Inhibition of Human Aldehyde Oxidase Activity by Diet-Derived Constituents: Structural Influence, Enzyme-Ligand Interactions, and Clinical Relevance^S

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ABSTRACT

The mechanistic understanding of interactions between diet-derived substances and conventional medications in humans is nascent. Most investigations have examined cytochrome P450-mediated interactions. Interactions mediated by other phase I enzymes are understudied. Aldehyde oxidase (AO) is a phase I hydroxylase that is gaining recognition in drug design and development programs. Taken together, a panel of structurally diverse phytoconstituents ($n = 24$) was screened for inhibitors of the AO-mediated oxidation of the probe substrate O⁶-benzylguanine. Based on the estimated IC₅₀ (<100 μ M), 17 constituents were advanced for K_i determination. Three constituents were described best by a competitive inhibition model, whereas 14 constituents were described best by a mixed-mode model. The latter model consists of two K_i terms, K_{is} and K_{ii} , which ranged from 0.26–73 and 0.80–120 μ M, respectively. Molecular

modeling was used to glean mechanistic insight into AO inhibition. Docking studies indicated that the tested constituents bound within the AO active site and elucidated key enzyme-inhibitor interactions. Quantitative structure-activity relationship modeling identified three structural descriptors that correlated with inhibition potency ($r^2 = 0.85$), providing a framework for developing in silico models to predict the AO inhibitory activity of a xenobiotic based solely on chemical structure. Finally, a simple static model was used to assess potential clinically relevant AO-mediated dietary substance–drug interactions. Epicatechin gallate and epigallocatechin gallate, prominent constituents in green tea, were predicted to have moderate to high risk. Further characterization of this uncharted type of interaction is warranted, including dynamic modeling and, potentially, clinical evaluation.

Introduction

At least 20% of patients acknowledge taking herbal products and other diet-derived substances with their conventional medications (Gardiner et al., 2006), leading to potential untoward interactions. Consequently, dietary substance–drug interactions are increasingly being recognized in clinical practice. Such interactions typically arise when phytoconstituents modulate (i.e., induce or inhibit) the activity of a drug-metabolizing enzyme or transporter, which can lead to alterations in the pharmacokinetics of a coadministered “victim” drug. Pharmacokinetic dietary substance–drug interactions have been studied extensively with respect to modulation of the cytochromes P450 (P450s), both in vitro and in vivo. Clinically relevant P450-mediated interactions have been reported; notorious “perpetrators” include grapefruit juice, a potent inhibitor of intestinal CYP3A4 (Bailey et al., 1998; Paine et al., 2004), and St. John’s wort, an inducer of both intestinal and hepatic CYP3A4 (Durr et al., 2000). More recently, the effects of phytoconstituents on other biochemical processes that influence drug disposition, including conjugative

enzymes (Mohamed and Frye, 2011; Li et al., 2012; Gufford et al., 2014) and transporters (Roth et al., 2011; Kock et al., 2013), have been examined. In contrast, effects on non-P450 phase I drug-metabolizing enzymes are understudied.

Aldehyde oxidase (AO) is a cytosolic molybdenum-containing hydroxylase that is gaining attention in drug discovery and development programs. AO comprises two identical 150-kDa subunits, each containing domains that house FAD, two iron-sulfur clusters, and molybdenum cofactor. Although human AO has not been crystallized, knowledge of enzyme structure has been gleaned from the crystal structure of highly homologous enzymes, including human xanthine oxidase and murine AOX3. Although the physiologic purpose of AO is not completely understood, enzymatic activity toward various xenobiotics has been well characterized (Garattini and Terao, 2012; Barr et al., 2014). AO has broad substrate selectivity, oxidizing aldehydes and a variety of heterocyclic compounds, including nitrogen-containing aromatic heterocycles. Such heterocycles frequently are used as scaffolds in the design of new chemical entities, leading to an increased number of AO drug substrates during drug development (Pryde et al., 2010; Hutzler et al., 2013).

In parallel with increasing candidate AO substrates is an impetus to identify AO inhibitors, elucidate the mechanism of inhibition, and understand clinical implications (e.g., potential drug–drug interactions). AO inhibitors include estrogens, phenothiazines, tricyclic antidepressants,

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ABBREVIATIONS: AO, aldehyde oxidase; BG, benzylguanine; DACA, *N*-[(2-dimethylamino)ethyl]acridine-4-carboxamide; DMSO, dimethylsulfoxide; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; HLC, human liver cytosol; 4MU-G, 4-methylumbelliferone glucuronide; P450, cytochrome P450; QSAR, quantitative structure-activity relationship.

and tricyclic atypical antipsychotic agents (Obach et al., 2004). Reversible inhibition often occurs via atypical modes, including uncompetitive and mixed (Obach, 2004; Barr and Jones, 2011, 2013). Although in vitro data suggest an interaction risk, clinical AO-mediated xenobiotic-drug interactions have not been reported. As a next step toward improving the mechanistic and clinical understanding of AO inhibition, a panel ($n = 24$) (Supplemental Table 1) of closely related yet chemically diverse diet-derived constituents was examined for AO inhibitors using a combination of in vitro and in silico methods.

A working systematic framework for assessing dietary substance–drug interaction risk involves isolating individual constituents from the dietary substance of interest, testing the constituents as modulators of specific drug-metabolizing enzyme/transporter activity, and identifying potential clinical risks via static and dynamic modeling (Brantley et al., 2014a; Gufford et al., 2014). The objective of the present study was to expand this working framework by adding a molecular modeling component to advance the mechanistic understanding of AO-mediated xenobiotic-drug interactions. The aims were to: 1) screen a panel of diet-derived constituents as AO inhibitors using the clinically relevant probe substrate O^6 -benzylguanine (O^6 -BG); 2) determine inhibition potency (K_i) of selected constituents; 3) relate inhibitor structural features to K_i and identify key ligand-enzyme binding interactions; and 4) identify constituents as potential perpetrators of clinically relevant interactions with conventional medications. This expanded working framework identified potent inhibitors of AO that warrant further evaluation via dynamic modeling and simulation, as well as provided new mechanistic insight into ligand-AO interactions.

Materials and Methods

Chemicals, Reagents, and Enzyme Source

Human liver cytosol (HLC), pooled from 200 donors of mixed gender, was purchased from Xenotech, LLC (Lenexa, KS). (–)-Epicatechin, (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), hesperidin, 4-methylumbelliferone, 4-methylumbelliferyl- β -D-glucuronide hydrate (4MU-G), naringin, psoralen, and tangeretin were purchased from Sigma-Aldrich (St. Louis, MO). Apigenin, kaempferol, 6',7'-dihydroxybergamottin, naringenin, and quercetin were purchased from Cayman Chemical Company (Ann Arbor, MI). O^6 -BG, 8-oxo-BG, and resveratrol were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Tolbutamide was purchased from Alfa Aesar (Ward Hill, MA). Raloxifene was purchased from BIOTANG, Inc. (Lexington, MA). Silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin, and taxifolin were purified as described previously (Graf et al., 2007) and were >97% pure as determined by ultra high-performance liquid chromatography (Napolitano et al., 2013). Acetonitrile (liquid chromatography/mass spectrometry grade), potassium phosphate (monobasic and dibasic), and formic acid were purchased from Fisher Scientific (Waltham, MA).

Evaluation of Diet-Derived Constituents as Inhibitors of AO Activity

General Incubation Conditions. Incubation mixtures consisted of substrate (O^6 -BG), inhibitor (or vehicle for controls lacking inhibitor), HLC, and potassium phosphate buffer (KP_i) (25 mM, pH 7.4). Substrate and inhibitor/vehicle working solutions were added to KP_i in 96-well plates. The mixtures were equilibrated at 37°C for 5 minutes. Reactions were initiated by adding HLC (0.1 mg/ml final concentration), yielding a final volume of 100 μ l; the final dimethyl sulfoxide (DMSO) concentration was 0.5% v/v. After 4 minutes, reactions were terminated by adding 300 μ l ice-cold acetonitrile containing tolbutamide as internal standard (100 nM final concentration). After centrifugation at 1350g for 10 minutes at 4°C, the supernatant was analyzed for 8-oxo-BG by liquid chromatography–tandem mass spectroscopy (see below). Under these experimental conditions, less than 10% of the substrate was consumed, and 8-oxo-BG formation was linear with respect to incubation time and HLC protein concentration (data not shown).

Saturation Kinetics of O^6 -BG. O^6 -BG was dissolved in DMSO to yield working solutions ranging from 6.3–200 mM. HLC was diluted in KP_i to yield

a working solution of 0.4 mg/ml. Incubations proceeded as described above; final concentrations of O^6 -BG ranged from 3–500 μ M. Kinetic parameters (K_m , V_{max}) were obtained by fitting the unienzyme Michaelis-Menten equation to substrate-versus-velocity data via nonlinear least squares regression using GraphPad Prism (San Diego, CA). The equation was selected on the basis of a linear Eadie-Hofstee ($v/[S]$ -versus- $[S]$) transformation of the data.

Screening. Incubation mixtures were prepared as described above using a concentration of O^6 -BG (125 μ M) that approximated the K_m . Each phytoconstituent was dissolved in DMSO to yield working concentrations of 4 and 40 mM and added to the mixture to yield final concentrations of 10 and 100 μ M. AO activity was expressed as the percent of the rate of 8-oxo-BG formation in the presence to absence of inhibitor.

K_i Determination for Selected Inhibitors. Incubation mixtures were prepared in a similar manner to that described above using a 6×6 matrix of O^6 -BG (16–500 μ M) and inhibitor concentrations. IC₅₀s were estimated from the initial two-point inhibitor screening results and were used to guide the range of inhibitor concentrations. Because estimating an IC₅₀ from the two-point data was not possible for some inhibitors (quercetin, kaempferol, EGCG, and ECG), an abbreviated IC₅₀ screen (singlet, six inhibitor concentrations) was conducted (data not shown). Constituents with an estimated IC₅₀ of 100 μ M or less were selected for K_i determination. Inhibitor concentrations were selected such that at least two concentrations were above and below the estimated IC₅₀. Reaction mixtures were processed further as described above. Initial estimates of apparent K_m and V_{max} were derived from Michaelis-Menten fits to the velocity-versus-[substrate] data in the absence of inhibitor. Initial estimates of apparent K_{is} and/or K_{ii} were derived from Lineweaver-Burk plots of velocity⁻¹-versus-[substrate]⁻¹. Kinetic parameters (K_m , V_{max} , K_{is} , K_{ii}) were obtained by fitting eqs. 1, 2, or 3 to untransformed data via nonlinear least squares regression using Phoenix WinNonlin (v. 6.3; Certara, St. Louis, MO):

$$\text{Competitive } v = \frac{V_{max} \times [S]}{K_m \left(1 + \frac{[I]}{K_{is}}\right) + [S]} \quad (1)$$

$$\text{Uncompetitive } v = \frac{V_{max} \times [S]}{K_m + [S] \left(1 + \frac{[I]}{K_{ii}}\right)} \quad (2)$$

$$\text{Mixed } v = \frac{V_{max} \times [S]}{K_m \left(1 + \frac{[I]}{K_{is}}\right) + [S] \left(1 + \frac{[I]}{K_{ii}}\right)} \quad (3)$$

where v denotes the velocity of 8-oxo-BG formation, $[S]$ denotes the substrate concentration, and $[I]$ denotes inhibitor concentration. K_{ii} denotes the affinity of inhibitor toward the enzyme-substrate complex, whereas K_{is} denotes the affinity of the inhibitor toward the “free” enzyme (Cook and Cleland, 2007). If K_{ii} or K_{is} is very large (approach infinity), the mixed equation simplifies to competitive or uncompetitive inhibition, respectively. The best-fit equation was determined by visual inspection of Lineweaver-Burk plots, corresponding slope and intercept replots, the randomness of the residuals, Akaike information criteria, and standard errors of the parameter estimates generated from the nonlinear regression procedure.

Quantitation of 8-Oxo-BG by Liquid Chromatography Tandem Mass Spectrometry. Chromatographic separation was achieved using a Phenomenex (Torrance, CA) Synergi MAX-RP 80A column (4 μ m, 2.0 $\times$ 50 mm) coupled to an Aquasil C18 precolumn (3 μ M; Thermo Scientific, Waltham, MA) heated to 40°C using a binary gradient at a flow rate of 0.65 ml/min. Initial conditions comprised 95% mobile phase A (0.1% formic acid in water) and 5% mobile phase B (0.1% formic acid in acetonitrile). Initial conditions were maintained for 0.4 minutes, then ramped linearly to 5% mobile phase A and 95% mobile phase B over 3.1 minutes. These conditions were held for 0.6 minutes before returning to initial conditions over 0.01 minutes; initial conditions were held for 0.9 minutes. The total run time was 5 minutes. Samples were analyzed (3- μ l injection volume) using a QTRAP 6500 UHPLC/MS/MS system (AB Sciex, Framingham, MA) with a turbo-electrospray source operating in positive ion mode. The declustering potential and collision energy were set at 30 V and 15 mV, respectively. The m/z transitions for 8-oxo-BG (258→91) and the internal standard, tolbutamide (271→172),

were detected in multiple reaction-monitoring mode. Concentrations of 8-oxo-BG were determined using MultiQuant software (v2.1.1; AB Sciex) by interpolation from matrix-matched calibration curves with a linear range of 0.2–5000 nM. The calibration standards were judged for batch quality based on the FDA guidance for industry regarding bioanalytical method validation (www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf).

Molecular Modeling

Molecular modeling was conducted using the Schrödinger Small-Molecule Drug Discovery Suite 2014-2 (New York, NY). Structures were imported from Chemdraw (Cambridgesoft, Cambridge, MA) into Maestro (v. 9.8, Schrödinger). Ligands were prepared using LigPrep (v. 3.0; Schrödinger). The energy for each structure was minimized using OPLS_2005 force field, and ionization states were determined at pH 7.0 $\pm$ 0.5 using the Epik algorithm.

Homology Modeling. The homology model for human AO (AOX1) protein was developed as described previously (Choughule et al., 2013). In brief, Schrödinger Prime module was used to generate a homology model using the solved crystal structure (PDB ID 3ZYV) for mouse AOX3 (Coelho et al., 2012) as a template. ClustalW was used to align the sequences, and adjustment was not necessary owing to the high homology between sequences (62% identity). Induced-fit docking workflow using the AO substrate *N*-(2-dimethylamino)ethyl]acridine-4-carboxamide (DACA) as the ligand was used to refine residues in the active site of AO within 5 Å of the ligand surface. For the portions of the mouse structure that did not have sufficient electron density, the human enzyme was modeled using the energy-based method in Prime; residues 168–200 could not be replaced and are not included in the model.

Docking. Docking experiments were conducted using GLIDE (v. 6.3; Schrödinger). A receptor grid for the active site was built around DACA as the reference ligand and with a grid size of 10 $\times$ 10 $\times$ 10 Å. Prepared ligands were docked into the receptor grid using standard precision, flexible ligand sampling, included Epik state penalties to the docking score, rewarded intramolecular hydrogen bonds, and imposed a scaling factor of 0.8 and partial charge cutoff of 0.15 for van der Waals radii. The poses were scored according to the Glide algorithm and displayed as “docking_score” in the Schrödinger results table. Docking scores were used to evaluate the best pose for each ligand and were used for comparison with experimentally measured K_{is} .

Quantitative Structure-Activity Relationship. Forty-four structural descriptors were generated using QikProp (v. 4.0; Schrödinger). Property-based quantitative structure-activity relationship (QSAR) modeling was conducted using these descriptors and Statgraphics Centurion (v. 16.2.04; Statpoint Technologies, Inc., Warrenton, VA). K_{is} s were converted to molar units and expressed as the negative base-10 logarithm (pK_{is}). The K_i for 6',7'-dihydroxybergamottin, hesperidin, naringin, psoralen, and tangeretin were not determined owing to low inhibitory potency (estimated $K_i > 100 \mu\text{M}$); pK_{is} 4, which corresponds to 100 μM , was assigned to these compounds for QSAR modeling purposes. A K_i for EGC could not be determined and was excluded from the analysis. A partial least squares regression method was used with a maximum number of descriptors (independent variables) set to three and pK_{is} as the activity property (dependent variable). The best fit model was selected based on the *r*-squared coefficient of determination and the *q*-squared cross validation leave-one-out results.

Statistical Analysis

Data are presented as means $\pm$ S.D.s of triplicate incubations unless noted otherwise. K_{is} s are presented as estimates $\pm$ S.E.s. Concentration-dependent inhibition was evaluated by a paired, one-tailed *t* test using a *P* < 0.05 as the threshold value for significance using GraphPad Prism (v.6).

Results

Michaelis-Menten Kinetics Describe the Oxidation of the AO-Specific Probe Substrate, O⁶-BG. A unienzyme Michaelis-Menten equation described the kinetics of 8-oxo-BG formation in HLC, producing a V_{max} and K_m of 0.59 $\pm$ 0.012 nmol/min per milligram and 120 $\pm$ 13 μM , respectively (Fig. 1).

Fig. 1. Saturation kinetics for the oxidation of O⁶-BG to 8-oxo-BG by HLC. Symbols and error bars denote means and standard deviations, respectively, of triplicate determinations. Curve denotes model-generated values using the Michaelis-Menten equation. The inset symbols denote the Eadie-Hofstee transformation of the data, and the line denotes the best-fit least squares regression.

Diet-Derived Constituents Vary Greatly in AO Inhibition Potency.

A panel of structurally diverse phytoconstituents (*n* = 24) was screened for AO inhibitory activity using two test concentrations (10 and 100 μM) and a substrate concentration (125 μM) that approximated K_m . Constituents were ranked according to inhibition potency at 10 μM (Fig. 2). Relative to vehicle, at 100 μM , all constituents with the exception of psoralen inhibited AO activity by >10% (*P* < 0.05). All constituents with the exception of quercetin, EGC, 4MU-G, psoralen, and tangeretin inhibited activity in a concentration-dependent manner (*P* < 0.05). Based on the estimated IC₅₀, 17 constituents were selected for K_i determination. EGC displayed unusual kinetics, having an inhibitory effect at lower concentrations but a stimulatory effect at higher concentrations, precluding recovery of K_i .

K_i Determination Reveals Varied Inhibition Mode and Approximate 300-Fold Variation in Potency for Selected Inhibitors. Apparent K_i was determined using a 6 $\times$ 6 matrix of substrate-inhibitor concentrations. The mode of inhibition was determined, in part, from a Lineweaver-Burk plot (Fig. 3). The inhibition kinetics of 14 constituents were described best by mixed-mode inhibition, whereas those for three constituents were described best by competitive inhibition (Table 1). The K_{is} for competitive inhibitors varied 21-fold, and the K_{is} and K_i for the mixed-mode inhibitors varied 280- and 150-fold, respectively. With the exception of 4-methylumbelliferone, the mode of inhibition was primarily competitive ($K_{ii} > K_{is}$). The green tea constituents EGC and EGCG were the most potent ($K_{is} < 0.5 \mu\text{M}$), inhibiting by a mixed and competitive mode, respectively.

Docking Reveals Key Enzyme-Ligand Interactions, and Docking Scores Correlate with Measured K_i . Molecular structures were minimized for energy conformations using an OPLS_2005 force field in LigPrep. Structures were docked into a human AO homology model that was constructed using mouse AOX3 as a template. The best docking pose was selected based on docking score (Supplemental Table 2), which is a mathematical prediction indicative of the binding affinity of the inhibitor. Docking score was plotted versus pK_{is} , the experimentally measured binding affinity. Generally, the docking score correlated with measured pK_{is} ($r^2 = 0.5$), with the two most potent inhibitors (EGC and EGCG) having the highest docking scores (Fig. 4A). Observed interactions included π -stacking between the phenyl rings of EGC and residues F923 and F885 and hydrogen bonding between two phenol groups and the carboxylate group on E882 (Fig. 5). The π -stacking between phenyl groups and F923 and the hydrogen bonding between phenol groups of the inhibitor and the carboxylate moiety on E882 were the most conserved, with the

Fig. 2. Screening of diet-derived constituents as inhibitors of AO-catalyzed oxidation of O⁶-BG. Values are expressed as a percent of the activity compared with vehicle control (0.5% DMSO). Bars (black, 100 μ M; gray, 10 μ M) and error bars denote means and standard deviations, respectively, of triplicate incubations. Relative to vehicle, at 100 μ M, all constituents with the exception of psoralen inhibited AO activity by >10%. All constituents with the exception of quercetin, EGC, tangeretin, 4MU-G, and psoralen showed concentration-dependent inhibition (paired, one-tailed *t* test, *P* < 0.05). DHB, 6',7'-dihydroxybergamottin; EC, epicatechin; 4MU, 4-methylumbelliferone.

docking pose for 9 of the 17 most potent inhibitors showing both of these interactions.

QSAR Relates Molecular Properties to Inhibition Potency. Schrödinger Qikprop generated 44 molecular descriptors for each constituent. A subset of descriptors was selected based on preliminary correlational analyses and removal of descriptors that did not vary between constituents. Partial least squares analysis was used to relate a maximum of three descriptors (independent variables) to the measured pK_{is} (dependent variable). The model that yielded the highest *r*-squared and *q*-squared cross validation leave-one-out values (0.85 and 0.78, respectively) contained three molecular descriptors: dipole, FISA, and acptHB; dipole is the computed dipole moment of the molecule, FISA is the hydrophilic component of the solvent-accessible surface area, and acptHB is the estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution. The raw and standardized regression line for the QSAR model was described by eqs. 4 and 5, respectively:

$$pK_i = 4.98 - 0.15 \times \text{dipole} + 0.00800 \times \text{FISA} - 0.139 \times \text{acptHB} \quad (4)$$

$$pK_i = -0.37 \times \text{dipole} + 0.91 \times \text{FISA} - 0.77 \times \text{acptHB} \quad (5)$$

The raw model is useful for making predictions, whereas the standardized model is useful for showing the relative contribution of

Fig. 3. Lineweaver-Burk plots showing inhibition of AO-catalyzed O⁶-BG oxidation by EGCG (A) and ECG (B). HLC was incubated with O⁶-BG (16–500 μ M) and inhibitor (0–10 μ M EGCG or 0–5 μ M ECG) for 4 minutes. Symbols denote means of duplicate incubations. Insets are replots of the slopes (x) and intercepts (*) of corresponding Lineweaver-Burk plots versus inhibitor concentration.

each descriptor to the model. The model-predicted pK_{is} versus the experimentally-derived pK_{is} is shown in Fig. 4B. The descriptors used for the model (dipole, FISA, and acptHB) are listed in Supplemental Table 2.

Discussion

As dietary supplement usage continues to increase, the understanding of the mechanisms underlying potential interactions with drugs must advance to make accurate predictions of interaction risk. A working framework for predicting metabolism-based dietary substance–drug interactions includes assessing the inhibitory effects on drug-metabolizing enzymes of individual constituents within a dietary substance mixture and identifying key marker constituents that can be used to predict the likelihood and magnitude of an interaction. Although interactions mediated by the P450s have predominated, recent investigations have applied similar approaches to UDP-glucuronosyl transferases and drug transport proteins (Roth et al., 2011; Gufford et al., 2014). Until now, dietary substance–drug interactions mediated by other phase I drug-metabolizing enzymes have not been systematically evaluated.

AO is a phase I molybdenum containing hydroxylase that is gaining attention in drug discovery and development. The mechanisms by which xenobiotics inhibit AO activity have been examined. AO inhibition is atypical in that the process often occurs via either a mixed or uncompetitive mode. Mixed inhibition occurs when the inhibitor

TABLE 1

K_i of diet-derived constituents toward the AO-mediated oxidation of O⁶-BG

Apparent K_i s were determined by fitting eqs. 1, 2, or 3 with observed 8-oxo-BG formation velocities in varying substrate and inhibitor conditions. Values represent the K_i estimate $\pm$ S.E (μ M) from nonlinear least-squares regression using Phoenix WinNonlin (version 6.3).

Inhibition Mode		K_{is}	K_{ii}
μ M			
Flavanonol			
Silybin A	Mixed	5.9 $\pm$ 0.90	9.4 $\pm$ 1.7
Silybin B	Mixed	29 $\pm$ 5.2	53 $\pm$ 9.6
Isosilybin A	Mixed	4.2 $\pm$ 0.60	4.1 $\pm$ 1.1
Isosilybin B	Mixed	3.0 $\pm$ 0.25	10 $\pm$ 1.6
Silydianin	Mixed	30 $\pm$ 2.4	120 $\pm$ 19
Silychristin	Mixed	7.3 $\pm$ 1.4	6.8 $\pm$ 2.0
Isosilychristin	Competitive	7.2 $\pm$ 0.70	—
Taxifolin	Mixed	6.5 $\pm$ 0.91	7.5 $\pm$ 1.6
Catechin			
EGCG	Competitive	0.34 $\pm$ 0.050	—
EGC	ND	ND	ND
ECG	Mixed	0.26 $\pm$ 0.038	0.80 $\pm$ 0.17
EC	Competitive	3.8 $\pm$ 0.65	—
Flavonol			
Kaempferol	Mixed	1.1 $\pm$ 0.28	2.9 $\pm$ 0.55
Quercetin	Mixed	1.2 $\pm$ 0.50	2.8 $\pm$ 0.73
Flavone			
Apigenin	Mixed	1.2 $\pm$ 0.11	2.1 $\pm$ 0.14
Flavanone			
Naringenin	Mixed	3.9 $\pm$ 0.61	9.3 $\pm$ 1.1
Stillbenoid			
Resveratrol	Mixed	4.8 $\pm$ 0.58	11 $\pm$ 1.2
Coumarin			
4MU	Mixed	73 $\pm$ 15	23 $\pm$ 4.2

—, indicates that K_i was not used in the best fit model; EC, epicatechin; 4MU, 4-methylumbelliferone; ND, values that could not be determined.

binds to both the “free” enzyme and the enzyme-substrate complex and can be explained mechanistically by multiple inhibitory binding sites, one where the inhibitor and substrate compete for the binding site, and another that is not competitive. As such, when determining the K_i for a mixed-mode inhibitor, two inhibition constants are generated: K_{is} , which describes the competitive inhibition component, and K_{ii} , which describes the uncompetitive component.

In the current work, 24 structurally diverse diet-derived constituents were screened as potential AO inhibitors, after which 17 were advanced for K_i determination. Three constituents inhibited AO activity in a competitive manner, whereas the remaining 14 inhibited activity via a mixed mode; with the exception of 4-methylumbelliferone, the K_{is} of each of these constituents was lower than that of the corresponding K_{ii} , implying that the affinity of the inhibitor for the competitive site was higher than that for the uncompetitive site. Since the substrate competes with inhibitor, the site of inhibitor binding was hypothesized to reside within the enzyme-active site. Accordingly, molecular docking approaches were used to glean mechanistic insight

into the enzyme-inhibitor interaction at the molecular level and determine if the interaction relates to inhibitory potency.

Interactions between the AO inhibitory constituents and specific amino acids within the enzyme active site were examined via docking studies. The inhibitors were docked into the homology model, generating a docking score for each constituent, which is indicative of the binding energy between the constituent and enzyme. Because the docking approach examines interactions within the enzyme-active site, K_{is} (competitive component) was used as the potency descriptor. The experimentally determined K_{is} correlated with the docking score ($r^2 = 0.5$), supporting validity of the approach. The highest-scoring docking poses revealed two key AO-inhibitor interactions: hydrogen bonding with E882 and π -stacking with F923 (Fig. 5). These interactions are consistent with previous molecular modeling studies involving the AO substrates zoniporide, DACA, and substituted phthalazines (Dastmalchi and Hamzeh-Mivehrod, 2005; Dalvie et al., 2012; Choughule et al., 2013), supporting the hypothesis that the inhibitors are competing with substrates within the active site. A recent report describing the X-ray crystal structure of quercetin bound to the active site of xanthine oxidase, which is highly homologous to AO, further supports this contention (Cao et al., 2014).

In addition to delineating the molecular enzyme-inhibitor binding interactions, *in silico* approaches can be used as tools to determine relationships between chemical structure and inhibitory potency. Diet-derived constituents offer a convenient source of closely related yet chemically diverse structures that are favorable for QSAR studies. Partial least squares multivariate analysis was applied to such constituents using simple property-based chemical descriptors. Dipole, the hydrophilic solvent-accessible surface area (FISA), and hydrogen bonding-accepting capacity (acptHB) were three descriptors that best explained inhibitory potency, generating a model with an r -squared of 0.85 and q -squared of 0.78. The standardized model revealed FISA contributed most to the model, with a coefficient of 0.91, followed by acptHB (−0.77) and dipole (−0.37). The negative correlation with acptHB may be influenced by weak-binding inhibitors such as hesperidin, 4MU-G, and naringin, which contain sugar moieties and thus many hydrogen bond acceptor groups. Likewise, the positive correlation with FISA may be partially explained by weak inhibitors that have very low hydrophilic solvent-accessible surface area, such as tangeretin and psoralen. The docking poses for tangeretin and psoralen also reflect the latter supposition, showing only hydrophobic interactions with the enzyme active site residues.

This work represents the first inhibitor QSAR study involving human AO. Previous QSAR studies using a series of flavonoid compounds involved rat AO (Hamzeh-Mivehroud et al., 2013, 2014); however, given the significant between-species variation in enzyme structure, inhibitory potency of compounds can vary widely (Sahi et al., 2008; Choughule et al., 2013). These results provide a first step toward developing *in silico* models to predict the human AO inhibitory activity of a xenobiotic solely

Fig. 4. Correlation plots for molecular docking (A) and QSAR (B) experiments. Docking scores versus pK_{is} were plotted; linear regression produced an r -squared of 0.5 (A). The best-fit QSAR model used the descriptors dipole, FISA, and acptHB (B); the r -squared and q -squared cross-validation leave-one-out values (0.85 and 0.78, respectively) contained three molecular descriptors. The solid line represents the line of unity.

Fig. 5. Molecular docking of ECG in the homology model of human AO. The homology model was constructed using the crystal structure for mouse AOX3 (PDB ID 3ZYV) as the template. Residues F885, F923, and E882 are highlighted as key interacting molecules with the ligand. Three-dimensional representation (A): Hatched lines represent hydrogen bonding interactions between the enzyme and inhibitor, and adjacent values indicate distances between interacting atoms (Å). Two dimensional depiction (B): Spheres represent the residues that are within a 3 Å radius to ECG (red indicates negatively charged, light blue indicates polar, green indicates hydrophobic, white indicates glycine). Green lines represent π -stacking and red dashed arrows represent hydrogen bonding interactions.

on the basis of chemical structure. Further studies are necessary to determine if the QSAR model can be expanded to compounds that inhabit different chemical space, including marketed drugs.

The final aim of this study was to identify dietary constituents as potential perpetrators of clinically relevant AO-mediated interactions with conventional medications. Static models, used routinely to predict the risk of drug-drug interactions, have been extended to dietary substance–drug interactions (Zhou et al., 2004; Brantley et al., 2013). For example, $[I]/K_i$ is a simple metric used to predict the magnitude of an interaction in vivo, where $[I]$ is the concentration of inhibitor in the systemic circulation. A value of 0.1 or lower is considered low risk, between 0.1 and 1 moderate risk, and greater than 1 high risk (Tucker et al., 2001). Applying this metric to dietary substances can be particularly challenging owing to the relative scarcity of human pharmacokinetic data for individual constituents. Studies also tend to report constituent concentrations as a conglomerate of the “parent” constituent and conjugated metabolites such as glycosides, glucuronides, and sulfates. The conjugated forms of these compounds typically predominate in the systemic circulation.

Accordingly, use of these concentrations may overpredict the magnitude of an interaction.

A literature search was conducted to obtain the maximum systemic concentrations ($C_{\max}$) of the dietary constituents examined in the current work. Such values ($n = 11$) were limited to healthy volunteer studies, the plasma from which the parent constituent was measured directly (i.e., independently from any of the metabolites). With the exception of silybin A, the interaction risk for the remaining milk thistle constituents (silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and taxifolin) was predicted to be low (Table 2). Likewise, the interaction risk for quercetin, a constituent in multiple foods including fruit juices, was predicted to be low. The interaction risk of the red wine component resveratrol, marketed as a supplement with potential as a cancer chemopreventative agent, was predicted to be moderate to high if ingested at “therapeutic” doses (0.5–5 g). EGCG and ECG, two major constituents in green tea, were predicted to have high and moderate interaction risk, respectively, if consumed as tea (Misaka et al., 2014) or a supplement (Chow et al., 2001). These

TABLE 2
Static model prediction of the AO-mediated interaction risk of diet-derived constituents

	$C_{\max}^a$ μM	$[I]/K_{is}$	Reference(s)
Milk thistle constituents			
Silybin A	0.6–0.8	0.1	Zhu et al. (2013), Brantley et al. (2014b)
Silybin B	0.3–0.4	0.01	Zhu et al. (2013), Brantley et al. (2014b)
Isosilybin A	0.02–0.07	0.005–0.01	Wen et al. (2008), Zhu et al. (2013)
Isosilybin B	0.02–0.2	0.007–0.07	Wen et al. (2008), Zhu et al. (2013)
Silydianin	0.01	0.0003	Zhu et al. (2013)
Silychristin	0.02	0.003	Zhu et al. (2013)
Taxifolin	0.02	0.003	Zhu et al. (2013)
Green tea constituents			
EGCG	0.4–1	1–3	Chow et al. (2001), Misaka et al. (2014)
ECG	0.1	0.3	Misaka et al. (2014)
Other dietary constituents			
Quercetin	0.03	0.03	Wang and Morris (2005)
Resveratrol	0.3–2	0.06–0.4	Boocock et al. (2007)

^a $C_{\max}$ values were limited to studies of healthy volunteers, from whose plasma the parent constituent was measured directly (i.e., independently from any of the metabolites). Suitable data were not available for six constituents.

observations substantiate a previous report showing marked inhibition of human AO activity by diluted green tea solutions (Tayama et al., 2011). However, a different probe substrate (*N*-1-methylnicotinamide) was used, and the teas were not quantified for individual constituents, precluding comparison with the current study.

A caveat to the present work is that the number of potential victim AO drug substrates currently marketed is few. Perhaps the best example is the sedative-hypnotic and sleep aid zaleplon (Sonata), which is 70% cleared by AO (Zientek et al., 2010). Coadministration of green tea and zaleplon could manifest as intensified and prolonged sedation, commonly referred to as the “hangover effect”. Despite the dearth of AO victim drugs, the number of AO substrates within the drug discovery pipeline is growing (Pryde et al., 2010). Kinase inhibitors, for example, are an increasingly frequent target in drug discovery and a class of compounds that often have nitrogen-containing heterocyclic backbones that are labile to AO-mediated oxidation. Lenvatinib, which underwent phase III clinical trials in 2014 for the treatment of radioiodine-refractory differentiated thyroid cancer, undergoes substantial AO-mediated oxidation (Inoue et al., 2014). Another kinase inhibitor, idelalisib (Zydelig), is metabolized primarily by AO (Robeson et al., 2013) and recently received FDA approval for the treatment of chronic lymphocytic leukemia. With the expected increase in AO victim drugs on the market, understanding the mechanisms and predicting the magnitude of AO-mediated interactions during early drug development is critical.

In summary, 17 diet-derived constituents were identified as AO inhibitors, with K_{is} s that varied approximately 300-fold. Docking studies supported the hypothesis that inhibitors bind within the active site and elucidated key enzyme-inhibitor interactions. QSAR modeling identified three structural descriptors that correlated with inhibition potency. These results provide a framework for developing in silico models to predict the AO inhibitory activity of a xenobiotic solely on the basis of chemical structure, which potentially can be expanded to include other chemical space, including marketed drugs. Finally, potential dietary substance–drug interactions were assessed, and two constituents in green tea, ECG and EGCG, were predicted to have moderate to high risk. Further characterization of this uncharted dietary substance–drug interaction is warranted, including dynamic modeling and, potentially, clinical evaluation.

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Authorship Contributions

Participated in research design: Barr, Jones, Paine.

Conducted experiments: Barr.

Contributed new reagents or analytic tools: Oberlies.

Performed data analysis: Barr, Paine.

Wrote or contributed to the writing of the manuscript: Barr, Jones, Oberlies, Paine.

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