JOURNAL OF NATURAL PRODUCTS

Article

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Selective Inhibition of Human Type-5 17β-Hydroxysteroid Dehydrogenase (AKR1C3) by Baccharin, a Component of Brazilian Propolis

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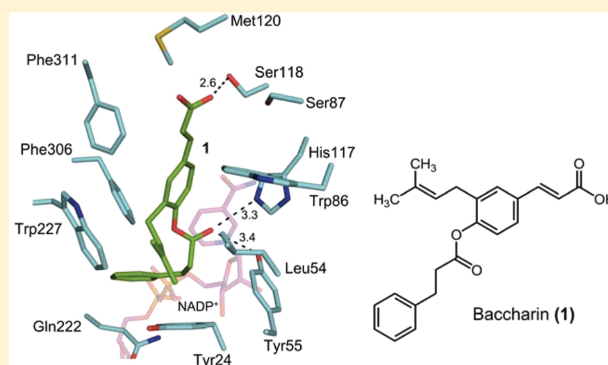
Supporting Information

ABSTRACT: The human aldo-keto reductase (AKR) 1C3, also known as type-5 17β-hydroxysteroid dehydrogenase and prostaglandin F synthase, has been suggested as a therapeutic target in the treatment of prostate and breast cancers. In this study, AKR1C3 inhibition was examined by Brazilian propolis-derived cinnamic acid derivatives that show potential antitumor activity, and it was found that baccharin (**1**) is a potent competitive inhibitor (K_i 56 nM) with high selectivity, showing no significant inhibition toward other AKR1C isoforms (AKR1C1, AKR1C2, and AKR1C4). Molecular docking and site-directed mutagenesis studies suggested that the non-conserved residues Ser118, Met120, and Phe311 in AKR1C3 are important for determining the inhibitory potency and selectivity of **1**. The AKR1C3-mediated metabolism of 17-ketosteroid and farnesal in cancer cells was inhibited by **1**, which was effective from 0.2 μM with an IC_{50} value of about 30 μM. Additionally, **1** suppressed the proliferation of PC3 prostatic cancer cells stimulated by AKR1C3 overexpression. This study is the first demonstration that **1** is a highly selective inhibitor of AKR1C3.



Mammalian hydroxysteroid dehydrogenases (HSDs) interconvert active and inactive steroid hormones in target tissues and belong to two protein superfamilies,[1,2] the aldo-keto reductases (AKRs)[3] and the short-chain dehydrogenases/reductases (SDRs).[4] HSDs belonging to the AKR superfamily share a >60% sequence identity and are classified in the AKR1C subfamily. In humans, four AKR1C isoforms, namely, AKR1C1, AKR1C2, AKR1C3, and AKR1C4, are known as NADP(H)-dependent 20α-HSD, type-3 3α-HSD, type-2 3α-HSD/type-5 17β-HSD, and type-1 3α-HSD, respectively.[2,3] Although the AKR1C isoforms share a >86% amino acid sequence identity and a common $(\alpha/\beta)_8$-barrel structural motif, they exhibit distinct positional and stereo preferences with respect to their steroid substrates and, thereby, play different physiological roles in modulating levels of active steroid hormones such as androgens, estrogens, and progestins at the prereceptor level.[2,5]

AKR1C3 is overexpressed in hormone-dependent prostate and breast cancers,[6−8] and other tumors such as hepatocellular and non-small-cell lung carcinomas.[9] The silencing of the AKR1C3 gene results in growth inhibition of prostatic cancer cells,[10] and its elevated expression in turn promotes progression of prostatic and breast cancer cells,[10−12] indicating that the enzyme participates in tumor development. AKR1C3 is involved in the synthesis of androgens and estrogen in the prostate[8,13] and breast,[7,11] respectively. In addition, AKR1C3 exhibits prostaglandin (PG) F synthase activity, which leads to decreases in the levels of antiproliferative PGD_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2.[7,10,11] Thus, AKR1C3 promotes the proliferation of prostate and breast cancers by both production of proliferative steroid hormones and a decrease in intratumoral levels of the antiproliferative PGs and, thereby, is a potential therapeutic target for the prevention and treatment of the above types of cancer. However, because of the high structural similarity of AKR1C3 to other AKR1C isoforms that play distinct roles in steroid metabolism, its selective inhibition over other AKR1C isoforms is required for the development of treatments targeting the types of cancers linked to AKR1C3.

Received: December 27, 2011
Published: April 16, 2012

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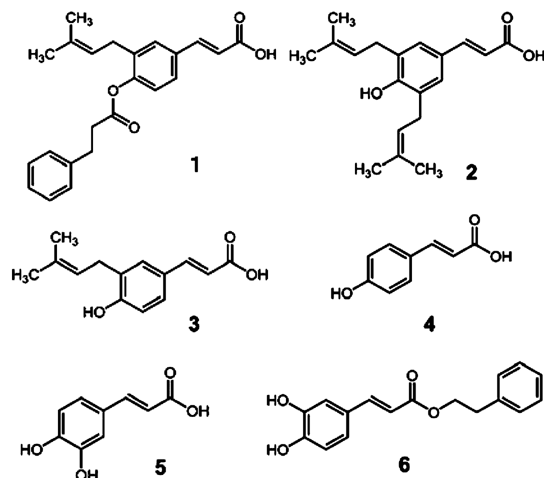
AKR1C3 is inhibited by several structurally different compounds, which are divided into steroidal and nonsteroidal inhibitors, as reviewed by Byrns et al.[14] Representative steroidal inhibitors are medroxyprogesterone acetate[15] and steroidal lactones that are estrogen derivatives bearing a lactone on the steroid D-ring.[16] Medroxyprogesterone acetate lacks AKR1C3 selectivity, and the inhibitory effects of the steroidal lactones on other AKR1C isoforms have not been tested. The nonsteroidal inhibitors are anti-inflammatory agents and their analogues, benzodiazepines, PG analogues, flavonoids, cyclopentane derivatives, and cinnamic acids.[14] Among the nonsteroidal inhibitors, highly selective inhibitors are indomethacin and its analogue, but their inhibition constant (K_i) values are in the micromolar range.[17] Recently, 3-(phenylamino)benzoic acids have been synthesized as potent and selective inhibitors, which show IC_{50} values of 36−62 nM for AKR1C3 with 94- to 360-fold less inhibition against AKR1C2.[18]

AKR1C3 inhibitor classes including nonsteroidal anti-inflammatory agents, flavonoids, and cinnamic acids have been described as being effective in the chemoprevention of cancer or having potential antitumor properties.[14,19] Cytotoxic properties are also detected for other phenolic and prenylated derivatives of cinnamic acid,[20−23] of which three prenylated cinnamic acids [baccharin (**1**), artepillin C (**2**), and drupanin (**3**)] are present as major constituents in the ethanol extract of Brazilian propolis,[22] which is a natural resinous substance collected by honeybees. The potential anticancer mechanism of **2** has been studied,[24,25] but those of **1** and **3** remain unknown.



Here, we have compared the inhibitory potencies against human AKR1C isoforms among the prenylated and phenolic cinnamic acids in Brazilian propolis[22,26] and show that, of these compounds, baccharin (**1**) is the most potent and selective inhibitor of AKR1C3, showing a K_i of 56 nM, with no inhibition against AKR1C1 and AKR1C2. Moreover, a structural rationale for the selectivity of **1** was investigated by molecular docking and site-directed mutagenesis of the binding-site residues. Furthermore, **1** was evaluated for its inhibitory effects on the proliferation of cancer cells and the metabolism of 17-ketosteroid and farnesal by cellular AKR1C3.

RESULTS AND DISCUSSION

Inhibitory Potency and Selectivity of Cinnamic Acid Derivatives. The compounds evaluated in this study were the prenylated cinnamic acids **1**−**3**, the phenolic cinnamic acids **4** and **5**, and the cinnamoyl ester **6**, and their inhibitory potency was compared with that of a known AKR1C3 inhibitor, indomethacin.[15,17] The three prenylated derivatives inhibited AKR1C3 and apparently had no inhibitory effects on the other AKR1C isoforms (Table 1). The two phenolic cinnamic acids showed low inhibition of AKR1C3, in agreement with a previous study on cinnamic acids.[27] The cinnamoyl ester **6** moderately inhibited AKR1C3, but lacked inhibitory selectivity to the enzyme. The IC_{50} value of **1** [3-prenyl-4-(dihydrocinnamoyloxy)cinnamic acid] for AKR1C3 was lower than those of **2** and **3**, suggesting that the dihydrocinnamoyloxy moiety is an important structural prerequisite for this potent inhibitor. The high inhibitory selectivity to AKR1C3 over other AKR1C isoforms was a common characteristic of the prenylated cinnamic acids, suggesting that the 3-prenyl moiety is an important structural factor for the selective binding to AKR1C3. In this respect, **1** showed the highest selectivity, as its IC_{50} ratios of AKR1C3 to other AKR1C isoforms were greater than 1000. The selectivity of **1** is also much higher than those of indomethacin and other known AKR1C3 inhibitors, for which the inhibitory effects on other AKR1C isoforms have been tested.[14,15,17,19,28]

The inhibition patterns of **1** and **2** were noncompetitive with respect to $NADP^+$ and competitive with respect to the alcohol substrate, *S*-(+)-1,2,3,4-tetrahydro-1-naphthol (*S*-tetralol), showing K_i values of 56 ± 6 and 680 ± 80 nM, respectively. Since the reaction catalyzed by AKR1C3 follows the ordered sequential mechanism,[2,17] **1** and **2** may bind to the substrate-binding site of the enzyme. The K_i value for **1** is higher than those of the steroidal lactone EM1404 (6.9 nM)[16] and tolfenamic acid (8 nM),[28] but lower than those of other known inhibitors.[14,17]

Structural Insight into Inhibitory Selectivity of Baccharin (1) to AKR1C3. In crystal structures of AKR1C3−coenzyme−inhibitor (or substrate) complexes, the binding site of the inhibitor or substrate is large and dissected

Table 1. Inhibitory Effects of Cinnamic Acid Derivatives on Human AKR1C Isoforms

inhibitor	IC_{50} (μM)[a] AKR1C1	AKR1C2	AKR1C3	AKR1C4
baccharin (**1**)	(0%)	(0%)	0.11 ± 0.01	(40 ± 6%)
artepillin C (**2**)	(29 ± 4%)	(13 ± 1%)	1.0 ± 0.1	(19 ± 3%)
drupanin (**3**)	(30 ± 5%)	108 ± 10	15 ± 1	(0%)
p-coumaric acid (**4**)	(22 ± 2%)	(0%)	(22 ± 4%)	(0%)
caffeic acid (**5**)	(6 ± 1%)	(19 ± 3%)	(36 ± 6%)	(0%)
phenethyl caffeate (**6**)	13 ± 2	6.0 ± 0.7	1.7 ± 0.2	2.3 ± 0.2
indomethacin	130 ± 10	75 ± 6	4.1 ± 1.1	54 ± 5

[a]Values in parentheses represent % inhibition at 100 μM.

dx.doi.org/10.1021/np201002x | J. Nat. Prod. 2012, 75, 716−721

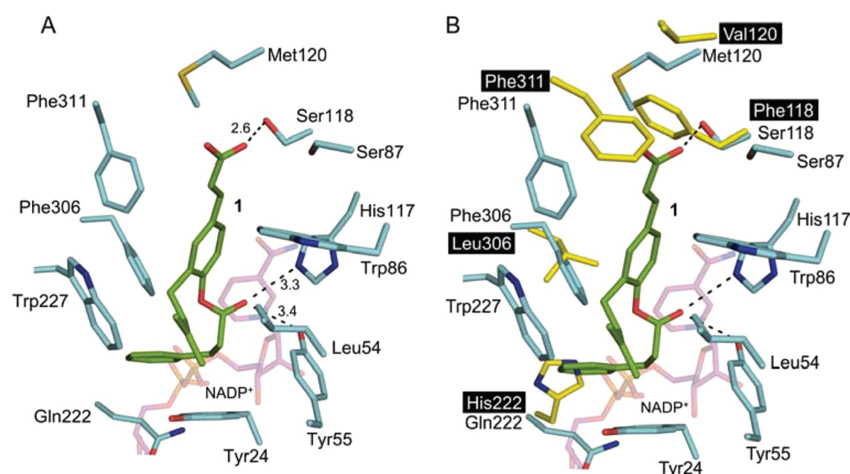


Figure 1. Binding mode of baccharin (**1**) to the AKR1C3-NADP$^+$ complex. (A) **1**-docked AKR1C3 model. NADP$^+$ (pink) and residues (sky-blue) within 3.6 Å from **1** (green) are depicted with possible hydrogen bonds (dotted line) and their corresponding distances shown in Å. (B) Differences between residues (at positions 118, 120, 222, and 306) and orientation of Phe311 in AKR1C3 (sky-blue) and AKR1C1 (yellow). The AKR1C1 structure (PDB code 3NTY) was superimposed on the **1**-docked AKR1C3 model. The orientations of the other amino-acid side chains are similar in the two enzymes.

into subsites: an oxyanion site (composed of Tyr55, His117, and coenzyme), a steroid channel (composed of residues that determine the steroid substrate specificity), and three subpockets (composed of residues that contribute to the binding for structurally different inhibitors).[14] The residue difference in the subpockets is suggested to be related to the inhibitory selectivity among human AKR1C isoforms. To investigate which residues in the subsites of AKR1C3 participate in the high selectivity of **1**, the nonconserved Ser118, Met120, Ser129, Gln222, and Phe306 were chosen among the residues in the subpockets of AKR1C3 and were replaced with the corresponding residues, Phe, Val, Ile, His, and Leu, respectively, of AKR1C1 and AKR1C2 (Figure 1). In addition, Phe311, another residue in the subpocket of AKR1C3, was replaced with the corresponding residue Leu in AKR1C4, and Trp227, a conserved substrate-binding residue in all isoforms, was replaced by a smaller aromatic residue, Tyr. With the exception of the Phe306Leu and Ser129Ile mutations, other mutations decreased the affinity for **1** by more than 3-fold compared to the wild-type enzyme (Table 2). The affinity for **1** was impaired greatly by the mutations of Ser118Phe and Phe311Leu, followed by Met120Val and Gln222His. Thus, the residue differences at positions 118, 120, 222, and 311 are probably related to the high selective inhibition of AKR1C3 by **1**.

Table 2. Effects of Mutations of AKR1C3 on the K_i Value of Baccharin (1)

enzyme	K_i (nM)[a]	Mu/Wt[a]
wild type	56 ± 6	
Ser118Phe	850 ± 100	15
Met120Val	550 ± 70	10
Ser129Ile	105 ± 11	2
Gln222His	300 ± 50	5
Trp227Tyr	148 ± 15	3
Phe306Leu	46 ± 8	0.8
Phe311Leu	1200 ± 90	21

[a]Inhibition patterns in the NADP$^+$-linked *S*-tetralol dehydrogenase activity were all competitive with respect to the substrate. Mu/Wt represents the ratio of the K_i value for the mutant enzyme to that for the wild-type enzyme.

The underlying structural reasons for the high selectivity of baccharin (**1**) were also examined by constructing models of docked **1** in the AKR1C3-NADP$^+$ complex, which were built on the basis of the crystal structure of a AKR1C3-NADP$^+$-flufenamic acid complex.[29] In all models showing low energy minimization scores, the carbonyl group of the esterified dihydrocinnamoyloxy moiety of **1** was in close proximity to the oxyanion site residues. Among these models, one that explains the results of the site-directed mutagenesis is depicted in Figure 1, in which the side chain of Ser118 formed a strong H-bond interaction with the carboxylic acid of **1** (2.6 Å), in addition to hydrophobic/van der Waals interactions of Met120 and Phe311 with the molecule of **1**. This H-bond interaction is missing in the Ser118Phe mutant enzyme, and as such, the affinity for **1** may be largely decreased. The side chain of Gln222 is within 3.6 Å from **1**, and its replacement with His would impair the proper orientation of the dihydrocinnamoyloxy moiety, an important structural prerequisite for the inhibition by **1**. Thus, the replacements of the residues at positions 118, 120, and 222 are responsible for the difference in sensitivity to **1** between AKR1C3 and AKR1C1/1C2. The largest impairment of the affinity for **1** by Phe311Leu may also result from the inability of AKR1C3 to undergo a proper induced-fit conformational change upon the binding of **1**, because Phe311 is one of the residues involved in the induced fit to accommodate several ligands in crystallographic studies of these isoforms.[14,16]

Inhibitory Effects of Baccharin (1) on Cellular Metabolism and Proliferation by AKR1C3. Compound **1**, artepillin C (**2**), and drupanin (**3**) were compared for their effects on cellular metabolism with an isoprenyl aldehyde, farnesal, which is rapidly reduced into farnesol by AKR1C3 in vitro and in MCF7 cells.[28] Since the three prenylated cinnamic acids exhibit growth inhibition of MCF7 cells by treatment for more than 48 h at high concentrations over 100 μM,[22,23] the cells were treated for 6 h with their concentrations lower than 100 μM, for which no apparent cytotoxicity was observed. In agreement with the data in Table 1, the cellular farnesal

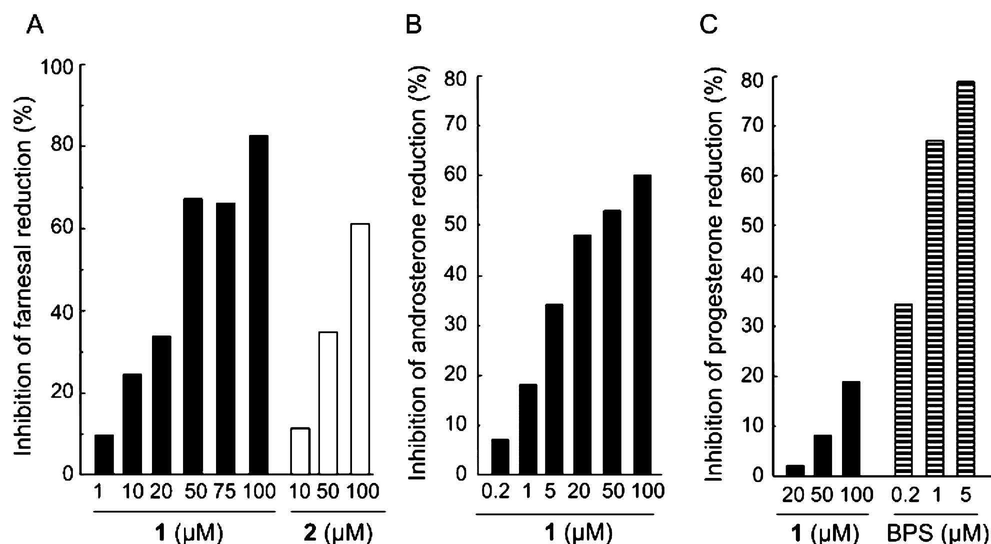


Figure 2. Inhibitory effects of baccharin (**1**) on cellular metabolism by AKR1C3. (A) Inhibition of farnesal reduction in MCF7 cells by **1** and artepillin C (**2**). The cells were pretreated with the indicated concentrations of inhibitors for 2 h and then incubated with 20 μM [1-^{14}C]farnesol for 6 h. The inhibition percentages of the farnesal reduction by the inhibitors are expressed as the mean of duplicate experiments. Drupanin (**3**) showed only 14% inhibition at its concentration of 100 μM. (B) Inhibition of reductive 17β-HSD activity in A549 cells by **1**. The cells were incubated with 50 μM androsterone for 24 h in the presence of the indicated concentrations of **1**, and the substrate and its metabolite 5α-androstane-3α,17β-diol in the medium were determined by LC/MS. The inhibition percentages of the reduction are expressed as the mean of duplicate experiments. (C) Effect of **1** on the progesterone metabolism in A549 cells. The cells were incubated with 30 μM progesterone for 6 h in the presence of **1** (■) and 3-bromo-5-phenylsalicylic acid (BPS, bars with lines). The substrate and its metabolite 20α-hydroxyprogesterone in the medium were determined by LC/MS, and inhibition percentages of the reduction are expressed as the mean of duplicate experiments.

metabolism was most potently inhibited by **1**, which was effective from 1 μM, showing an IC_{50} value of 30 μM (Figure 2A). Although few AKR1C3 inhibitors have been evaluated for their cellular efficacy, the inhibitory potency of **1** is lower than those of tolfenamic acid[28] and steroidal lactones,[16] but is higher than that of jasmonic acid, a PG analogue.[30]

The inhibitory selectivity of baccharin (**1**) to AKR1C3 was examined for the modulation of cellular steroid metabolism, using A549 lung cancer cells expressing AKR1C1, AKR1C2, and AKR1C3 exhibiting overlapping 3α-, 17β-, and 20α-HSD activities.[5,31] Western blot analysis confirmed the higher expression of AKR1C3 in A549 cells than in MCF7 and PC3 cells, which was not affected by the treatment of **1** (Supporting Information, Figure S1). 5α-Androstan-3α-ol-17-one (androsterone) and progesterone are good substrates for reductive 17β- and 20α-HSD activities, respectively, of AKR1C3, but also reduced to 5α-androstane-3α,17β-diol and 20α-hydroxyprogesterone, respectively, by AKR1C2 and AKR1C3.[5] When the cellular metabolism of the two ketosteroids was analyzed by LC/MS (Supporting Information, Figure S2), A549 cells metabolized androsterone more rapidly than the other two cell lines (data not shown). The androsterone reduction in A549 cells was inhibited by **1**, which was effective from 0.2 μM, but showed 60% inhibition at a 100 μM concentration (Figure 2B). The incomplete inhibition might be due to involvement of other 17β-HSD isoenzymes belonging to the SDR superfamily[4] in the cellular androsterone metabolism (Supporting Information, Table S1 and Figure S3). The IC_{50} value calculated from the dose−response curve was 29 μM, which is almost the same as that determined in the above inhibition of farnesal metabolism, supporting the specific inhibition of AKR1C3 by **1**. In contrast, the progesterone reduction was not suppressed significantly by **1** and was potently inhibited by the AKR1C1 inhibitor 3-bromo-5-phenylsalicylic acid (Figure 2C), which is not inhibitory to AKR1C3,[32] suggesting that AKR1C1, with high 20α-HSD activity,[5,15] is the major progesterone-metabolizing enzyme in the cells. The results demonstrate the high inhibitory selectivity of **1** to AKR1C3 and suggest that **1** can be used as the selective inhibitor for the elucidation of the biological roles of AKR1C3 in extrahepatic cells that express AKR1C1 and AKR1C2.

In contrast to the multifunctional artepillin C (**2**) and drupanin (**3**), showing both cytotoxic[21,24,25] and antioxidant activities,[26,33] baccharin (**1**) shows only growth suppression of several human cancer cell lines.[22,23] Indeed, **1** (50 μM) suppressed proliferation of PC3 prostatic carcinoma cells that express AKR1C3 when the cells were cultured for 72 h (Figure 3). To test for the possibility that the inhibition of AKR1C3 is related to growth suppression, the effect of **1** was examined on cell proliferation of AKR1C3-overexpressing PC3 cells, in which AKR1C3 was expressed 5-fold more highly than the control cells transfected with the vector alone. The AKR1C3-overexpressing cells exhibited an increase in cell growth compared to the control cells. This is in agreement with the promotion of proliferation of prostatic and breast cancer cells by elevated expression of AKR1C3.[10−12] Compared to the untreated cells, the treatment of the AKR1C3-overexpressing cells with **1** (50 μM) for 72 h resulted in 43% reduction of proliferation, which was larger than a 35% reduction in the control cells. The data show collectively the involvement of AKR1C3 in PC3 cell proliferation, which is effectively inhibited by **1**.

In conclusion, the present study has revealed that baccharin (**1**) is the most selective inhibitor of AKR1C3 among the existing inhibitors known and raises the intriguing possibility that the inhibition of this enzyme is a novel mechanism of the tumor-suppressive action of **1**. Due to its high inhibitory selectivity to AKR1C3 versus other AKR1C isoforms, **1**

dx.doi.org/10.1021/np201002x | J. Nat. Prod. 2012, 75, 716−721

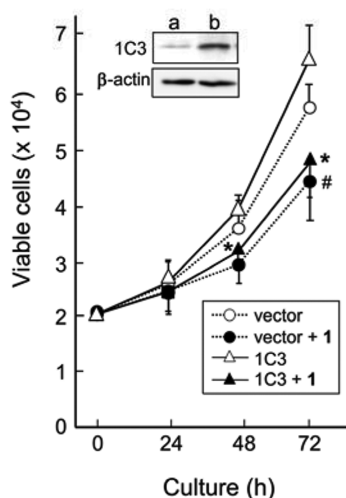


Figure 3. Suppression of PC3 cell proliferation by baccharin (**1**). Together with the control cells transfected with vector alone (vector), AKR1C3-overexpressing cells (1C3) were incubated without or with 50 μM **1**, and then the viable cell numbers were estimated at the indicated times. #Significant difference for vector + **1** from vector. *Significant difference for 1C3 + **1** from 1C3. The inset shows the Western blot analysis of AKR1C3 expression in the extracts (each 20 μg) of the control (lane a) and AKR1C3-expressed cells (lane b), in which β-actin was used as the loading control.

represents a promising lead for the development of more potent and specific agents targeting AKR1C3, which is involved in the proliferation of prostate and breast cancers.[7,8] Structural knowledge of the type of interactions between AKR1C3 and **1** suggested by the present molecular docking and site-directed mutagenesis studies may facilitate the design of novel anticancer agents in the future.

■ EXPERIMENTAL SECTION

Compounds and Materials. The prenylated cinnamic acids **1**−**3** were isolated from Brazilian green propolis by the methods described previously,[22] and their purities by HPLC analysis[22] were 98.4%, 99.0%, and 99.7%, respectively. The phenolic cinnamic acids **4** and **5** were obtained from Wako Pure Chemical Industries (Osaka, Japan), and the cinnamoyl ester **6** was from Bachem AG (Bubendorf, Switzerland). [1-^{14}C]Farnesol and the steroids used were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA) and Steraloids (Newport, RI, USA), respectively. 3-Bromo-5-phenylsalicylic acid was synthesized as described previously.[32] Human A549, MCF7, and PC3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

Preparation of Recombinant Enzymes. The recombinant AKR1C1,[34] AKR1C2,[35] AKR1C3,[36] and AKR1C4[34] were prepared and purified to homogeneity, as described previously. Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) and the pKK223-3 expression plasmid harboring the cDNA for AKR1C3[36] as the template, according to the protocol described by the manufacturer. The primer pair used for the mutagenesis was composed of sense and antisense oligonucleotides to alter one codon of the cDNA. The 26- to 30-mer primers were synthesized to give the Ser118Phe, Met120Val, Ser129Ile, Gln222His, Trp227Tyr, Phe306Leu, and Phe311Leu mutant enzymes. The coding regions of the cDNAs in the expression plasmids were sequenced in order to confirm the presence of the desired mutation and ensure that no other mutation had occurred. The mutant enzymes were expressed in *Escherichia coli* JM109 cells and purified to homogeneity as described above for the wild-type AKR1C3.[36]

Assay of Enzyme Activity. The dehydrogenase activities of the enzymes were determined at 25 °C by measuring the rate of change in NADPH fluorescence (at 455 nm with an excitation wavelength of 340 nm).[32] The IC_{50} values for inhibitors were determined in the reaction mixture that consisted of 0.1 M potassium phosphate, pH 7.4, 0.25 mM $NADP^+$, *S*-tetralol (0.1 mM for AKR1C1 and 1 mM for other AKR1C isoforms), and enzyme, in a total volume of 2.0 mL. Kinetic studies in the presence of inhibitors were carried out in the $NADP^+$-linked *S*-tetralol oxidation over a range of five substrate concentrations (0.33−2 mM) at a saturating concentration (0.25 mM) of $NADP^+$. The IC_{50} and K_i values are expressed as the means ± SD of at least three determinations.

Molecular Modeling. Atomic coordinates for the AKR1C3-$NADP^+$-flufenamic acid complex (PDB code 1S2C)[29] were obtained from the RCSB Protein Data Bank. The structure was prepared using the Maestro (Schrödinger, LLC) software package, version 8.5, as described previously.[37] The ligand, baccharin (**1**), was subjected to a full minimization using the program LigPrep prior to the docking. The docking calculations were performed using the program Glide 5.0[38] on a Linux workstation under the conditions described previously.[37] Among 30 poses generated, the poses showing a distance of less than 3.5 Å between the docked **1** molecule and the catalytically important residues Tyr55 and His117 were selected initially. Finally, the selected pose was the highest scoring pose supported by the site-directed mutagenesis results. The figure showing the docked model was generated using PyMOL (DeLano Scientific).

Cell Culture Experiments. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator containing 5% CO_2. The transfection of the pGW1 plasmids harboring the cDNA for AKR1C3[28] into PC3 cells was performed using Lipofectamine 2000 reagent (Invitrogen) 24 h after the cells were seeded into a 48-well multiplate at a density of 1.5 × 10^4 cells/well. The transfection efficacy was evaluated by Western blotting using the antibody against AKR1C3 and density measurement of the immunoreactive protein,[39] in which anti-human β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as loading control. The transfected cells were then treated for 0, 24, 48, or 72 h with **1**. The number of viable PC3 cells was measured by the MTT method using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium.[40] Data are expressed as means ± SD of at least three independent experiments. Statistical evaluation of the data was performed by using the unpaired Student's *t*-test and ANOVA followed by Fisher's test. A p value < 0.05 was considered statistically significant.

The analysis of the metabolism of 20 μM [1-^{14}C]farnesol in MCF7 cells was carried out as described previously.[28] The metabolism of androsterone and progesterone in A549 cells were analyzed by determining the steroids and their reduced metabolites by a LC/MS system using a Chiralcel OJ-H 5 μm column, as described previously.[41] The cells were pretreated for 2 h with various concentrations of inhibitors in serum-free growth medium prior to incubating with 50 μM androsterone and 30 μM progesterone for 24 and 6 h, respectively. The steroids in the culture medium were extracted twice by ethyl acetate. In the LC/MS analysis (Supporting Information, Figure S2), progesterone and its metabolite 20α-hydroxyprogesterone were detected by monitoring their total ions.[32] Androsterone and its metabolite 5α-androstane-3α,17β-diol were detected in the positive-ion mode by monitoring their fragment ions (m/z 256.5 and 258.5, respectively) and eluted at the retention times of 24 and 27 min, respectively, which were identical to those of the two authentic steroids. Data are expressed as the means of two independent experiments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was partly funded by grants-in-aid for Young Scientists (B) and Scientific Research (C) from the Japan Society for the Promotion of Science.

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