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Phytoestrogens as inhibitors of the human progesterone metabolizing enzyme AKR1C1

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Abstract

Phytoestrogens are plant-derived, non-steroidal constituents of our diets. They can act as agonists or antagonists of estrogen receptors, and they can modulate the activities of the key enzymes in estrogen biosynthesis. Much less is known about their actions on the androgen and progesterone metabolizing enzymes. We have examined the inhibitory action of phytoestrogens on the key human progesterone-metabolizing enzyme, 20α-hydroxysteroid dehydrogenase (AKR1C1). This enzyme inactivates progesterone and the neuroactive 3α,5α-tetrahydroprogesterone, to form their less active counterparts, 20α-hydroxyprogesterone and 5α-pregnane-3α,20α-diol, respectively. We overexpressed recombinant human AKR1C1 in *Escherichia coli*, purified it to homogeneity, and examined the selected phytoestrogens as inhibitors of NADPH-dependent reduction of a common AKR substrate, 9,10-phenantrenequinone, and progesterone. The most potent inhibitors were 7-hydroxyflavone, 3,7-dihydroxyflavone and flavanone naringenin with IC_{50} values in the low μM range. Docking of the flavones in the active site of AKR1C1 revealed their possible binding modes, in which they are sandwiched between the Leu308 and Trp227 of AKR1C1.

Keywords: 20α-Hydroxysteroid dehydrogenases; Aldo-keto reductases; Progesterone; 3α,5α-Tetrahydroprogesterone; Neurosteroids

1. Introduction

Phytoestrogens are plant-derived, non-steroidal constituents of our diets. They are structurally divided into four main groups: flavonoids, coumestans, stilbenes and lignans, where the flavonoids comprise flavones, isoflavones and flavanones (Rosselli et al., 2000; Cos et al., 2003). Accumulating evidence from molecular and cellular biology investigations suggests that phytoestrogens may confer health benefits with respect to cardiovascular diseases, cancer, osteoporosis and menopausal syndromes (Tham et al., 1998). On the other hand, exposure to phytoestrogens during the critical early stages of human development (through the maternal diet, or infant soya formulas) may also have deleterious endocrine-disrupting effects (Jefferson and Newbold, 2000; Yellayi et al., 2002).

Of the hormone-dependent forms of cancer, huge geographical differences in incidence have been reported for breast, colorectal, endometrial and prostate cancers (Adlercreutz and Mazur, 1997; Carrusi, 2000). The incidence of these diseases is much higher in Northern Europe and the United States of America than in Asia (Adlercreutz and Mazur, 1997; Tham et al., 1998; Mage and Rowland, 2004). These differences have been correlated with environmental and dietary factors, and especially with phytoestrogens, which are abundant in soya products, a major component of the Asian diet (Adlercreutz and Mazur, 1997; Tham et al., 1998; Carrusi, 2000).

Phytoestrogens can act in different ways (Rosselli et al., 2000): they are agonists or antagonists of estrogen receptors (ERα and ERβ), the pregnane X receptor and the constitutive androstane receptor (Jacobs and Lewis, 2002; Wuttke et al., 2002). They have stimulatory effects on hepatic sex-hormone-binding globulin (SHBG), they inhibit tyrosine kinases, and thus prevent growth-factor-mediated stimulation of proliferation, and they can also modulate the activities of key enzymes in estrogen biosynthesis, such as aromatase, sulfatase, sulfotransferases, 3β-hydroxysteroid dehydrogenases (3β-HSDs) and 17β-hydroxysteroid dehydrogenases (17β-HSDs); in this manner, they may act at a pre-receptor level (Wuttke et al., 2002).

Cell-specific metabolic activation of inactive hormone precursors represents a novel level of hormonal regulation. Steroid

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hormones exist in active and inactive forms that can be enzymatically interconverted (Nobel et al., 2001). The active forms have high affinities towards their corresponding receptors, while the inactive forms have very low affinities. The enzymes that interconvert the active and inactive forms, and that thus act as molecular switches, are pre-receptor regulatory enzymes (Nobel et al., 2001; Penning, 2003). Tissue-specific expression of these enzymes allows for the regulation of local concentrations of the active steroid hormones. These pre-receptor regulatory enzymes include different conjugating phase II enzymes, cytochrome P450 enzymes and hydroxysteroid dehydrogenases (HSDs; Nobel et al., 2001).

The HSDs that have been implicated in such regulation at the pre-receptor level belong either to the short-chain dehydrogenase/reductase (SDR) superfamily or to the aldo-keto reductase (AKR) superfamily (Penning, 1997). The majority of the HSDs belong to the SDR family (Peltoketo et al., 1999; Adamski and Jakob, 2001; Mindnich et al., 2004). SDR proteins are monomers of 25–35 kDa that usually function as dimers or tetramers, and that are often membrane bound (Adamski and Jakob, 2001; Mindnich et al., 2004). The AKR members are cytosolic proteins with molecular masses of between 34 and 37 kDa (Penning et al., 2000). These AKRs now follow a standard nomenclature: following the AKR abbreviation, there is a number that designates the family, then a letter that designates the subfamily, and then another number that designates a specific protein within that subfamily (Jez et al., 1997; Hyndman et al., 2003).

Four human HSDs, AKR1C1-AKR1C4 (hence members of the AKR1C subfamily), function *in vitro* as 3-keto, 17-keto and 20-ketosteroid reductases, or as 3α, 3β, 17β and 20α-hydroxysteroid oxidases, to varying degrees (Penning et al., 2000; Steckelbroeck et al., 2004). These AKR1C isoenzymes are expressed in different tissues: AKR1C4 is liver specific, while AKR1C1-AKR1C3 are expressed ubiquitously, and have been detected at different levels in liver, lung, prostate, mammary gland, uterus, brain, small intestine, testis and other tissues (Penning et al., 2000). In intact cells, all of the AKR1C isozymes preferentially work as reductases, and can either form potent androgens (testosterone from androstenedione) and estrogens (estradiol from estrone), or convert the potent androgen 5α-dihydrotestosterone (5α-DHT) into the less potent 3α- or 3β-androstandiol, and the potent progesterone into its less active metabolite 20α-hydroxyprogesterone (20α-OHP) (Penning et al., 2000; Lanišnik Rižner et al., 2003; Steckelbroeck et al., 2004). In this manner, many AKR1Cs regulate the occupancy and *trans*-activation of androgen, estrogen and progesterone receptors (Penning, 2003; Bauman et al., 2004; Steckelbroeck et al., 2004). AKR1Cs have important roles also in the production and inactivation of neuroactive allopregnanolone (3α,5α-tetrahydroprogesterone, 5α-THP), which allosterically modulates the activity of the γ-aminobutyric acid $(GABA)_A$ receptor, and thus exhibits anesthetic, analgetic, anxiolytic and anticonvulsant effects (Griffin and Mellon, 1999; Penning et al., 2000; Nobel et al., 2001). Among these AKR1C isoforms, AKR1C1 acts preferentially as a 20α-HSD and inactivates progesterone by its conversion to 20α-OHP, which has a low affinity for progesterone receptors; it also converts 5α-THP into 5α-pregnane-3α,20α-diol, which has a weak affinity for the $(GABA)_A$ receptor. AKR1C1 thus diminishes the levels of progesterone and 5α-THP in peripheral tissue (Higaki et al., 2003).

Among the steroid metabolizing enzymes, the inhibitory effects of phytoestrogens have been studied against aromatase, sulfatase, sulfotransferases, 5α-reductase, 3β-HSD Δ^5/Δ^4 isomerase, 11β-HSD type 1 and type 2, and 17β-HSD isoenzymes (Weber et al., 1999; Le Bail et al., 2000; Kirk et al., 2001; Lephart et al., 2001; Ohno et al., 2002, 2004; Schweitzer et al., 2003). Phytoestrogens have been shown to inhibit the human 17β-HSD types 1, 2, 3 and 5 (Makela et al., 1995, 1998; Krazeisen et al., 2001, 2002; Le Bail et al., 2001; Le Lain et al., 2001, 2002; Poirier, 2003). There are, however, no reports of phytoestrogen inhibition of other human 17β-HSD isoforms (types 4, 7, 8, 10, 11, 12 or 13) or 20α-HSDs (Poirier, 2003; Mindnich et al., 2004; Deluca et al., 2005).

We have focused our attention on AKR1C1, which is regarded as the dominant form of human 20α-HSD and has an important role in progesterone and 5α-THP inactivation. Thus it may be involved in the development of breast and endometrial cancers, as well as in conditions such as premenstrual syndrome, catamenial epilepsy and depressive disorders (Higaki et al., 2003; Ji et al., 2004; Lanišnik Rižner et al., 2006).

2. Materials and methods

2.1. Phytoestrogens, substrates and coenzymes

Phytoestrogens were originally purchased from ICN Biochemicals GmbH, Steraloids Inc. and Sigma Aldrich Chemie GmbH, and were a kind gift from Dr. Jerzy Adamski (GSF, Neuherberg, Germany). The substrates progesterone and 9,10-phenantrenequinone, and coenzyme NADPH, were from Sigma Aldrich Chemie, GmbH.

2.2. Construction of pGex-AKR1C1

pGex-AKR1C1 was constructed from a pcDNA3-AKR1C1 vector (provided by Dr. Trevor M. Penning). The AKR1C1 coding sequence was amplified with oligonucleotide primers that added *Sac*I and *Xho*I restriction sites at the 5′- and 3′-ends, respectively. The primers were as follows: forward, 5′-TTTTGAGCTCAATGGATTCGAAATATCAG-3′, and reverse, 5′-AAACTGGAGTTAATATTCATCAGAAAATGGATAATT-3′. The PCR product and pGex vector (Pharmacia, Orsay, France) were digested with *Sac*I and *Xho*I, gel purified, and ligated. The nucleotide sequence of the construct was confirmed by dideoxysequencing.

2.3. Expression and purification of recombinant AKR1C1

The pGex-AKR1C1 construct was transferred into the BL21 *Escherichia coli* strain. The cells were then grown in Luria-Bertani medium containing 100 μg/ml ampicilin at 37 °C in a rotary shaker until an OD_{600} of 1.0 had been reached. Expression was induced by IPTG at a final concentration of 1 mM, and the incubation was continued for 16 h at 24 °C (Couture et al., 2002). The preparation of cell extracts, purification of the glutathione-*S*-transferase (GST)-fusion protein by affinity binding to glutathione-Sepharose, and the cleavage with thrombin were performed as described in the GST Gene Fusion System Handbook. The protein concentrations of samples were determined using the Bradford method, with BSA as the standard (Bradford, 1976), and the homogeneity of the proteins was checked by SDS PAGE followed by Coomassie Blue staining.

2.4. Enzyme activity measurements

Recombinant AKR1C1 catalyzes the reduction of progesterone to 20α-OHP in the presence of the coenzyme NADPH. This reaction was carried out in the absence and presence of different phytoestrogens (from 3 to 100 μM), and was followed by incubation of 3 μM recombinant enzyme with 100 μM progesterone and 2.3 mM NADPH in PBS, pH 7.3, for 20 min at 37 °C in a reaction volume of 200 μl. In each instance, samples were extracted with ethyl acetate, dried, resuspended in 40 μl chloroform, and applied to TLC plates (Kieselgel $60F_{254}$ or aluminium oxide 60F254, both Merck, Darmstadt, Germany). The TLCs were run in the following solvent systems: chloroform:ethyl acetate = 4:1; diethylether:petrolether = 2:1 (3,7-dihydroxyflavone) or diethylether (flavone, genistein). The bands were identified by co-migration with standards and quantified by densitometry (Camag TLC Scanner II).

AKR1C1 also catalyzes the reduction of 9,10-phenantrenequinone (9,10-PQ) in the presence of the coenzyme NADPH. This reaction was followed spectrophotometrically by measuring the differences in NADPH absorbance ($\varepsilon_{\lambda 340} = 6270\,M^{-1}\,cm^{-1}$) in the absence and presence of each phytoestrogen. The assays were carried out in a 0.6-ml volume that included 100 mM phosphate buffer (pH 6.5) and 0.9% DMF as a co-solvent. A substrate concentration of 5 μM was used, with 200 μM coenzyme and 0.5 μM enzyme. The concentrations of phytoestrogens were from 0.5 to 100 μM. Initial velocities were calculated and IC_{50} values were determined graphically from a plot of $\log_{10}$ (inhibitor concentration) versus % inhibition, using GraphPad Prism Version 4.00 (GraphPad Software Inc.).

2.5. Molecular docking

Automated docking was used to locate the appropriate binding orientation of inhibitors within the active site of human AKR1C1. The genetic algorithm method implemented in the AutoDock 3.0 program was used (Morris et al., 1998). The structures of the inhibitors were prepared using HyperChem 7.5 (HyperChem, version 7.5 for Windows. Hypercube Inc.: Gainesville, FL, 2002). The crystal structure of AKR1C1 was retrieved from the RCSB protein database (PDB entry 1MRQ), and the steroid ligand and water molecules were removed. Polar hydrogen atoms were added, and Kollman charges (Weiner et al., 1984), atomic solvation parameters and fragmental volumes were assigned to the protein using AutoDock Tools (ADT). For docking calculations, Gasteiger–Marsili partial charges (Gasteiger and Marsili, 1980) were assigned to the coenzyme molecule, and the ligands and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map, which was centered on His117 of the protein, was generated with the auxiliary program AutoGrid. The grid dimensions were large enough to cover the inhibitors and the enzyme active site. The Lennard–Jones parameters 12-10 and 12-6, supplied with the program, were used for modeling H-bonds and van der Waals interactions, respectively. The distance-dependent dielectric permittivity of Mehler and Solmajer was used for the calculation of the electrostatic grid maps (Mehler and Solmajer, 1991). For all ligands, random starting points, random orientation, and torsions were used. The translation, quaternion, and torsion steps were taken from default values in AutoDock. The Lamarckian genetic algorithm and the pseudo-Soils and Wets methods were applied for minimization, using default parameters. The number of docking runs was 250, the population in the genetic algorithm was 250, the number of energy evaluations was 500,000, and the maximum number of iterations was 27,000.

3. Results and discussion

3.1. Purification to homogeneity of recombinant AKR1C1

We overexpressed recombinant human AKR1C1 in *E. coli* in the form of a GST-fusion protein, and then purified it by affinity chromatography on glutathione-Sepharose, from where it was recovered by thrombin cleavage. Next, we examined the purity of AKR1C1 by SDS PAGE followed by Coomasie Blue staining, which revealed a band with an approximate molecular weight of 37 kDa (Fig. 1). The recombinant protein was obtained in milligram quantities.

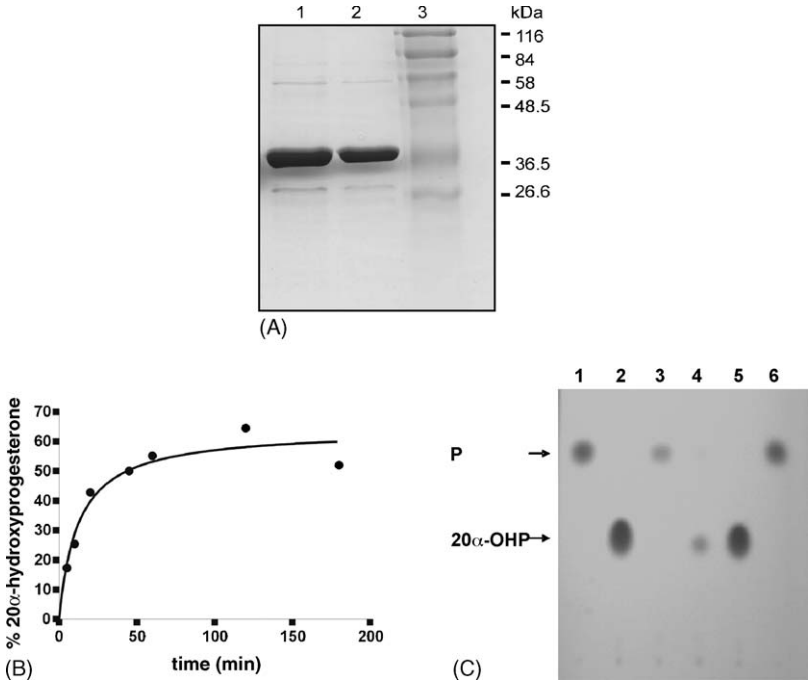


Fig. 1. Homogeneity and 20-ketosteroid reductase activity of purified recombinant AKR1C1. (A) Ten (lane 1) and five (lane 2) μg recombinant AKR1C1 were applied to SDS PAGE and stained with Coomassie Blue. The molecular mass markers (lane 3) were of 116, 84, 58, 48.5, 36.5 and 26.6 kDa. (B) Reduction of 100 μM progesterone by recombinant AKR1C1 (3 μM) in the presence of 2.3 mM NADPH. The reactions were stopped after 5, 10, 20, 45, 60, 120 and 180 min. (C) Identification of the reaction products for the reduction of progesterone: lanes 1 and 6, standard progesterone (P); lanes 2 and 5, standard 20α-OHP; lane 3, 100 μM progesterone + 2.3 mM NADPH; lane 4, 100 μM progesterone + 2.3 mM NADPH + 3 μM AKR1C1.

The homogenous recombinant AKR1C1 converted progesterone to 20α-OHP (Fig. 1) with a specific activity of 37.5 nmol progesterone reduced/min/mg, and an apparent *Km* of 4.6 ± 1.6 μM, which is in agreement with values that have been reported by others (Penning et al., 2000; Couture et al., 2003; Jin and Penning, 2006). The specific activity for 9,10-PQ reduction was 0.55 μmol of 9,10-PQ reduced/min/mg.

3.2. Phytoestrogens as inhibitors of 9,10-PQ and progesterone reduction

We next examined 25 compounds for inhibition of recombinant AKR1C1: 21 plant-derived estrogenic compounds (flavones, flavanones, isoflavones, coumestans, coumarin, stilben resveratrol and organic acids); one myco-estrogen (zearalenone); three synthetic estrogens/antiestrogens (diethylstilbestrol, equilin and tamoxifen). We tested the inhibitory effects of phytoestrogens on two NADPH-dependent reactions: the reduction of the common AKR substrate 9,10-PQ, and the reduction of progesterone. The first reaction was followed spectrophotometrically and enabled fast screening and IC_{50} determinations. The second was performed to simulate the physiological activity of AKR1C1, and its products were identified and quantified by TLC analysis and correlated with the results obtained using the non-physiological substrate. The percentages of inhibition at 5 μM 9,10-PQ and 50 μM inhibitors, and at 100 μM progesterone and 100 μM inhibitors, were determined (Tables 1 and 2). We also determined the IC_{50} values (Tables 1 and 2) for those phytoestrogens that showed more than 50% inhibition of 9,10-PQ and progesterone reduction.

3.3. The most potent inhibitors of AKR1C1: flavones hydroxylated at positions 3 and 7 and flavanone naringenin

The most potent inhibitors of 9,10-PQ reduction were 7-hydroxyflavone and 3,7-dihydroxyflavone, with IC_{50} values of 2.3 and 4.9 μM, respectively (Table 1). An additional hydroxyl group at position 5 decreased the inhibitory potential (5,7-dihydroxyflavone; 28% inhibition) and a flavone with one hydroxyl group at position 5 (5-hydroxyflavone) had no inhibitory effects. The replacement of the hydroxyl at the same position with a metoxyl group (5-metoxyflavone) enhanced the inhibition (33%). Of the other flavones, kaempferol (4′,3,5,7-tetrahydroxyflavone) with hydroxyl groups at positions 3 and 7 was very potent (9.3 μM IC_{50}), quercetin (4′,5′,3,5,7-pentahydroxyflavone), with additional hydroxyl groups at position 5′ was quite potent (60% inhibition; 26.8 μM IC_{50}) while luteolin (4′,5′,5,7-tetrahydroxyflavone; 44% inhibition) and apigenin (4′,5,7-trihydroxyflavone; 19.8% inhibition), which both have only the 7-hydroxyl group, were less potent inhibitors.

The absence of hydroxyl groups (flavone) led to a very weak inhibitory effect (7.4%), and the lack of one double bond in ring C in flavanone abolished the inhibitory action. Also in flavanones the presence of hydroxyl groups increased the inhibitory effect and naringenin possessing hydroxyl groups at positions 4′, 5 and 7 was a very potent inhibitor with IC_{50} value of 2.6 μM. Similar results were obtained for progesterone reduction. Our results thus show that the hydroxyl groups at positions 3 and 7 are important for efficient inhibition by flavones, while the hydroxyl groups at positions 4′, 5 and 7 determine the inhibitory effect of flavanones.

3.4. Isoflavones, coumestans, coumarins, stilbene, organic acids and zearalenone as inhibitors of AKR1C1

The isoflavones genistein (4′,5,7-trihydroxyisoflavone) and biochanin A (4-metoxy-5,7-dihydroxyisoflavone) were potent inhibitors of 9,10-PQ reduction, with IC_{50} values of 5.0 and 5.7 μM, respectively (Table 2). The absence of the 5-hydroxyl group (4′,7-dihydroxyisoflavone-daidzein) decreased the inhibitory activity (40% inhibition), indicating that for isoflavones, the hydroxyl groups at positions 5 and 7 are important for efficient inhibition of 9,10-PQ reduction. Interestingly, isoflavones had almost no inhibitory effects on the reduction of progesterone (Table 2). Inhibition by genistein, biochanin A and daidzein was also tested in the spectrophotometric assay, which confirmed, as previously shown by TLC assay, that these compounds are not inhibitors of progesterone reduction. The differences between inhibition of 9,10-PQ and of progesterone reduction can be explained by the different binding modes of these two substrates within the active site. It has been shown previously that the mutation of two tryptophanes within the steroid-binding pocket of AKR1C9 (Trp86 and Trp227) differently affected binding of small non-steroidal and steroidal-based substrates, supporting the concept of ligand-dependent binding modes (Jez et al., 1996). Docking simulation of 9,10-PQ and progesterone into the AKR1C1 active site revealed that both bind close to the catalytic Tyr55 and His117 and the coenzyme nicotinamide moiety, but because of their different orientations (they are almost perpendicular to one another) and sizes, they interact with different amino-acid residues within the active site (Fig. 2).

Coumestrol and coumarin were less effective as inhibitors of 9,10-PQ reduction than of progesterone reduction. Stilben resveratrol was less potent inhibitor showing about 30% and 10% inhibiton of 9,10-PQ and P reduction, respectively. The plant organic acids glycyrrhetinic and abitinic acid were effective inhibitors of 9,10-PQ reduction, with IC_{50} values of 12.9 and 33.7 μM, respectively. However, only glyrrhetinic acid showed inhibitory action on progesterone reduction, again probably because of the different binding modes of 9,10-PQ and progesterone. The myco-estrogen zearalenone, which is contained in mould-infected food, showed about 40% inhibition of 9,10-PQ and progesterone reduction. As a comparison, for AKR1C3, IC_{50} values of 2 and 4 μM were reported for androstanediol oxidation and androstanedione reduction, respectively, at 30 nM substrate concentrations (Krazeisen et al., 2001). Zearalenone has been reported to have no inhibitory action towards the reductive human 17β-HSD type 1, a member of the SDR superfamily (Makela et al., 1995), while it has been shown to inhibit the oxidative human 11β-HSD type 2, as well as the oxidative activity of human 11β-HSD type 1 and fungal 17β-HSD (Schweitzer et al., 2003; Kristan et al., 2005).

Table 1
Inhibiton of human AKR1C1 by flavones and flavanones

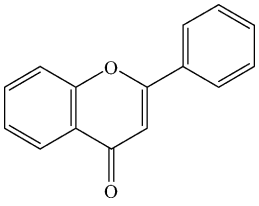
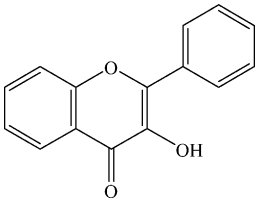
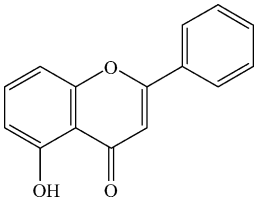
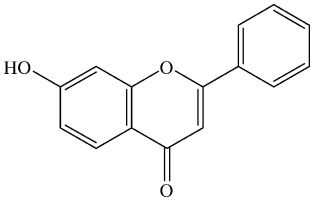
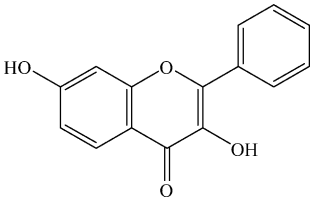
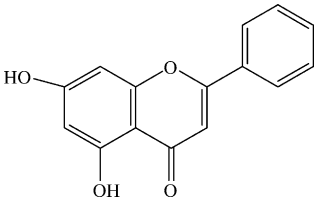
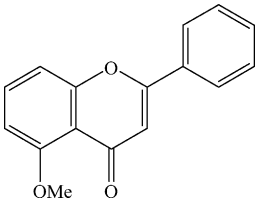
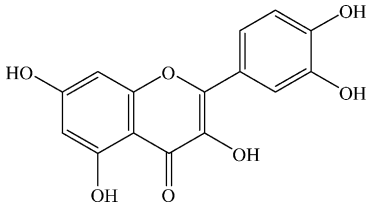
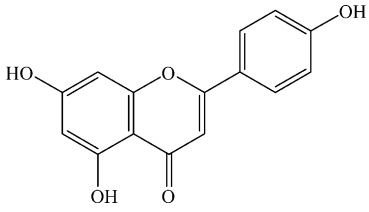
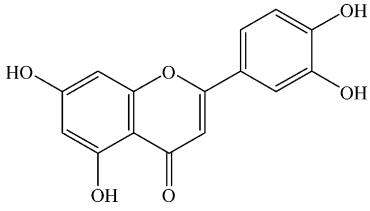
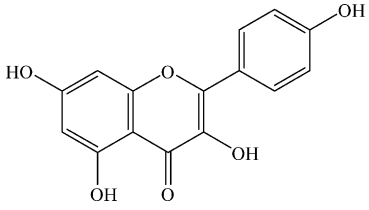
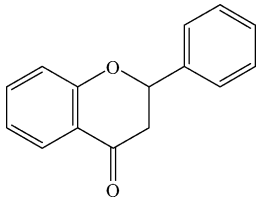
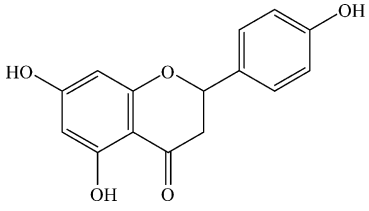
Compound	Main sources	Structure	9,10-PQ[a]		Progesterone[b]	
			Inhibition (%)	IC_{50} (μM)	Inhibition (%)	IC_{50} (μM)
Flavone	Sources of flavones: yellow/red fruits, vegetables		7.4 ± 0.1		13	
3-Hydroxyflavone			76.1 ± 4.5	8.8 ± 1.4	40.4	
5-Hydroxyflavone			0		0	
7-Hydroxyflavone			85.9 ± 0.4	2.3 ± 0.2	74.9	14.0 ± 1.3
3,7-Dihydroxyflavone			87.5 ± 2.2	4.9 ± 0.2	40	
5,7-Dihydroxyflavone	*Passiflora coerulea*		28.1 ± 10.2		22.8	
5-Metoxyflavone			33.3 ± 3.6		ND	

Table 1 (*Continued*)

Compound	Main sources	Structure	9,10-PQ[a]		Progesterone[b]	
			Inhibition (%)	IC_{50} (μM)	Inhibition (%)	IC_{50} (μM)
Quercetin	Chamomille, red onions, apples, tea, endive		60.1 ± 7.1	26.8 ± 4.2	24.4	
Apigenin	Snapdragon, chamomille		19.8 ± 11.7		23	
Luteolin	Parsley, artichoke, basil, celery		44.1 ± 4.5		34.6	
Kaempferol	Beans		74.5 ± 3.7	9.3 ± 1.3	34.8	184 ± 41
Flavanone	General sources of flavanones: yellow/red fruits, vegetables		0		0	
Naringenin	Grapefruit		87.9 ± 2.2	2.6 ± 0.5	74	14.2 ± 0.7

ND, not determined.

[a] Percentage of enzyme inhibition at 5 μM 9,10-PQ and 50 μM of each inhibitor. The results of two independent experiments are shown as means ± S.D. IC_{50} values were determined as described in Section 2, with values representing the means of at least two independent experiments ± S.D.

[b] Percentage of enzyme inhibition at 100 μM progesterone and 100 μM of each inhibitor. The results of two independent experiments are shown as means. IC_{50} values were determined as described in Section 2, with values representing the means ± S.D.

Table 2
Inhibiton of human AKR1C1 by isoflavones, plant organic acids, coumestrol, coumarin, resveratrol, synthetic estrogens/antiestrogens and myco-estrogen

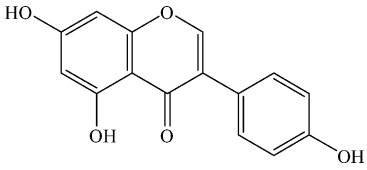
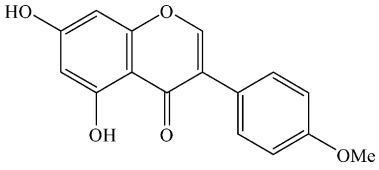
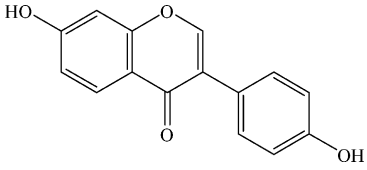
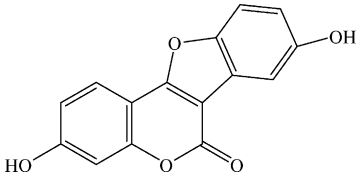
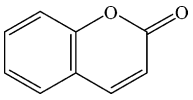
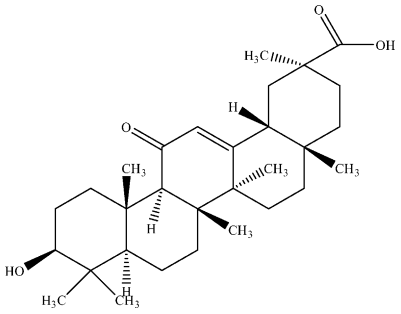
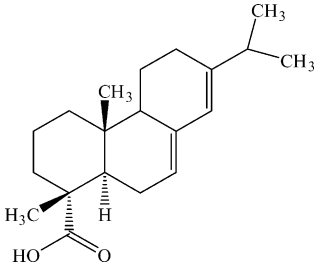
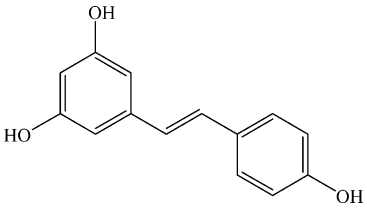
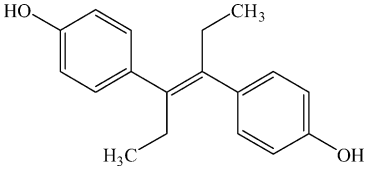
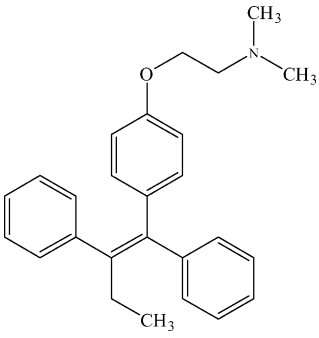
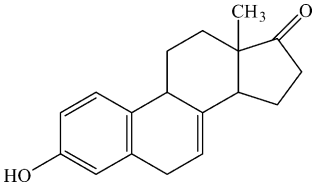
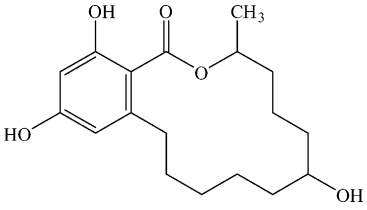
Compound	Main sources	Structure	9,10-PQ[a]		Progesterone[b]	
			Inhibition (%)	IC_{50} (μM)	Inhibition (%)	IC_{50} (μM)
Genistein	Soybeans, beer		97.9 ± 2.9	5.0 ± 0.5	0	
Biochanin A	Red clover, beer, bourbon		88.7 ± 4.3	5.7 ± 1.6	3.3	
Daidzein	Soybeans, beer		39.5 ± 4.5		2.5	
Coumestrol	Sprouts of alfalfa, beans		2.3 ± 3.2		17.9	
Coumarin	Woodruff, vanilla, lavender oil, tonka bean, minor constituent in cherries, strawberries, apricots		7.2 ± 7.8		15.3	
Glycyrrhetinic acid	Licorice		89.3 ± 6.5	12.9 ± 3.4	75.8	36.2 ± 3.5
Abietic acid	Pine wood		60.8 ± 2.3	33.7 ± 4.4	0	

Table 2 (*Continued*)

Compound	Main sources	Structure	9,10-PQ[a]		Progesterone[b]	
			Inhibition (%)	IC_{50} (μM)	Inhibition (%)	IC_{50} (μM)
Resveratrol	Skins of certain red grapes, in peanuts, blueberries, pines, roots and stalks of knotweed		27.8 ± 1.8		10.2	
Diethylstilbestrol	Synthetic estrogen; estrogen replacement therapy		46.1 ± 0.1		14.7	
Tamoxifen	Synthetic antiestrogen; estrogen replacement therapy		0		0	
Equilin	Horse estrogen; estrogen replacement therapy		63.6 ± 2.8	23.7 ± 1.5	39.6	286 ± 2.3
Zearalenone	Mold-infected grain and feeds		42.5 ± 2.2		40	

[a] Percentage of enzyme inhibition at 5 μM 9,10-PQ and 50 μM of each inhibitor. The results of two independent experiments are shown as means ± S.D. IC_{50} values were determined as described in Section 2, with values representing the means of at least two independent experiments ± S.D.

[b] Percentage of enzyme inhibition at 100 μM progesterone and 100 μM of each inhibitor. The results of two independent experiments are shown as means. IC_{50} values were determined as described in Section 2, with values representing the means ± S.D.

3.5. Synthetic estrogens/antiestrogens as inhibitors of AKR1C1

Among the synthetic estrogens/antiestrogens, tamoxifen was not inhibitory for 9,10-PQ and progesterone reduction, while diethylstilbestrol showed higher inhibitory effect than natural stilben resveratrol; 46% and 15% inhibition, respectively. Tamoxifen has been reported previously to be a weak inhibitor of AKR1C3 and 17β-HSD types 1 and 3, members of the SDR superfamily (Santner and Santen, 1993; Krazeisen et al., 2001; Le Lain et al., 2002) while diethylstilbestrol has also been shown to inhibit AKR1C3 (Krazeisen et al., 2001), but to have no effects on 17β-HSD type 1 (Makela et al., 1995). Equilin, a potent inhibitor of human 17β-HSD type 1 (Sawicki et al., 1999), showed 64% and 40% inhibition of AKR1C1, and IC_{50} values of 24 and 286 μM for 9,10-PQ and progesterone reduction, respectively (Tables 1 and 2). For equilin, which is often used for estrogen replacement therapy (Brinton et al., 1997), this is

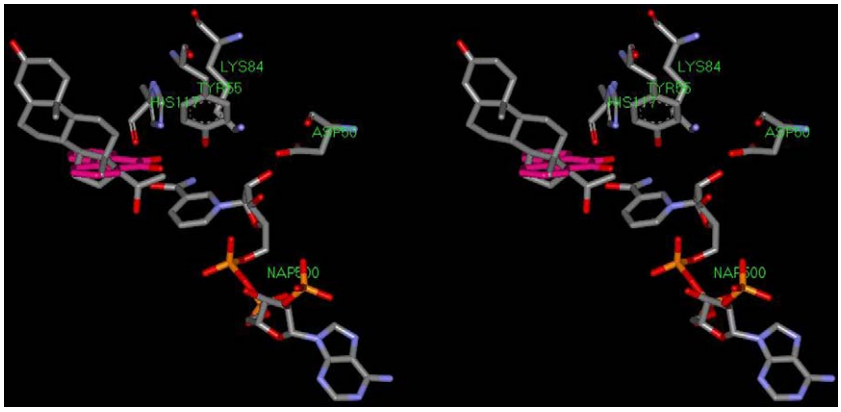


Fig. 2. Docking of 9,10-PQ into the active site of AKR1C1. Stereo view showing NADPH, 20α-OHP, 9,10-PQ and the catalytic amino-acid residues.

the first report showing that it is not SDR specific, and that it inhibits at least one of the human AKR1C isoforms.

3.6. Docking flavones into the active site of AKR1C1

We performed computational simulations of the docking of the most potent inhibitors of 9,10-PQ and progesterone reduction into the crystal structure of AKR1C1 (1MRQ), using AutoDock 3.0. Simulations revealed that flavones can enter the substrate-binding region of the active site. In the position with the lowest docking energy, the flavone rings A and C are sandwiched between Leu308, Trp307 and Trp227, and its ring B points toward the oxyanion hole composed of the catalytic amino-acid residues Tyr55 and His117, and the nicotinamide moiety of NADPH (Fig. 3). This type of binding supports the importance of a hydroxyl group at position 7, since this group can form H-bonds with the amide backbone of Leu308 and with the Thr307 side chain (Fig. 3). The proposed binding of 7-hydroxyflavone resembles the hypothetical catalytic orientation of progesterone in the AKR1C1 active site (Couture et al., 2003), although ring B is not close enough to interact with Tyr55 and His117; the A and C rings mimic the positions of the steroidal A and B rings.

For 3,7-dihydroxyflavone, the binding may be similar to that of 7-hydroxyflavone (data not shown). Also, the crystal structure of the homologous AKR1C3 with NADPH and rutin (PDB code 1RY8), the glycoside of quercetin, revealed binding where ring B orients into the oxyanion hole towards the catalytic His117 and Tyr55 (Komoto et al., 2004), and thus supports this orientation of the flavones within the active site of the AKR1C isozymes.

For the isoflavone genistein, computer simulations suggested several possible orientations. In the position with the lowest docking energy, genistein would lie near the β1–α1 loop and form three H-bonds, two between the 5-hydroxyl group and Thr23 and/or Tyr272, and one between the 7-hydroxyl group and Pro30 (Fig. 4). This position supports the inhibition data, which showed that the 5-hydroxyl and 7-hydroxyl groups are important for potent inhibition of 9,10-PQ reduction. The unhindered conversion of progesterone (genistein is not an inhibitor of progesterone reduction) can also be explained by this mode of binding. However, the results of docking simulation failed to explain how genistein impedes binding and reduction of 9,10-PQ. Although we may speculate the presence of an allosteric effect, the crystal structure of AKR1C1 and genistein would be indispensable to answer the question of this selective inhibition.

3.7. Comparison to known inhibitors of AKR1C1

There are a very limited number of studies on inhibitors of AKR1C1. Usami et al. reported benzodiazepines, especially diazepam and medazepam, are efficient inhibitors of AKR1C1 and IC_{50} values in low micromolar range were determined when following oxidation of 1 mM *S*-tetralol (Usami

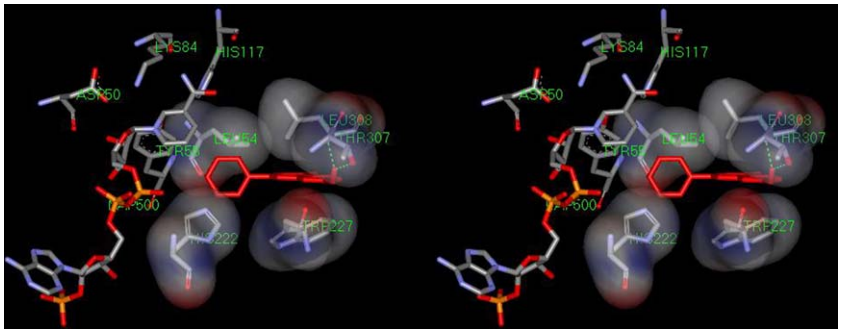


Fig. 3. Docking of 7-hydroxyflavone into the active site of AKR1C1. Stereo view of the phytoestrogen binding site of AKR1C1, showing NADPH, 7-hydroxyflavone (in red) and the amino-acid residues of the active site.

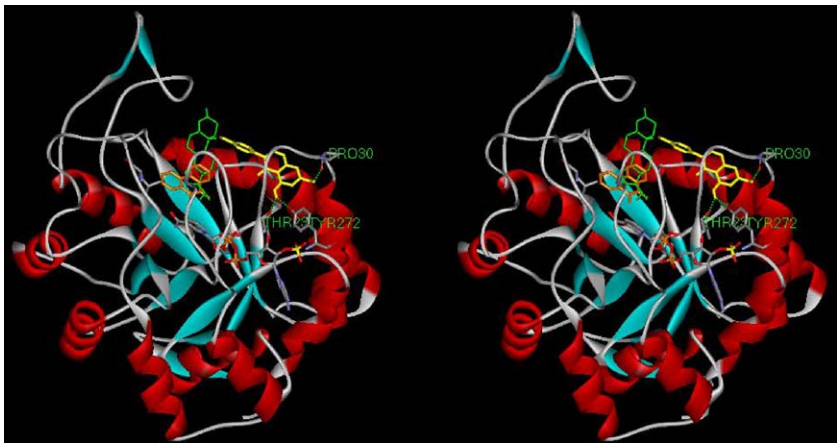


Fig. 4. Docking of genistein into the crystal structure of AKR1C1. Stereo view of AKR1C1 crystal structure, showing the positions of NADPH, 20α-hydroxyprogesterone (green), 9,10-PQ (orange), genistein (yellow), and the amino-acid residues involved in the binding.

et al., 2002). Later the same group found benzbromarone (BZB) and 3′,3″,5′,5″-tetrabromophenolphthalein (TBPP) to be selective and more potent inhibitors with IC_{50} values in nM range (Higaki et al., 2003). Lately, Bauman et al. presented a group of non-steroidal anti-inflammatory drug (NSAID) analogs as inhibitors of AKR1C1, the best inhibitor of oxidation of 100 μM 1-acenaphthenol was 5-methyl-*N*-phenylantranilic acid with 3.2 μM IC_{50} and $K_I = 0.88$ μM (Bauman et al., 2005). Also phytoestrogens revealed IC_{50} values in the low micromolar range. Lower substrate concentrations used in our enzyme assay (5 μM 9,10-PQ) suggest phytoestrogens are less potent inhibitors than benzodiazepines, BZB, TBPP and NSAID analogs, however, one should take into account that an average individual is not constantly exposed to the later substances.

3.8. Comparison of phytoestrogens as inhibitors of AKR1C1 and AKR1C3

Of the AKR1C isozymes, phytoestrogens have already been tested as inhibitors of AKR1C3. The reduction of androstenedione to testosterone and oxidation of androstanediol to androsterone were studied (Krazeisen et al., 2001, 2002). Since the AKR1C isoforms act as reductases *in vivo* (Steckelbroeck et al., 2004), we focussed here on the inhibitors of reduction. The reduction of 30 nM androstenedione to testosterone was potently inhibited by zearalenone, coumestrol, quercetin and biochanin A, all with IC_{50} values below 15 μM (Krazeisen et al., 2001). For 7-hydroxyflavone, naringenin, 3,7-dihydroxyflavone, kaempferol, genistein, biochanin A and glycyrrhetinic acid, which are the best inhibitors of AKR1C1, these were not so effective on AKR1C3, with IC_{50} values 20 μM and above (Krazeisen et al., 2001, 2002). Although 5-hydroxyflavone, tamoxifene and flavanone showed no inhibition of AKR1C1, they still had some inhibitory action on AKR1C3, with IC_{50} values above 20 μM. Thus, despite an 87% identity of their amino acids, AKR1C1 and AKR1C3 show distinct structural requirements for potent inhibition.

3.9. Could phytoestrogens inhibit AKR1C1 in vivo?

Our results show phytoestrogens, and especially the flavones, can also inhibit recombinant human AKR1C1, so could phytoestrogens affect this enzyme also *in vivo*? The IC_{50} values we determined for reduction of 9,10-PQ and progesterone were in the micromolar range, from 2 to 300 μM. Considering 5 and 100 μM concentrations of these two substrates in our enzyme assays, we would expect lower IC_{50} values with the physiological nM concentrations of steroids. Also, the individual phytoestrogen concentrations in human serum extend from nM up to μM, depending on diet; the average European will have low nM concentrations, while Asian populations show up to μM concentrations of isoflavones (Adlercreutz et al., 1993). The average plasma concentrations of the flavones are lower, but can be increased by a plant-based diet or by dietary supplements. For quercetin and naringenin, for instance, up to 1.3 and 6 μM concentrations were reported, respectively (Erlund et al., 2001; Paganga and Rice-Evans, 1997). From these data, we would expect that in certain individuals the concentrations of flavones, especially naringenin, and quercetin may reach sufficient levels to affect AKR1C1 *in vivo*.

AKR1C1 is expressed in the breast, uterus and other peripheral tissues, where it regulates progesterone action. Thus, its inhibition may have profound effects in these tissues. Recent findings suggest that P endogenously produced or exogenously administered does not affect the risk for breast cancer (Campagnoli et al., 2005a). The increase in breast cancer risk found in women receiving estrogen and progestin, compared with those receiving estrogen alone, may be explained by the fact that some progestins exert non-progesterone-like effects, such as androgenic, estrogenic or glucocorticoid (Sitruk-Ware and Plu-Bureau, 2004; Campagnoli et al., 2005b). But also progesterone metabolites may influence proliferation of breast cells; higher levels of 5α-reduced progesterone metabolites and lower levels of Δ^4-metabolites in tumor breast tissue suggest 5α-pregnanes (5α-P), acting through specific 5α-P receptors (5α-PR), stimulate cell proliferation, while 4-pregnanes (including 20α-OHP)

down regulate expression of these receptors and have the opposite effect (Wiebe et al., 2000; Wiebe and Lewis, 2003; Lewis et al., 2004; Pawlak et al., 2005; Wiebe et al., 2005). Although loss of AKR1C1 expression has been reported in breast cancer (Wiebe and Lewis, 2003; Lewis et al., 2004; Ji et al., 2004), inhibition of AKR1C1 may still affect the ratio between progesterone and 20α-OHP, but this may have no effect on 5α-PR, but rather on the occupancy of PRAB and receptor mediated action of progesterone (Wiebe et al., 2000; Pawlak et al., 2005; Gizard et al., 2005).

In the uterus, inhibition of AKR1C1, which is upregulated in endometrial cancer (Lanišnik Rižner et al., 2006), can result in a higher concentration of progesterone and may thus protect the endometrium from the mitotic activity of exogenous and endogenous estrogens (Akhmedkhanov et al., 2001). AKR1C1 is also important in the brain, where it regulates the action of neurosteroids (Penning et al., 2000; Steckelbroeck et al., 2004). Inhibition of AKR1C1 with phytoestrogens that readily pass across the blood–brain barrier could result in higher concentrations of the neuroactive 5α-THP, and may thus influence mood, memory, cognition, neuroendocrine and reproductive behaviors (Lephart et al., 2000, 2001).

4. Conclusions

We have shown here that phytoestrogens inhibit recombinant AKR1C1. The most potent inhibitors of progesterone reduction revealed IC_{50} values in the low micromolar range. Phytoestrogens may thus affect the whole range of steroid metabolizing enzymes, and may in this manner influence not only estrogen and androgen action, but also progesterone action in peripheral tissues, such as in the breast and endometrium. In addition, phytoestrogens may also affect the synthesis and inactivation of neurosteroids, which are also catalyzed by the AKR1C isozymes.

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