5α-reductase activity in the presence of 100 μM concentration of the indicated compound.

[b] ND: not determined.

activity against the type 1 isoenzyme, perhaps indicating limited penetration of these polyhydroxy compounds across the cell membrane or enzymatic or non-enzymatic changes in the structure of these compounds in assays using whole-cell cultures. In contrast to the results with the type 1 enzyme, four flavonoids, biochanin A, daidzein, genistein, and kaempferol, had significant inhibitory activities against the type 2 isozyme in the whole-cell assay. The most active of these, biochanin A and daidzein, have only two and three free hydroxyl groups, respectively. These flavonoids may be active in whole cells because they may penetrate cells easier than other flavonoids that have more hydroxyl groups. These flavonoids also may be less susceptible to modification in cell cultures.

Since flavonoids that had catechol moieties were potent inhibitors of the type 1 5α-reductase, a variety of naturally occurring and synthetic compounds that have catechol groups as part of their structure were tested for inhibitory activity against 5α-reductase (Tables 4 and 5, Fig. 3). Sixteen compounds had IC_{50} values below 100 μM and five compounds had IC_{50} values below 10 μM. All were more active against the type 1 than the type 2 isozyme. Several of these compounds, including alizarin, anthrarobin, dodecyl gallate, gossypol, octyl gallate, caffeic acid phenethyl ester, and nordihydroguaiaretic acid also were inhibitors of 5α-reductase in whole-cell assays with IC_{50} values of less than 20 μM. In the whole-cell assay, anthrarobin and alizarin were much more effective against the type 1 than the type 2 isozyme, whereas the other five inhibitors were equally effective inhibitors of both iso-

zymes. Alizarin (1,2-dihydroxyanthraquinone) and purpurin (1,2,4-trihydroxyanthraquinone), which contain a catechol group on an anthraquinone backbone, were potent inhibitors of 5α-reductase. The structural isomers of alizarin, anthraflavic acid (2,6-dihydroxyanthraquinone), anthrarufin (1,5-dihydroxyanthraquinone), and quinizarin (1,4-dihydroxyanthraquinone), which do not have catechols in their structure, were weak inhibitors, highlighting again the importance of catechols for inhibitory activity against 5α-reductase. The *p*-quinone structure found in alizarin may not be necessary for inhibitory activity, since anthrarobin (1,2,10-anthracenetriol) had inhibitory activity

Table 5
Inhibition of 5α-reductase isozymes by hydroxyanthraquinones[a]

Anthraquinone	5α-Reductase			
	Cell-free assay IC_{50} (μM)		Whole-cell assay IC_{50} (μM)	
	Type 1	Type 2	Type 1	Type 2
Purpurin	2 (95)	>100 (20)	ND[b]	ND
Alizarin	3 (95)	>100 (54)	6 (75)	>100 (27)
Alizarin red S	30 (91)	>100 (8)	>100 (22)	>100 (1)
Anthrarufin	40 (67)	>100 (13)	ND	ND
Anthraflavic acid	>100 (27)	>100 (22)	ND	ND
Ouinizarin	>100 (26)	>100 (7)	ND	ND

[a] IC_{50}: concentration (μM) of compound producing 50% inhibition of 5α-reductase activity. Values in parentheses are percent inhibition of 5α-reductase activity in the presence of 100 μM concentration of the indicated compound.

[b] ND: not determined.

equivalent to that of alizarin (Tables 4 and 5). Alizarin red S, which is structurally similar to alizarin, but has an additional 3-sulfate group adjacent to the catechol, was 10–20 times less potent than alizarin (Table 5). The charged sulfate group on alizarin red S may interfere with binding to 5α-reductase, a hydrophobic enzyme, as well as interfere with the transport of this class of molecule across the cell membrane. The difference in the activities of caffeic acid ($IC_{50} > 100\,\mu M$) and caffeic acid phenethyl ester ($IC_{50} = 25\,\mu M$) (Table 4) may have a similar explanation. Gossypol, a potent inhibitor in both whole-cell and cell-free assays, contains two catechol moieties. Both of these groups could be contributing to the inhibitory activity of this compound. The methyl and propyl esters of gallic acid were much less potent inhibitors of 5α-reductase than the octyl and dodecyl esters. The latter are more hydrophobic than the former and may interact more readily with microsomal 5α-reductase because of their hydrophobic nature. Dodecyl and octyl gallate were more potent inhibitors in the whole-cell than in the cell-free assay (Table 4). The long fatty acid esters on dodecyl and octyl gallate may enhance uptake of these compounds in whole cells, and these compounds may concentrate in cell membranes leading to inhibition of 5α-reductase. Hydroxydopamine (3,4,5-trihydroxyphenethylamine), which is structurally similar to the short-chain esters of gallic acid, but is positively charged at physiological pH, had inhibitory activity in the cell-free assay that was similar in potency to that of dodecyl gallate. The compounds catechol (1, 2-dihydroxybenzene), pyrogallol (1,2,3-trihydroxybenzene), and gallic acid (3,4,5-trihydroxybenzoic acid), which have catechols in their structure, had weak inhibitory activity in both cell-free and whole-cell assay systems. Three dyes, bromopyrogallol red (5′,5″-dibromopyrogallolsulfonephthalein), pyrocatechol violet (pyrocatecholsulfonephthalein), and pyrogallol red (pyrogallolsulfonephthalein), each containing catechol groups, were potent inhibitors of 5α-reductase in the cell-free assay. In contrast to alirazin red S, all three of these dyes are effective inhibitors, even though they contain a charged sulfate group. This difference in inhibitory potency may be because the sulfate in alirazin red S is adjacent to the catechol group, while the sulfate group in the other dyes that are active inhibitors is located some distance from the catechol group. Three naturally occurring catechol-containing compounds, ellagic acid, a condensation product of two gallic acid molecules, and the coumarins, esculetin (6,7-dihydroxycoumarin) and fraxetin (7,8-dihydroxy-6-methoxycoumarin), had little activity ($IC_{50} > 100\,\mu M$) in the cell-free assay.

4. Discussion

We performed this structure–activity relationship study of 5α-reductase inhibitors in an effort to provide some insight into the mechanism by which EGCG, a green tea polyphenol, inhibits 5α-reductase, as well as to identify other natural product inhibitors of this enzyme. Inhibition studies were conducted using a cell-free assay, as well as assays with intact cells. The latter assay may provide some estimate of the potential of a particular compound to inhibit 5α-reductase activity *in vivo*. This study identified several natural products that were inhibitors of 5α-reductase. Since some of these compounds were effective on whole cells, they may be capable of modulating the activity of 5α-reductase *in vivo*. Activity *in vivo* will ultimately be dependent upon attaining pharmacologically active levels of these agents in target tissues, which will depend on absorption, distribution, metabolism, and excretion of the agents.

There is the possibility that diets containing natural inhibitors of 5α-reductase may adversely affect processes dependent upon 5α-reductase activity, such as male sexual differentiation. Mutations in the type 2 5α-reductase gene have been linked to isolated cases of hypospadias, a common developmental abnormality of the male reproductive tract [50]. It also has been reported that a maternal vegetarian diet during pregnancy is associated with hypospadias [51]. The authors of this study speculated that phytoestrogens in the maternal diet may have a deleterious effect on the developing male reproductive system. It also seems possible that natural or synthetic dietary components that are potent inhibitors of 5α-reductase may have adverse effects on male sexual development when consumed in high levels at critical periods during pregnancy.

A variety of natural and synthetic polyphenolic compounds were found to be inhibitors of 5α-reductase. Many of these compounds were better inhibitors of the type 1 than the type 2 isozyme, while a few inhibited both isozymes equally. Biochanin A, daidzein, genistein, and kaempferol were the only polyphenols tested that were better inhibitors of the type 2 than the type 1 isozyme. The first three compounds are isoflavones, while kaempferol is a flavonol. These compounds are found in a variety of plant dietary products including soybean-based products. Since the type 2 isozyme of 5α-reductase has a critical role in prostate development and is the predominant isozyme present in the adult human prostate [52], diets rich in these particular compounds have the potential to affect the development and function of the prostate by modulating the activity of 5α-reductase. Since excessive 5α-reductase activity has been proposed to be a possible contributing factor in prostate cancer development or progression [19], the development and progression of prostate cancer may also be affected by diets containing inhibitors of 5α-reductase. Certain natural dietary agents may have the ability to act as prostate cancer chemopreventative agents by modulating 5α-reductase activity. If some of these compounds are active *in vivo*, they may be important candidates for prostate cancer chemopreventative agents, either taken in pure or enriched formulations or as components of natural dietary products. Differences in the

intake of dietary inhibitors of 5α-reductase also may be responsible, in part, for geographical and racial differences in prostate cancer mortality.

A consistent observation in this study was that polyphenolic inhibitors of the type 1 5α-reductase had a catechol in their structure. Flavonoids that were better inhibitors of the type 2 than the type 1 5α-reductase, however, did not contain catechols. Although a catechol group was necessary for potent inhibition of the type 1 isozyme by polyphenols, it was not always sufficient. For instance, the natural product ellagic acid contains two catechol groups and was a weak ($IC_{50} > 100 \mu M$) inhibitor of 5α-reductase. The proximity of the catechols in ellagic acid to other molecular groups may have steric effects, and the highly constrained structure of ellagic acid may prevent inter- and intra-molecular interactions necessary for inhibition by catechol-containing compounds.

Inhibition of the type 1 5α-reductase by EGCG, which contains two separate catechol/pyrogallol groups in its structure, was competitive with the substrate NADPH. Therefore, the catechol groups in EGCG may be interacting with amino acid residues important for binding of this cofactor by 5α-reductase. Several studies, based upon characterization of naturally occurring mutants [53,54], site-directed mutagenesis [55–57], and photoaffinity labeling [58] of 5α-reductase, have identified certain amino acid residues that may have a role in substrate and cofactor binding. NADPH-binding is altered by certain amino acid changes in the carboxyl-terminal half of the protein, while substrate (testosterone) or inhibitor (finasteride) binding is affected predominantly by changes in the amino-terminal half of the protein, although some changes in the carboxyl-terminal half also affect binding of substrate. Photoaffinity labeling of the rat type 1 5α-reductase with 2-azido $NADP^+$ modifies a portion of the carboxyl-terminal half of the protein that is conserved among human and rat 5α-reductase isoforms [58]. Since EGCG was a competitive inhibitor of NADPH, it may have inhibited the enzyme by interactions with residues in the carboxyl-terminal portion of the protein. Although the inhibition of 5α-reductase by EGCG was determined to be competitive, we have observed that inhibition of the type 1 5α-reductase by 100 μM EGCG could not be reversed by pelleting microsomes exposed to EGCG and then resuspending them in new reaction buffer without EGCG. EGCG either must be strongly bound to microsomes or must permanently alter 5α-reductase causing irreversible inhibition. Inhibition of 5α-reductase by EGCG also did not increase when microsomes containing the type 1 or 2 5α-reductase were incubated with EGCG for 15–60 min prior to the start of the assay (unpublished observations).

Natural polyphenols, such as EGCG and certain other flavonoids, have been shown to inhibit a variety of enzymes [59,60]. Three properties of these compounds that may be responsible for their biological activity are their ability to form complexes with certain metal ions, their anti-oxidant and pro-oxidant activities, and their ability to form complexes with proteins [59]. Given our current understanding of the mechanism of 5α-reductase, it does not appear likely that metal ion complexation or anti- or pro-oxidant activity would be responsible for inhibition of 5α-reductase by EGCG and other polyphenols. The tea catechins ECG and EGCG will form precipitates with soybean lipoxygenase and yeast alcohol dehydrogenase [61]. We also have observed that EGCG will rapidly precipitate certain proteins, such as chicken egg white lysozyme (unpublished observation). The basis for this precipitation activity has not been defined thoroughly, but it may be due to the ability of certain polyphenols to form both numerous H-bonds with a protein, as well as unselective association of the aromatic nuclei of a polyphenol with certain amino acids, especially prolines [59]. Types 1 and 2 human 5α-reductases contain 14 (5.4%) and 17 (6.7%) proline residues, respectively; hence, these enzymes are not proline-rich proteins. Also, only five of these proline residues are in the carboxyl-terminal half of the protein containing the putative NADPH-binding site.

Catechols can form *o*-quinones, which are known to react covalently with both primary amines and sulfhydryls [62]. There are reports that EGCG [63] and other green tea catechins [64] react covalently with sulfhydryls. Both the type 1 and 2 5α-reductases are inhibited by sulfhydryl modifying agents, such as *N*-ethylmaleimide, 5,5′-dithiobis(2-nitrobenzoic acid), 2,2′-bispyridyldisulfide, *p*-hydroxymercuribenzoate, and mercuric chloride (unpublished observation). However, inclusion of 0.1–10 mM dithiothreitol or β-mercaptoethanol in assays did not prevent inhibition of 5α-reductase by EGCG (unpublished observations). Therefore, it is unlikely that EGCG inhibited 5α-reductase by covalently modifying essential sulfhydryl groups.

Acknowledgments

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