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Biochemistry

Daidzin: A potent, selective inhibitor of human mitochondrial aldehyde dehydrogenase

(acetaldehyde/ethanol/alcohol abuse)

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ABSTRACT **Human mitochondrial aldehyde dehydrogenase (ALDH-I) is potently, reversibly, and selectively inhibited by an isoflavone isolated from *Radix puerariae* and identified as daidzin, the 7-glucoside of 4',7-dihydroxyisoflavone. Kinetic analysis with formaldehyde as substrate reveals that daidzin inhibits ALDH-I competitively with respect to formaldehyde with a K_i of 40 nM, and uncompetitively with respect to the coenzyme NAD^+. The human cytosolic aldehyde dehydrogenase isozyme (ALDH-II) is nearly 3 orders of magnitude less sensitive to daidzin inhibition. Daidzin does not inhibit human class I, II, or III alcohol dehydrogenases, nor does it have any significant effect on biological systems that are known to be affected by other isoflavones. Among more than 40 structurally related compounds surveyed, 12 inhibit ALDH-I, but only prunetin and 5-hydroxydaidzin (genistin) combine high selectivity and potency, although they are 7- to 15-fold less potent than daidzin. Structure–function relationships have established a basis for the design and synthesis of additional ALDH inhibitors that could both be yet more potent and specific.**

The NAD^+-dependent aldehyde dehydrogenase (ALDH) catalyzes the oxidation of acetaldehyde, the primary product of ethanol metabolism, and a wide spectrum of other aldehyde metabolites including biogenic amines such as dopamine, serotonin, and norepinephrine (1). Human liver contains at least four ALDH isozymes, but their physiological roles in the metabolism of specific aldehydes are still not defined completely (1, 2). The two most abundant ALDH isozymes found in human liver are ALDH-I in mitochondria and ALDH-II in the cytosol. Of these, the affinity of acetaldehyde for ALDH-I is much greater than that for ALDH-II (1, 2) and hence, ALDH-I is thought to be the one principally responsible for the detoxification of acetaldehyde. This is confirmed by the fact that in ≈50% of Asians, liver ALDH-I is present as a catalytically inactive genetic variant of this enzyme (2). The capacity of individuals with this trait to metabolize acetaldehyde is markedly impaired (3) as signaled by facial flushing soon after ingestion of ethanol (4). Interestingly, alcoholism and alcohol abuse are virtually nonexistent among this Asian population (1, 5). The absence of ALDH-I activity in these individuals is not known to generate any other deleterious metabolic consequences. Such findings suggest the use of a specific inhibitor of this enzyme as an approach to the treatment of alcohol abuse in a population with the active form of ALDH-I.

While disulfiram (Antabuse) is an ALDH inhibitor, it primarily inhibits ALDH-II and does so by covalently modifying sulfhydryl groups (6). More importantly, it is nonspecific and also covalently modifies the catalytically essential sulfhydryl groups of many other physiologically important enzymes, thereby irreversibly inactivating some that are crucial to neurotransmitter metabolism (dopamine β-hydroxylase), drug metabolism and detoxification (microsomal mixed-function oxidases), and multiple pathways of intermediary metabolism (glyceraldehyde-3-phosphate dehydrogenase, hexokinase, etc.) (6). Indeed, the reaction is so nonspecific that it has led to the use of this agent as a general reagent for the determination of sulfhydryl groups in proteins (7). In 1948 it became the first—and last—ALDH inhibitor ever to be approved for the therapeutic treatment of alcohol abuse in the United States. The mode of action and its toxic manifestations were demonstrated only long thereafter. Disulfiram suppresses alcohol intake in laboratory animals (8, 9) and significantly reduces short-term alcohol consumption in a subset of alcoholic patients undergoing aversion therapy (6). Its broad and deleterious action and lack of specificity would seem largely responsible for the substantial toxicity that accompanies and limits its therapeutic use.

A number of traditional remedies for the treatment of alcohol abuse and alcoholism have been described in the ancient Chinese materia medica *Beiji-Qianjin-Yaofang* (≈600 A.D.) and *Lan-Tai Kuei-fan* (≈1600 A.D.). Among them only *Radix puerariae*- and *Flos puerariae*-based medications are still prescribed by some herbalists in China for the treatment of alcohol abuse. We have explored the biochemical basis for the putative effects of these medications by fractionating *R. puerariae* extracts chromatographically, assaying for active principles that modulate the activities of ALDH isozymes, and characterizing the resultant products. We here describe the isolation and characterization of daidzin—a specific, potent, reversible inhibitor of human ALDH-I.

MATERIALS AND METHODS

ALDH Inhibition Assay. Extracts of *R. puerariae* were monitored for the presence of an ALDH inhibitor by measuring ALDH activities in the presence and absence of 50 μl of each chromatographic fraction added to 1 ml of standard pH 9.5 ALDH assay medium [0.1 M glycine/NaOH or $NaPP_i$ buffer containing 0.15 M KCl, 0.6 mM NAD^+ (grade III, Sigma), 30 μM acetaldehyde (Aldrich), and 5–10 nM ALDH-I or an unresolved mixture of ALDH-I and ALDH-II purified by AMP-agarose (Sigma) chromatography]. Enzyme reaction rates were measured at 340 nm (ε_{NADH} = 6.22 $mM^{-1} \cdot cm^{-1}$) with a Varian Cary 219 spectrophotometer at 25°C. ALDH inhibition was expressed in percent as $(v_0 - v_i) \times 100/v_0$, where v_0 and v_i are the enzyme reaction rates measured in the absence and presence of a fraction sample, respectively. For convenience, the kinetic mechanism of daidzin inhibition of ALDH-I and ALDH-II was studied with formaldehyde as substrate. Initial velocities were determined at various concentrations of formaldehyde and daidzin in a mixture of 3 mM NAD^+, 5 nM ALDH-I or ALDH-II, 0.15 M

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Abbreviation: ALDH, aldehyde dehydrogenase.

KCl, and 0.1 M $NaPP_i$ buffer (pH 9.5) at 25°C. Inhibition constants were estimated from Dixon plots.

Purification of ALDH-I and ALDH-II. ALDH isozymes were purified by the procedure of Ikawa *et al.* (10) except that a DEAE 5PW HPLC column (Waters) was used to resolve ALDH-I and ALDH-II isozymes eluted from the AMP-agarose column. The isozymes obtained were homogeneous as indicated by starch gel and SDS/polyacrylamide gel electrophoresis.

Mass and ^1H NMR Spectroscopy. Samples of ALDH inhibitor and daidzin (Indofine, Somerville, NJ), authenticated by decomposition temperature and mass and UV spectra, were introduced with a direct insertion probe in a Hewlett Packard 5985B gas chromatograph/mass spectrometer operating at 70 eV in an electron ionization mode, and probe temperature was raised from ambient to 295°C. ^1H NMR spectra were acquired at 25°C in perdeuterated dimethyl sulfoxide (Sigma) by using a 30-degree excitation pulse in a Varian VXR 300s NMR spectrometer operating at 299.949 MHz.

RESULTS

Isolation and Characterization of ALDH-I Inhibitor. Dried *R. puerariae* (Vinh-Ken Ginseng, Boston), 10 g, was ground to a powder in a domestic food processor and extracted with 100 ml of methanol for 10 hr in a Soxhlet extractor equipped with an all-glass extraction thimble (Kontes). Methanol was removed by vacuum evaporation and the resultant syrup was dissolved in 5 ml of 10 mM NaP_i at pH 7.5. About 60% of the ALDH inhibitory activity was soluble. Chromatography on Bio-Gel P-4 separated the ALDH inhibitor from most of the materials that absorbed strongly at 214 nm (Fig. 1*A*). Reverse-phase HPLC yielded ALDH inhibitory activity that was eluted as a distinct peak at 87 min (Fig. 1*B*). Rechromatography on the same column in 20% methanol/80% water yielded a virtually pure inhibitor that subsequently was shown to be coeluted with authentic daidzin.

The inhibitor has UV absorption maxima at 230 and 260 nm, with shoulders at 249 and 304 nm, and decomposes at 235°C (authentic daidzin, dec 234–236°C). The electron-ionization mass spectrum of the inhibitor exhibits fragment peaks at m/z 118 and 136 and an apparent molecular ion peak at m/z 254, properties identical to those of authentic daidzin. The ^1H NMR spectrum of the ALDH inhibitor [δ 9.54 (4′-OH), 8.38 (H 2); 8.036 (d, J = 8.79 Hz, H 5), 7.396 (d, J = 8.79 Hz, H 2′ and H 6′), 7.222 (d, J = 1.95 Hz, H 8), 7.13 (q, J = 2.44, 8.79 Hz; H 6), 6.804 (d, J = 8.79 Hz, H 3′ and H 5′), multiplets at 5.43, 5.0–5.2, and 4.6] is also identical to that of daidzin.

The identity of the ALDH inhibitor as daidzin was confirmed by comparison of their acid-hydrolyzed products on TLC. Table 1 shows the R_f values of daidzin, daidzein, D-glucose, ALDH inhibitor, and acid-hydrolyzed ALDH inhibitor and daidzin obtained in different solvent systems. Authentic daidzin and unhydrolyzed ALDH inhibitor ran as single spots with identical R_f values between those of D-glucose and daidzein. A mixture of daidzin and ALDH inhibitor comigrated as a single spot (result not shown). Hydrolysis cleaved both daidzin and ALDH inhibitor into two components. One was detectable only under UV light and had R_f values identical to those of daidzein in all three solvent systems tested. The other was detectable only with anisaldehyde reagent and had R_f values identical to those of D-glucose in solvent systems I and II. These results demonstrate that the ALDH inhibitor isolated from *R. puerariae* is daidzin, whose chemical structure is shown in Fig. 2.

Inhibition of ALDH by Daidzin and Related Compounds. *R. puerariae*-derived daidzin inhibits human ALDH-I and ALDH-II in a concentration-dependent manner (Fig. 3*A*). The IC_{50} for ALDH-I determined with 5 μM acetaldehyde is 150 nM, while that for ALDH-II measured with 200 μM acetaldehyde is 20 μM. For comparison, the inhibition of ALDH-I and ALDH-II by disulfiram (Antabuse, Sigma) was

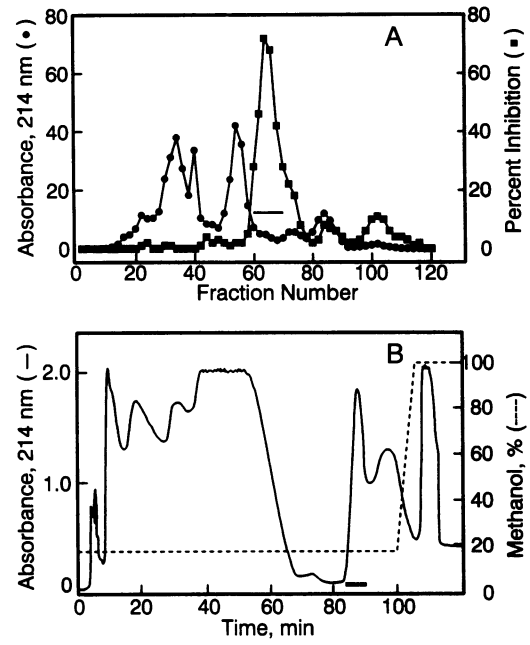

FIG. 1. Purification of ALDH inhibitor from *R. puerariae*. Undissolved materials from the concentrated methanol extract (see text) were removed by centrifugation. The supernatant solution was fractionated (*A*) on a Bio-Gel P-4 column (3.5 × 55 cm; Bio-Rad) equilibrated with 10 mM NaP_i (pH 7.5). The column was eluted at 55 ml/hr and fractions of 11 ml were collected. The horizontal line indicates fractions that exhibited ALDH inhibitory activity (assayed as described in *Materials and Methods*) and were pooled, lyophilized, and redissolved in 15% methanol/85% water (vol/vol). The solution was filtered (Millipore Millex filter, 0.45 μm) and injected onto a NovaPak C_{18} column (6–8 μm, 7.8 mm × 30 cm; Waters). The column was eluted at 2 ml/min with 15% methanol/85% water and fractions of 4 ml were collected. Fractions that inhibited ALDH activity (bar) were pooled, dried by vacuum evaporation, and rechromatographed on the same HPLC column in 20% methanol/80% water to yield pure inhibitor.

Table 1. TLC of glucose, daidzein, unhydrolyzed, and acid-hydrolyzed daidzin and ALDH inhibitor

Compound	R_f value × 100		
	I	II	III
Glucose*	40	73	—†
Daidzein‡	85	96	43
Daidzin*‡	69	91	2
Hydrolyzed daidzin*	40	73	—†
Hydrolyzed daidzin‡	85	96	43
ALDH inhibitor*‡	69	91	2
Hydrolyzed ALDH inhibitor*	40	73	—†
Hydrozyled ALDH inhibitor‡	85	96	43

Daidzin and ALDH inhibitor were hydrolyzed in 2 M HCl for 15 hr at 70°C. Hydrolyzed products were spotted onto silica gel 60F-254 precoated plates, layer thickness 0.2 mm (Merck), and the plates were developed in three different solvent systems: I (ethyl methyl ketone/glacial acetic acid/methanol, 6:2:2), II (benzene/glacial acetic acid/methanol, 2:2:6), and III (chloroform/glacial acetic acid/methanol, 75:8.5:16.5). Daidzin, ALDH inhibitor, and daidzein were visualized by fluorescence quenching under UV (254-nm) light. D-glucose, ALDH inhibitor, and daidzin were visualized with an anisaldehyde spray (11).

*Detected by anisaldehyde reagent.

†Not developed with anisaldehyde because of high background.

‡Detected under UV.

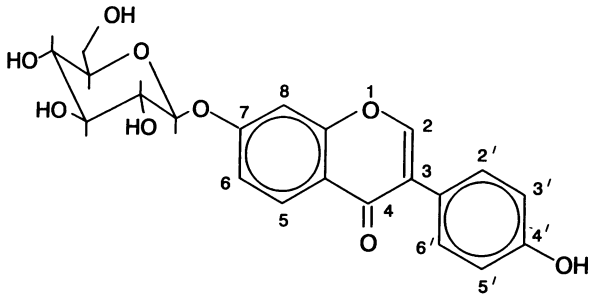

FIG. 2. Structure of daidzin. The ring numbering system shown in this structure should be used in evaluating the data in Tables 2 and 3. Isoflavone and flavone are 3- and 2-phenylchromone, respectively. Chromone is 4*H*-benzo[*b*]pyran-4-one, whereas coumarin is the lactone, 2*H*-benzo[*b*]pyran-2-one.

studied under the same conditions (Fig. 3*B*). Unlike daidzin, disulfiram inhibits ALDH-II (IC_{50} = 10 nM) more potently than ALDH-I (IC_{50} > 20 μM). A survey of commercially available compounds structurally related to daidzin identified only a few that inhibit ALDH (Table 2). Among these, only genistin (the 7-glucoside of genistein) and prunetin (7-*O*-methylgenistein) are selective inhibitors of ALDH-I, albeit an order of magnitude less potent than daidzin. None of the others examined are as potent as daidzin, nor do they inhibit ALDH-I selectively. Ononin, a 4'-*O*-methyl derivative of daidzin, is much less potent and inhibits ALDH-I and ALDH-II with similar potency. Most of the flavones and isoflavones tested do not inhibit ALDH-I or ALDH-II to any significant extent (Table 3). Allantoin, a constituent of *R. puerariae* extracts; 1-methylhydantoin, a structural analog of 5-methylhydantoin; and 2-phenylquinoline, a steric analog of isoflavone, fail to inhibit either ALDH-I or ALDH-II.

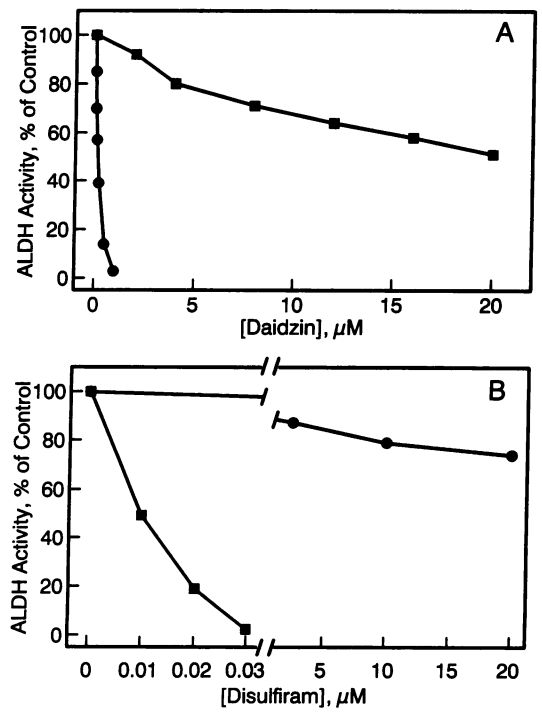

FIG. 3. Inhibition by daidzin (*A*) and disulfiram (*B*) of human ALDH-I (●)- and ALDH-II (■)-catalyzed oxidation of acetaldehyde. ALDH activities were measured at 25°C in 0.1 M $NaPP_i$ buffer (pH 9.5) containing 0.15 M KCl, 1 mM NAD^+, various concentrations of inhibitors, 5–10 nM of ALDH-I or ALDH-II, and 5 and 200 μM acetaldehyde for ALDH-I and ALDH-II, respectively. Reactions were initiated by the addition of enzyme and initial velocities were measured immediately. ALDH-I was chromatographed through Sephadex G-10 to remove thiols prior to disulfiram inhibition studies.

The kinetics of daidzin inhibition of human ALDH-I and ALDH-II were measured with formaldehyde as substrate because the extremely low K_m of ALDH-I for acetaldehyde, <1 μM, precludes accurate kinetic measurements by the initial-velocity method. The K_m values with formaldehyde are 600 μM and 9.2 mM for ALDH-I and ALDH-II, respectively. Daidzin inhibits both ALDH-I and ALDH-II in an apparently competitive manner with K_i values of 40 nM and 20 μM, respectively, and it inhibits ALDH-I uncompetitively with respect to NAD^+ (data not shown).

Daidzin inhibition of ALDH-I is reversible. In the presence of 100 nM daidzin ALDH-I is inhibited by 70%. A 100-fold dilution restores activity to 98% (result not shown). Inhibition kinetic studies of ALDH-I and ALDH-II by genistin and ononin indicate that, like daidzin, both are competitive inhibitors with respect to formaldehyde. The K_i values estimated for genistin and ononin are 360 nM and 14 μM for ALDH-I, and >20 μM and 11 μM for ALDH-II, respectively.

DISCUSSION

We have isolated a potent, selective, and reversible inhibitor of human ALDH-I, daidzin, by monitoring fractions from an extract of *R. puerariae* with a simple ALDH activity assay. Daidzin inhibits ALDH-I at very low concentrations (K_i = 40 nM), but it is at least 500 times less effective against ALDH-II, the cytosolic isozyme (K_i = 20 μM). To our knowledge, daidzin is the first isoflavone found to inhibit human ALDH and the first compound found to inhibit ALDH-I selectively and reversibly at nanomolar concentrations.

Most of the potent ALDH inhibitors described previously—e.g., disulfiram, (*Z*)-hexadeca-1,11-dien-3-one, bromoacetophenone—are irreversible inactivators that abolish ALDH activity by covalent modification of amino acid residues critical to catalysis. Cys-302 has been implicated as a component of the active site of ALDH by selective modification with iodoacetamide (12) and by the fact that it is the only cysteine conserved among all ALDHs sequenced to date, including human ALDH-I and -II (2). Disulfiram inactivates ALDH by promoting the formation of a disulfide bond between Cys-302 and a vicinal sulfhydryl group in the enzyme active site (13), whereas the vinyl ketone (*Z*)-hexadeca-1,11-dien-3-one inactivates by forming a Michael addition adduct with Cys-302 (14).

Daidzin bears neither a structural nor a chemical resemblance to disulfiram (tetraethylthiuram disulfide), though its α,β-unsaturated carbonyl function, by analogy with the vinyl ketone, could conceivably undergo adduct formation with Cys-302. However, ALDH inhibition by daidzin is readily reversible by dilution, thus precluding covalent modification as the basis for this inhibition. Furthermore, flavones, which are structurally related to the isoflavones, also contain α,β-unsaturated carbonyls and yet exhibit little or no inhibitory activity.

Daidzin selectively inhibits ALDH-I, whereas disulfiram preferentially inactivates ALDH-II (Fig. 3). ALDH-I is normally resistant to disulfiram inhibition *in vitro* but is readily inactivated by it *in vivo* or in the presence of trace amounts of thiol. With 2-mercaptoethanol, for example, both isozymes are inactivated via thiol–disulfide interchange with the intermediate diethyldithiocarbamic acid/2-mercaptoethanol mixed disulfide (15). The preformed mixed disulfide with methanethiol, a putative intermediate of the transamination pathway for the degradation of methionine, also inactivates ALDH-I very rapidly. Rate constants for the inhibition of ALDH-I activity by mixed disulfides of increasing chain length suggest that steric hindrance plays a role in resisting inhibition by disulfiram itself (15). While the physiological

Table 2. Structurally related compounds that inhibit ALDH

Type	Substituents 2	3	4	5	7	8	4′	Name	K_i, μM ALDH-I	ALDH-II	Selectivity, $K_{i,II}/K_{i,I}$
Isoflavone	H		=O	H	OGlc	H	OH	Daidzin	0.02	10	500
	H		=O	OH	OMe	H	OH	Prunetin	0.15	6	40
	H		=O	OH	OGlc	H	OH	Genistin	0.3	>20*	≫70
	H		=O	H	H	H	i-Pr	4′-Isopropylisoflavone	0.8	0.75	0.9
	H		=O	H	OGlc	H	OMe	Ononin	>3	10	<3
Flavone		H	=O	H	H	H	H	Flavone	1.5	2.5	1.6
		Ph	=O	H	OH	H	H	3-Phenyl-7-hydroxyflavone	1.5	1	0.7
		H	=O	OH	OH	H	OMe	Acacetin	0.8	2.5	3
Chromone	Me	Bz	=O	H	OAc	OAc		3-Benzyl-7,8-diacetoxy-2-methylchromone	1.5	1.5	1
Coumarin	=O	H	Ph	H	OH	H		7-Hydroxy-4-phenylcoumarin	1.5	0.15	0.1
Dihydrocoumarin	=O	H	Ph	H	Me	H		7-Methyl-4-phenyl-3,4-dihydrocoumarin	1.5	0.25	0.2
Hexahydrocoumarin	=O	Ph	Me	H	Cl	H		7-Chloro-4A,5,6,7,8,8A-hexahydro-4-methyl-3-phenylcoumarin	0.15	0.5	3

ALDH activities were assayed at 25°C in 0.1 M $NaPP_i$ buffer (pH 9.5) containing 0.15 M KCl, 1 mM NAD^+, various concentrations of inhibitors, and 5 and 200 μM acetaldehyde for ALDH-I and ALDH-II, respectively. K_i values shown were calculated from IC_{50} values based on the formula $K_i = IC_{50}/\{(3[S_0]/K_m) - 1\}$ for competitive inhibition. K_m values of 2 μM and 200 μM were used in the calculation for ALDH-I and ALDH-II, respectively.
*Up to 20 μM does not inhibit ALDH-II.

function of ALDH-II is yet to be revealed, its irreversible inactivation by disulfiram may contribute to the side effects associated with the use of the drug.

The kinetic mechanism of human ALDH-II has been shown to follow a strictly ordered pathway with NAD^+ binding first, followed by the aldehyde (16). ALDH-I follows a random kinetic mechanism in which initial binding of NAD^+ is preferred but not obligatory (17). Analysis of the kinetics of ALDH-I and -II inhibition by daidzin reveals that for both enzymes, inhibition is competitive with respect to aldehyde and uncompetitive with respect to NAD^+. Since daidzin inhibits both isozymes by the same competitive mechanism, this suggests that a sequential ordered Bi–Bi mechanism may pertain in both cases with NAD^+ binding to the coenzyme site first followed by either aldehyde or daidzin binding to the aldehyde site.

Daidzin at concentrations up to 20 μM does not affect any of the isozymes of alcohol dehydrogenase. Its effect on other enzymes has not been tested systematically. However, it has little or no effect on biological systems that are known to be

Table 3. Structurally related compounds that do not inhibit ALDH

Type	Substituents 2	3	4	5	6	7	8	2′	3′	4′	5′	Name
Isoflavone	H		=O	H	H	OH	H	H	H	OH	H	Diadzein*
	H		=O	H	H	OH	H	H	H	OMe	H	Formononetin*
	H		=O	OH	H	OH	H	H	H	OH	H	Genistein*
	H		=O	OH	H	OH	H	H	H	OMe	H	Biochanin A*
	H		=O	H	H	OH	OGlc	H	H	OH	H	Puerarin*
	Me		=O	H	H	OAc	H	H	H	H	H	7-Acetoxy-2-methylisoflavone
	Me		=O	H	H	OAc	OAc	H	H	H	H	7,8-Diacetoxy-2-methylisoflavone
Isoflavan	H		H	H	H	OH	H	H	H	OH	H	Equol
Flavone		H	=O	H	H	H	H	Cl	H	H	H	2′-Chloroflavone
		H	=O	H	H	OH	H	H	H	H	H	7-Hydroxyflavone
		H	=O	H	H	O_2CPh	H	H	H	H	H	7-Benzoyloxyflavone
		H	=O	H	H	OH	OH	H	H	H	H	7,8-Dihydroxylfavone
		H	=O	OH	H	OH	H	H	H	H	H	Chrysin
		H	=O	OH	H	OMe	H	H	H	H	H	Techtochrysin
		H	=O	OH	H	OH	H	H	H	OH	H	Apigenin
		OH	=O	H	H	H	H	H	H	H	H	3-Hydroxyflavone
		OH	=O	OH	H	OH	H	H	H	H	H	Galangin
		OH	=O	OH	H	OH	H	H	H	OH	H	Kaempferol
		OH	=O	H	H	OH	H	H	OH	OH	H	Fisetin
		OH	=O	OH	H	OH	H	OH	H	OH	H	Morin
		OH	=O	OH	H	OH	H	H	OH	OH	H	Quercitin
		O-rutinose	=O	OH	H	OH	H	H	OH	OH	H	Rutin
		OH	=O	OH	H	OH	H	H	OH	OH	OH	Myricetin
Flavan		H	=O	H	H	H	H	H	H	H	H	Flavanone
		H	=O	OH	H	OH	H	H	H	OH	H	4′,5,7-Trihydroxyflavanone
		OH	H	OH	H	OH	H	H	OH	OH	H	(±)-Catechin
		OH	H	OH	H	OH	H	H	OH	OH	H	(−)-Epicatechin
Coumarin	=O	†	OH	H	H	H	H					Warfarin
	=O	H	H	H	OMe	OMe	H					6,7-Dimethoxycoumarin*

For assay conditions, see Table 2.
*Present in *R. puerariae*.
†2-Acetyl-1-phenylethyl.

inhibited by such isoflavones as genistein, daidzein, formononetin, and puerarin (e.g., tyrosine-specific protein kinase, topoisomerase, estrogenic, β-adrenergic) (18–21).

An assessment of the active-site structures of ALDH-I and -II must await the results of crystallographic studies yet to be reported. However, a survey of commercially available compounds that resemble the structure of daidzin has revealed some structure–activity relationships associated with ALDH inhibition. Flavones, in general, are not good ALDH inhibitors. Even polyhydroxyflavones such as quercitin and myricetin, which are known to be potent inhibitors of aldehyde reductases, do not inhibit ALDH (22, 23). A 3-phenyl substituted chromone structure is most likely important for ALDH inhibition as seen by comparison of the inhibitory isoflavone prunetin (Table 2) with its inactive flavone analog techtochrysin (Table 3). Among the coumarin derivatives surveyed, three are very good ALDH-II inhibitors (Table 2), but none inhibits ALDH-I with the same potency as daidzin. A major constituent of *R. puerariae*, 6,7-dimethoxycoumarin, inhibits neither ALDH-I nor ALDH-II.

The characteristics of the isoflavones tested show that the substituents at the 4′, 5, and 7 positions are important for inhibition. A free 4′ hydroxyl group seems to bind to ALDH-I more favorably than a 4′ methoxy group (compare daidzin and ononin). In contrast, ALDH-II seems to accommodate 4′ hydroxyl and 4′ methoxy groups equally well. Hydroxyl substitution at the 5 position decreases potency by at least an order of magnitude but does not seem to abolish selectivity. A blocked hydroxyl group at the 7 position is essential for ALDH-I inhibition: the aglycones daidzein and genistein do not inhibit. The 7-*O*-glucosyl derivatives are the most potent yet selective inhibitors of ALDH-I. Potency is improved by replacing the 7-*O*-glucosyl moiety with a 7-*O*-methyl group (compare genistin and prunetin), but selectivity toward ALDH-I is somewhat reduced.

Based on these relationships, it would appear that the daidzin binding sites of ALDH-I and ALDH-II differ substantially. In ALDH-I, the 4′ position of a bound inhibitor is likely to be located in a relatively restricted area, whereas in ALDH-II its environment is likely to be more accessible. Furthermore, the binding pocket for daidzin in ALDH-I seems to accommodate a hydrophilic or bulky substituent on the 7 position of the inhibitor better than that in ALDH-II. Hence, a variety of 7-*O*-substituted daidzein derivatives would seem to be appropriate targets for designing novel daidzin analogs that could be more potent and selective for ALDH-I, and perhaps therapeutically useful for the treatment of alcohol abuse and alcoholism.

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