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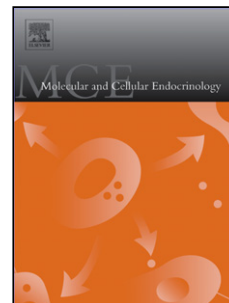
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Flavonoids and cinnamic acid derivatives as inhibitors of 17 β -hydroxysteroid dehydrogenase type 1

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Summary

17 β -Hydroxysteroid dehydrogenase (17 β -HSD) type 1 converts estrone to estradiol, a potent ligand for estrogen receptors. It represents an important target for the development of drugs for treatment of estrogen-dependent diseases. In the present study, we have examined the inhibitory activities of some flavonoids, their biosynthetic precursors (cinnamic acids and coumaric acid), and their derivatives. The proliferative activity of flavonoids on the T-47D estrogen-receptor-positive breast cancer cell line was also evaluated. Among 10 flavonoids, 7,4'-dihydroxyflavone, diosmetin, chrysoeriol, scutellarein, genkwanin and fisetin showed more than 70% inhibition of 17 β -HSD type 1 at 6 μ M. In a series of 18 derivatives of cinnamic acid, the best inhibitor was 4'-cyanophenyl 3,4-methylenedioxycinnamate, with more than 70% inhibition of 17 β -HSD type 1. None of flavonoids affected the proliferation of T-47D breast cancer cells.

Key words: 17 β -hydroxysteroid dehydrogenase type 1; inhibitors; flavonoids; cinnamic acid derivatives

Introduction

17 β -hydroxysteroid dehydrogenase (17 β -HSD) type 1 (EC 1.1.1.62) catalyzes the interconversion of the less active estrogen estrone (E₁) to the potent estradiol (E₂), using NADPH as a cofactor (Miettinen et al., 1996). It is expressed in ovaries, where it affects circulating levels of estradiol, and in peripheral tissue, where it regulates ligand occupancy of estrogen receptors (ERs), and thus acts at the pre-receptor level (Mäentausta et al., 1991; Zhang et al., 1996; Takase et al., 2006). The balance between E₁ and E₂ is also regulated by other reductive 17 β -HSDs (types 7 and 12) that form the potent E₂, and by enzymes with the opposite, oxidative, activity (17 β -HSD types 2, 4 and 14) (Miettinen et al., 1996; Möller and Adamski, 2006; Song et al., 2006; Lukacik et al., 2007). As an increased local concentration of E₂ leads to different pathophysiological conditions, 17 β -HSD type 1 represents an interesting drug target, and its potent inhibitors constitute a class of selective intracrine modulators with potential for the treatment of estrogen-dependent diseases (Brožič et al., 2008).

Flavonoids are plant-derived secondary metabolites that form non-steroidal constituents of our diets. They exert different biological actions in the human body. Among these, they can interfere with the human endocrine system by binding to ERs and key enzymes in estrogen biosynthesis, such as aromatase, sulfatase, sulfotransferases, 3 β -HSDs and 17 β -HSDs (Jacobs and Lewis, 2002; Wuttke et al., 2002). General flavonoid biosynthesis starts from phenylalanine, and proceeds via cinnamic acid and p-coumaric acid to 4-coumaroyl-CoA. Condensation of 4-coumaroyl-CoA and malonyl-CoA gives the intermediate chalcones, the precursors of different flavonoid subgroups, like flavones, flavanones, isoflavones, flavonols, proanthocyanidins and anthocyanidins (Winkley-Shirley, 2001; Treutter, 2005; Miyahisa et al., 2006).

Flavonoids are known inhibitors of the human 17 β -HSD types 1, 2, 3 and 5 (Mäkelä et al., 1995; Le Bail et al., 1998; Mäkelä et al., 1998; Krazeisen et al., 2001; Le Bail et al., 2000; Le Lain et al., 2001; Krazeisen et al., 2002; Poirier, 2003; Brožič et al., 2008). Inhibitory effects of cinnamic acids and coumaric acids and their derivatives have till now been evaluated against 17 β -HSD type 5 (Brožič et al., 2006) and inhibitory effect of chalcones was shown against 17 β -HSD type 1 (Le Bail et al., 2001). In this study, we have evaluated the inhibitory activities of 10 flavonoids and a series of related cinnamic acid derivatives that have not been tested for inhibition of human recombinant 17 β -HSD type 1 to date. We have also investigated the *in-vitro* proliferative effects of the flavonoids, to evaluate their potential agonist activities on ERs.

Materials and Methods

Materials

7,4'-Dihydroxyflavone, diosmetin, chrysoeriol, eupatorin, scutellarein and genkwanin were obtained from TransMIT GmbH Flavonoidforschung (Giessen/Marburg, Germany). Fisetin, 6-hydroxyflavone and baicalein were from Sigma Aldrich Chemie GmbH (Deisenhofen, Germany), and myricetin was from Carl Roth GmbH (Karlsruhe, Germany). [³H]-labelled estrone (2,4,6,7-[³H](N)) was obtained from Perkin Elmer (Boston, MA, USA). Trans-cinnamic acid derivatives with free carboxylic groups were purchased from Fluka Chemie, Acros Organic and Sigma Aldrich Chemie. *Trans*-cinnamic acid esters were synthesized at the Faculty of Pharmacy, University of Ljubljana (Ljubljana, Slovenia) (Sova et al., 2006). The T-47D hormone-sensitive

breast cancer cell line was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

Inhibition assay

Human 17 β -HSD type 1 was overexpressed in the BL21-CodonPlus (DE3)-RIL strain of *Escherichia coli* containing the pQE30-type 1 17 β -HSD construct (prepared at Institute of Experimental Genetics, Neuherberg, Germany). The bacteria were resuspended in PBS and sonicated; the resultant cell homogenate was used as the source of the recombinant enzyme. Inhibition assays were carried out in 100 mM phosphate buffer (pH 6.5) in the presence of 1% acetonitrile as the co-solvent. The concentration of the substrate ([³H]-labelled E₁ [2,4,6,7-³H(N)] and unlabelled E₁) in the reaction solution was 62 nM, and the concentration of NADPH was 100 μ M. The reactions were carried out at 37 °C and stopped with ethyl acetate after the time needed to convert approximately 30% of the substrate in a control assay (in the absence of inhibitor). Substrate and product were extracted from the reaction mixture in ethyl acetate. The organic phase was removed, the residue was dissolved in acetonitrile and separated on a reverse-phase (C18) HPLC column with a mobile phase of acetonitrile and water (40:60, v/v) at 1 mL/min. The assays were performed in triplicates and the results are expressed as the mean values.

Cell culture and cell proliferation assay

T-47D cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin in a humidified atmosphere of 5% CO₂ in air, at 37 °C. For the proliferation assay, charcoal-stripped FBS was used, as this is free of estrogens.

The effects of flavonoids on T-47D breast cancer cells was evaluated using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer instructions. The assay is based on conversion of [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; inner salt] (MTS) into the soluble coloured formazan product by mitochondrial dehydrogenase enzymes in metabolically active cells. The absorbance intensity of the sample serves as an indicator of cell viability. The cells were seeded (4 x 10³ per well) in 70 μ L medium in 96-well plates, and left for 24 h to adhere. After this time, 30 μ L 16.7 nM E₁ and 20 μ M flavonoid solution in 0.2% (v/v) DMSO in growth medium were added. The final concentrations in the assays were 6 μ M flavonoids and 5 nM estrone. After 72 h, the medium was changed for a fresh solution of flavonoids and incubated for a further 72 h. Then, medium containing inhibitors was removed and 90 μ L of fresh DMEM and 10 μ L of MTS reagent was added per well. Following 3 h incubation at 37 °C in humidified, 5% CO₂ atmosphere, the absorbance was measured at 490 nm using a microplate reader (Safire²™ Tecan, Switzerland). Cell viability was expressed as percentage of the absorbance of control cells:

$$\text{Cell viability (\%)} = ((A_S - A_M) / (A_C - A_M)) \times 100$$

where A_S is the absorbance of treated cells, A_C is the absorbance of control cells and A_M is the absorbance of the medium without cells. The cells were grown in six parallel experiments over six days in the presence of the test compounds. The control cells were only treated with 0.2% (v/v) DMSO in growth medium.

Statistically significant differences between control and treated cells were determined using one-way analysis of variance (ANOVA) and the Bonferroni *post-hoc* test. Significance was tested at the 0.05 level of probability, with the statistical analysis performed with the SPSS® software package.

Molecular docking

Automated docking was used to locate the possible binding orientations of 7-hydroxyflavone within the active sites of human 17 β -HSD type 1, using the program AutoDock 3.05 (Morris et al., 1998). The structure of 7,4'-dihydroxyflavone was prepared using HyperChem 7.5 (HyperChem, version 7.5 for Windows; Hypercube, Inc., Gainesville, FL, 2002). The crystal structure of 17 β -HSD type 1 was retrieved from the RCSB protein database (PDB entry 1EQU, Berman et al., 2000). The Lamarckian genetic algorithm was applied using the default parameters. The analysis used: number of docking runs, 250; population in the genetic algorithm, 250; number of energy evaluations, 500,000; and maximum number of iterations, 27,000.

Results and Discussion

Flavonoids as inhibitors of 17 β -HSD type 1

The best inhibitors of 17 β -HSD type 1 among the tested flavonoids (Table 1) were 7,4'-dihydroxyflavone and diosmetin (3',5,7-trihydroxy-4'-methoxy-flavone), with both showing more than 40% and 90% inhibition at 600 nM and 6 μ M, respectively. The other good inhibitors at 600 nM and 6 μ M, respectively, were: scutellarein (4',5,6,7-tetrahydroxyflavone), 43% and 88%; genkwanin (3',5-dihydroxy-7-methoxyflavone), 37% and 81%; and chrysoeriol (4',5,7-trihydroxy-3'-methoxyflavone), 33% and 82%. Our data thus show the importance of substituents at the 7, 5, 3' and 4' positions, and they are in agreement with previously published data showing the importance of substituents on the B ring for inhibitory activity (Mäkelä et al., 1998; Le Bail et al., 1998; Le Bail et al., 2000). Fisetin, with two hydroxyl groups on the B ring (3,3',4',7-tetrahydroxyflavone), showed 20% and 72% inhibition, respectively. Fisetin has previously been tested as an inhibitor of 17 β -HSD type 1 and our data are in agreement with that published (Mäkelä et al., 1998). Myricetin (3, 3',4',5',5,7-hexahydroxyflavone) showed low inhibition (less than 10% at 600 nM and 52% at 6 μ M); this compound has three hydroxyl groups on the B ring and a hydroxyl group at position 3. It has previously been shown that the inhibitory activities of flavones with a 3-hydroxyl group (a subgroup of flavonols) decreased with increasing numbers of hydroxyl groups. Compounds without a substituent on the B ring, 6-hydroxyflavone and baicalein (5,6,7-trihydroxyflavone) both showed less than 10% inhibition at 600 nM, and 53%, and 61% inhibition at 6 μ M, respectively. The former compound here has previously been tested as an inhibitor of 17 β -HSD type 1, and our data are in agreement with that published (Mäkelä et al., 1998). Eupatorin (3',5-dihydroxy-4',6,7-trimethoxy-flavone), with three methoxy substituents on the flavone ring, showed the lowest inhibitory activity. Our data indicate that potent inhibitors possess one or two substituents on the B ring and not more than one methoxy group on the flavone ring. For all of the tested compounds except eupatorin, we can estimate that 50% inhibition of 17 β -HSD type 1 is achieved in low micromolar range.

Cinnamic acid and its derivatives as inhibitors of 17 β -HSD type 1

First, we tested cinnamic acid derivatives with a free carboxylic group (Table 2): cinnamic acid, seven derivatives with substituents on the aromatic ring (compounds **1a-g**), and coumarin-3-carboxylic acid (compound **2**). None of these inhibited 17 β -HSD type 1 at 6 μ M. Next, we tested esters of cinnamic acid (compounds **3a-h**), esters of 3,4-methylenedioxycinnamic acid (compounds **4a-d**), esters of 3,4,5-trimethoxycinnamic acid (compounds **5a-d**), cinnamamide **6**, and an amide of coumarin-3-carboxylic acid (compound **7**) (Table 3). Among the cinnamic acids **3a-h**, the best inhibitor was **3e**, with 45% inhibition at 6 μ M. The other seven cinnamic acids were less active, and three of them even showed less than 10% inhibition. Compound **4d** was the most potent among all of the 3,4-methylenedioxycinnamic acid esters evaluated, with 76% inhibition at 6 μ M. The other three of these esters showed less than 20% inhibition. Compounds **5a-d** appeared to be the best starting points for further development, as they showed 35% (**5b**) to 60% (**5c**) inhibition at 6 μ M. A comparison of compounds **3** (**3a**, **3b**) and compounds **4** (**4a**, **4b**) with compounds **5** (**5a**, **5b**) suggests that the presence of three methoxy groups increases the inhibitory activity. Cinnamide **6** showed 30% inhibition and coumarin-3-carboxylic acid amide **7** showed less than 10% inhibition of enzyme, both at 6 μ M.

Docking of 7,4'-dihydroxyflavone into the active site of 17 β -HSD type 1

We performed computational simulations of the docking of 7,4'-dihydroxyflavone into the crystal structure of 17 β -HSD type 1 co-crystallized with inhibitor equilin (PDB entry 1EQU), using AutoDock 3.05. Equilin occupies the steroid binding pocket and is stabilized by H-bonds with Ser142, His221 and /or Glu282. Surprisingly, the position with the lowest docking energy shows 7,4'-dihydroxyflavone orientated perpendicular to equilin. The 7-hydroxyl group points toward the catalytic amino acids: Asn114, Ser142, Tyr155 and Lys159 and also forms H-bonds with Ser142 and Val143. Similarly to 3-hydroxy group of equiline, 4'-hydroxyl group may be additionally stabilized since it is close to Leu197 and Val196 and may even be involved in H-bond formation (Fig. 1).

Effects of flavonoids on proliferation of T-47D cells

Flavonoids may act as ER agonists as well, and therefore we tested their effects on cell proliferation using the T-47D breast cancer cell line, which is known for its high expression of both 17 β -HSD type 1 and ER α (Möller and Adamski, 2006). At 6 μ M (the same concentration as used for the *in-vitro* enzyme inhibitory activity measurements), none of the flavonoids significantly influenced the T-47D cell proliferative activity (Fig. 2).

Conclusions

We have evaluated the inhibitory activities of 10 flavonoids towards human recombinant 17 β -HSD type 1 and investigated their *in-vitro* proliferative effects using T-47D cells, to evaluate their potential estrogenic activities. Five tested flavonoids are good inhibitors (7,4'-dihydroxyflavone, diosmetin, scutellarein, chrysoeriol and genkwanin) demonstrating more than 80% inhibition of 17 β -HSD type 1 activity at 6 μ M. Four flavonoids are moderate inhibitors (fisetin, baicalein, myricetin, and 6-hydroxyflavone) with more than 50% of inhibition and only eupatorin showed less than 40% of inhibition at 6 μ M. None of tested flavonoids significantly influenced T-47D cell proliferation. We also evaluated the inhibitory activities of a series of cinnamic acid derivatives, which have not been tested as inhibitors of 17 β -HSD type 1 to date. Among 18 tested derivatives, 4'-cyanophenyl 3,4-methylenedioxycinnamate (**4d**) was the most active revealing more than 70% inhibition at 6 μ M. Four compounds showed about 50% inhibition and 13 compounds less than 40% of inhibition. Although esters of 3,4,5-trimethoxycinnamic acid possess moderate activity, they may serve as very interesting starting points for further development of 17 β -HSD type 1 inhibitors.

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Figure Legends

Figure 1. Docking of 7,4'-dihydroxyflavone (white) into the active site of 17 β -HSD type 1 (PDB entry 1EQU). The inhibitor equilin (pink) and the coenzyme NADP⁺ are also shown, along with the amino-acid residues of the catalytic tetrad: Asn114, Ser142, Tyr155 and Lys159, and additional amino-acid residue Val143 (blue).

Figure 2. Effects of flavonoids in 6 μ M concentration on the growth of T-47D cells after 6 days of treatment.

Cell viability is expressed as percentage of the absorbance of the control (cells treated with 0.2% (v/v) DMSO in growth medium): $\text{Cell viability (\%)} = ((A_S - A_M)/(A_C - A_M)) \times 100$ where A_S is the absorbance of treated cells, A_C is the absorbance of untreated cells (control) and A_M is the absorbance of the medium without cells.

Figure 1

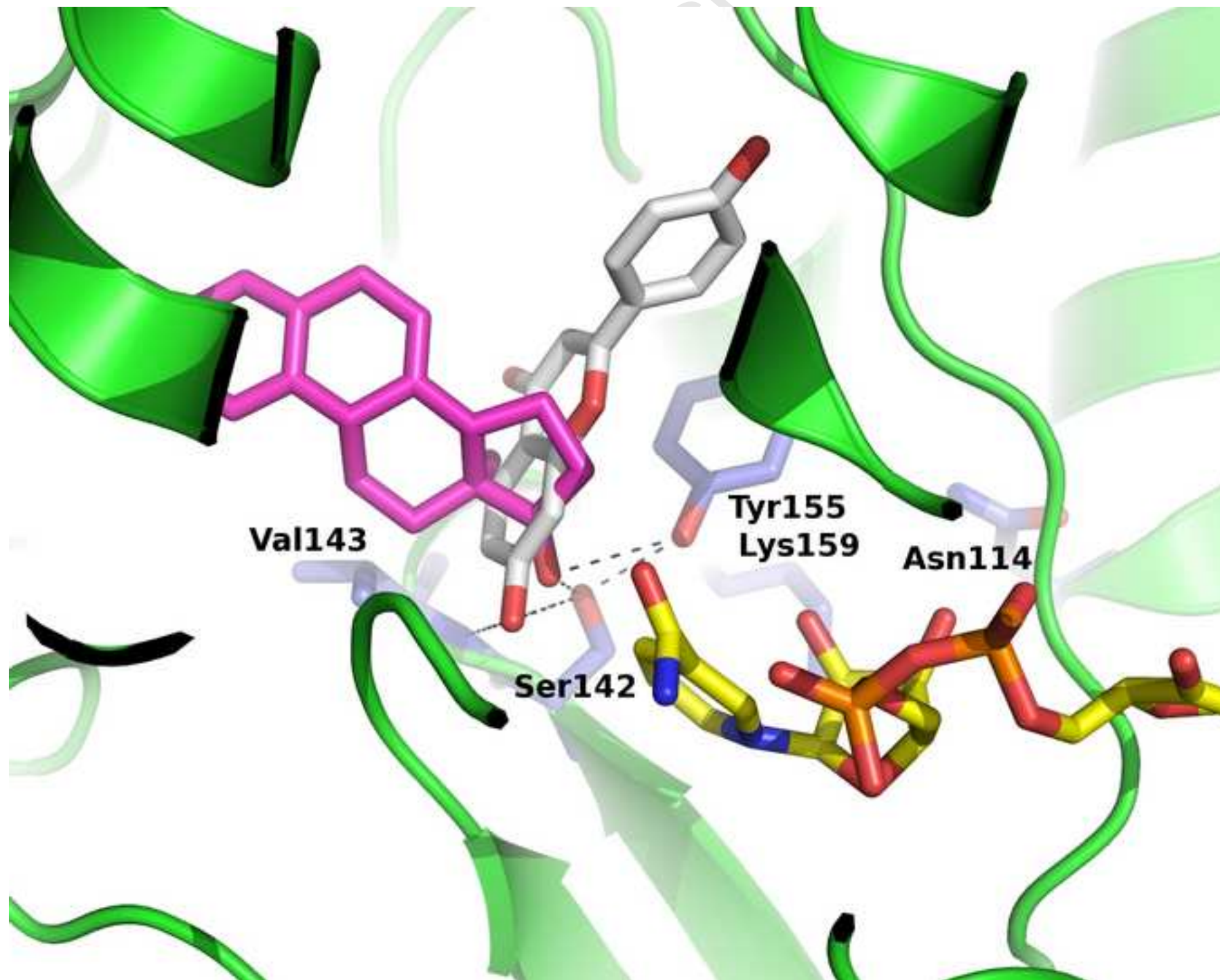


Figure 2

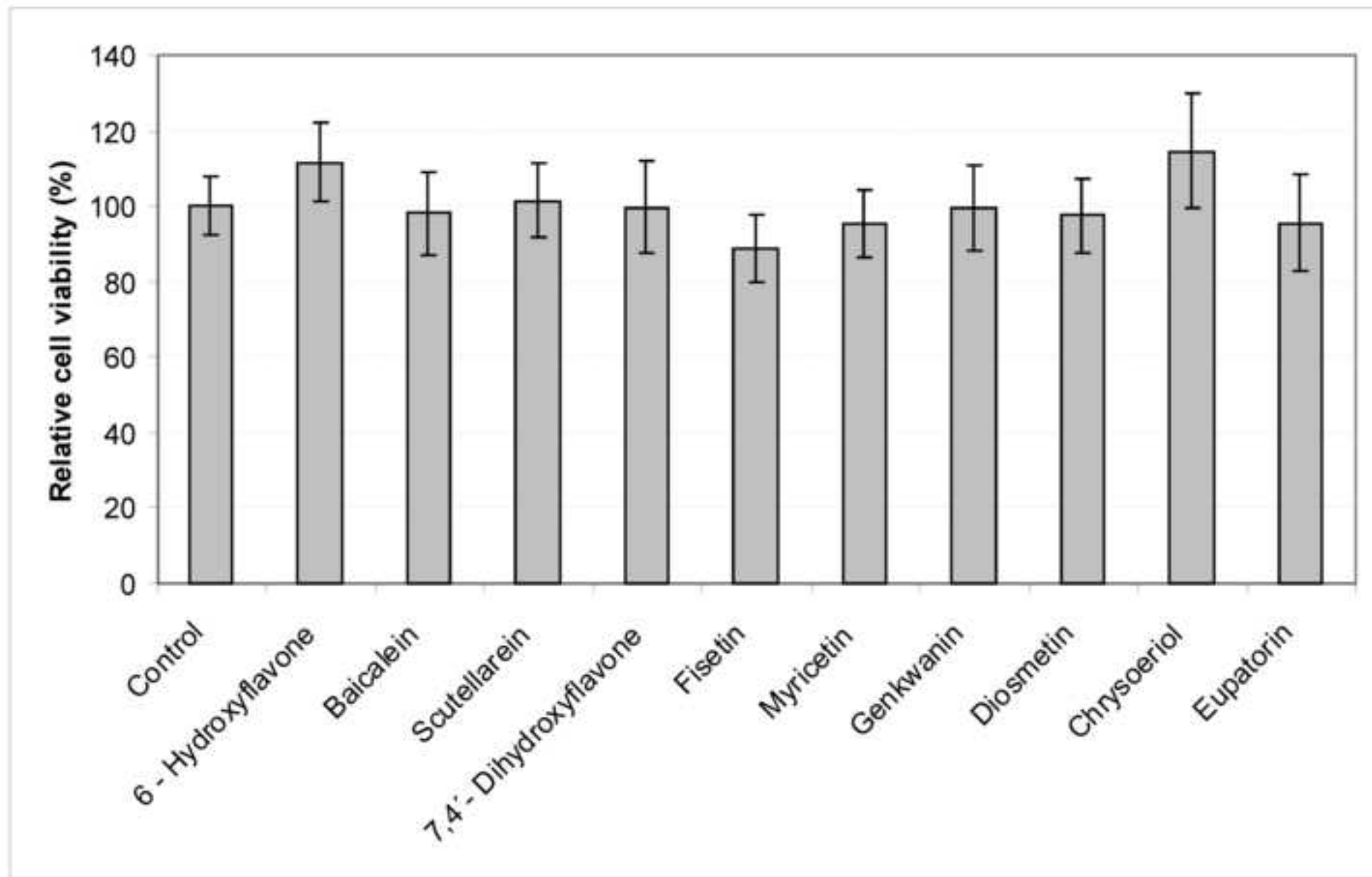
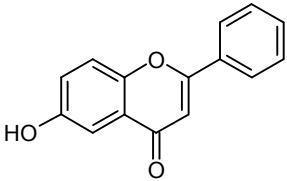
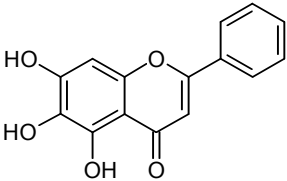
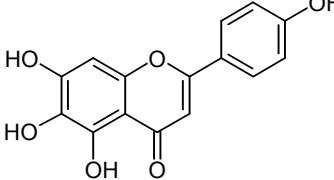
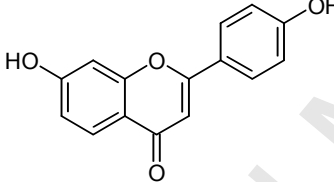
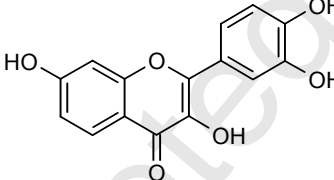
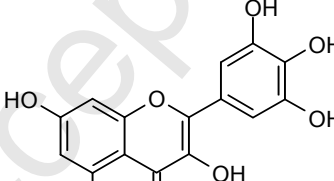
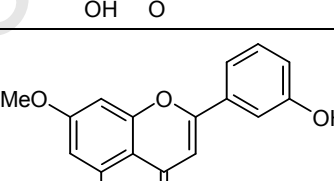
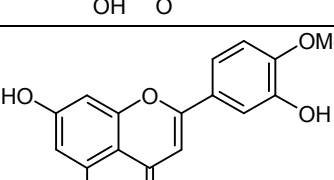
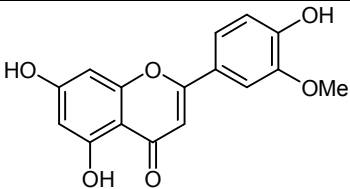
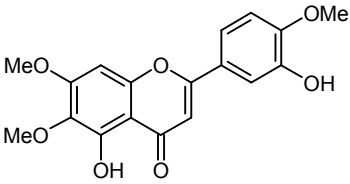


Table 1. Flavonoids evaluated for inhibition of 17 β -HSD type 1.

Compound	Structure	Inhibition ^a (% \pm SD at 600 nM)	Inhibition ^a (% \pm SD at 6 μ M)
6 - Hydroxyflavone		NI	53 \pm 3
Baicalein		NI	61 \pm 3
Scutellarein		43 \pm 1	88 \pm 1
7,4'- Dihydroxyflavone		41 \pm 4	>90
Fisetin		20 \pm 5	72 \pm 2
Myricetin		NI	52 \pm 4
Genkwanin		37 \pm 2	81 \pm 2
Diosmetin		45 \pm 3	>90

Chrysoeriol		33 ±1	82 ±1
Eupatorin		NI	37 ±4

NI: less than 10% inhibition

^a mean of at least three experiments

Table 2

Cinnamic acid and its derivatives with free caboxylic group.

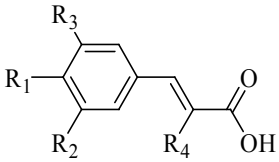
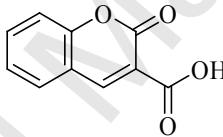
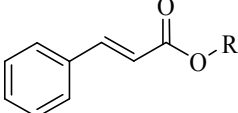
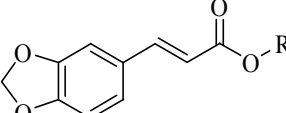
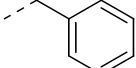
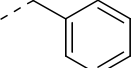
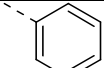
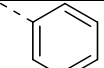
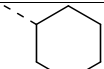
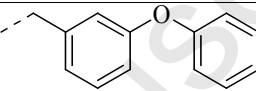
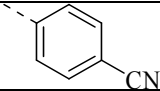
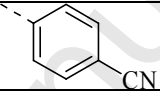
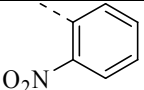
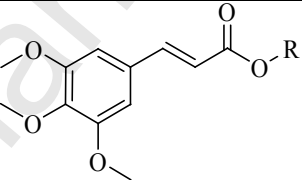
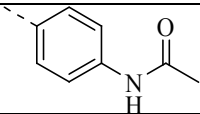
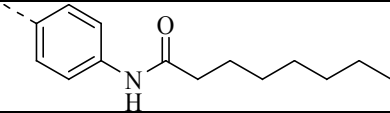
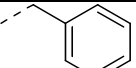
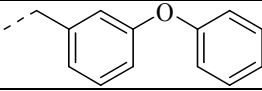
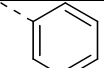
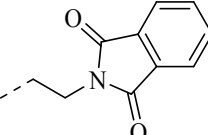
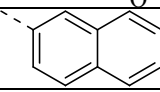
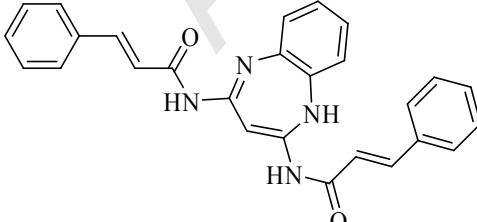
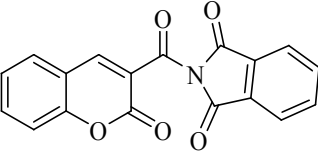
	Name	Structure			
1					
		R ₁	R ₂	R ₃	R ₄
1a	cinnamic acid	H	H	H	H
1b	3-(trifluoromethyl)-cinnamic acid	CF ₃	H	H	H
1c	p-aminocinnamic acid	NH ₂	H	H	H
1d	p-carboxycinnamic acid	COOH	H	H	H
1e	p-nitro-cinnamic acid	NO ₂	H	H	H
1f	m-coumaric acid	H	OH	H	H
1g	3,4,5-trimethoxycinnamic acid	OCH ₃	OCH ₃	OCH ₃	H
2	coumarin-3-carboxylic acid				

Table 3. Cinnamic acid esters and amides evaluated for inhibition of 17 β -HSD type 1.

	Structure	Inhibition ^a (% \pm SD at 6 μ M)		Structure	Inhibition ^a (% \pm SD at 6 μ M)
3			4		
	R			R	
3a		35 \pm 6	4a		NI
3b		NI	4b		NI
3c		25 \pm 6	4c		14 \pm 5
3d		NI	4d		76 \pm 2
3e		45 \pm 1	5		
3f		NI		R	
3g		15 \pm 5	5a		60 \pm 6
3h		13 \pm 5	5b		35 \pm 5
			5c		44 \pm 5
			5d		56 \pm 4
6		30 \pm 2	7		NI

NI: less than 10% inhibition

^a mean of at least three experiments